CLINICAL SCIENCE

M2-polarized macrophages promote metastatic behavior of Lewis lung carcinoma cells by inducing vascular endothelial growth factor-C expression

Bicheng Zhang,[I] Yafei Zhang,[I,II] Guoqing Yao,[I] Juan Gao,[III] Bo Yang,[I] Yong Zhao,[I] Zhiguo Rao,[I] Jianfei Gao[I]

[I] Wuhan General Hospital of Guangzhou Command, Department of Oncology, People's Liberation Army, Wuhan, Hubei Province, China. [II] Third Military Medical University Southwest Hospital, Department of Gastroenterology, Chongqing, China. [III] Wuhan General Hospital of Guangzhou Command, Department of Gastroenterology, People's Liberation Army, Wuhan, Hubei Province, China.

OBJECTIVES: Tumor-associated macrophages that generally exhibit an alternatively activated (M2) phenotype have been linked to tumor progression and metastasis. However, the role of M2-polarized macrophages in the growth and metastasis of lung adenocarcinoma remains enigmatic. The aim of this study was to explore the effect of M2 macrophages on the proliferation and migration of mouse Lewis lung carcinoma cells and tumor-induced lymphangiogenesis.

METHODS: Trypan blue staining and the Transwell migration assay were performed to evaluate the effects of activated (M1 or M2) macrophages on the proliferation and migration of Lewis cells. Furthermore, vascular endothelial growth factor-C expression in Lewis cells and nitric oxide secretion from activated macrophages were detected during the co-culture assay. Following treatment with activated macrophages, lymphatic endothelial cells differentiated into capillary-like structures, and the induction of Lewis cell migration was assessed using a two-dimensional Matrigel-based assay.

RESULTS: In the co-culture Transwell system, the proliferation and migration of Lewis cells were promoted by M2 macrophages. Moreover, the co-culture significantly increased the expression of vascular endothelial growth factor-C by Lewis cells and reduced the secretion of nitric oxide from M2 macrophages, which subsequently led to the capillary morphogenesis of lymphatic endothelial cells. Interestingly, following co-culture with Lewis cells, the function of RAW264.7 cells was polarized toward that of the M2 macrophage phenotype.

CONCLUSION: M2-polarized macrophages promoted the metastatic behavior of Lewis cells by inducing vascular endothelial growth factor-C expression. Thus, the interruption of signaling between M2 macrophages and Lewis cells may be considered to be a new therapeutic strategy.

KEYWORDS: M2-polarized macrophages; Lewis lung carcinoma; Proliferation; Migration; Lymphangiogenesis.

INTRODUCTION

Metastasis is the main cause of cancer-related mortality secondary to malignancy. It has been clearly demonstrated that inflammatory cells in the tumor microenvironment play an important role in tumor growth, progression, and metastasis. Within the tumor stroma, tumor-associated macrophages (TAMs) constitute a pivotal class of inflammatory cells, and compelling evidence has emerged to suggest that TAMs play a promoting role in processes such as carcinogenesis,

tumor growth, angiogenesis, and lymphangiogenesis and are the key regulators of the metastatic phenotype of cancer cells (1-3). Moreover, in lung adenocarcinoma, this pro-tumor role of TAMs is further supported by clinical studies demonstrating a correlation between high macrophages numbers in tumor tissue and poor patient prognosis (4-7). However, the activated phenotype and polarization status of TAMs in solid tumors are not well-understood, and the results of previous reports have often been contradictory.

There appear to be at least two different subpopulations of activated macrophages coexisting in the tumor micro-environment (8,9). The first subpopulation is referred to as classically activated macrophages (M1 macrophages), which are characterized by the IL-12^{high}, IL-23^{high}, and IL-10^{low} phenotype, and these macrophages can produce tumor necrosis factor (TNF)-α and nitric oxide (NO). Traditionally, M1 macrophages are regarded as potent effector cells that

are able to kill microorganisms and tumor cells. The other subpopulation is termed alternatively activated macrophages (M2 macrophages). Exposure to IL-4, IL-13, vitamin D3, glucocorticoids, or transforming growth factor-β (TGF-β) decreases the antigen-presenting capability of macrophages and up-regulates the expression of macrophage mannose receptors (MMR, also known as CD206), scavenger receptors (SR-A, also known as CD204), CD163, dectin-1 and DC-SIGN (10). M2-polarized macrophages have the IL-12^{low}, IL-23^{low}, and IL-10^{high} phenotype and are involved in stromal formation, tissue repair, tumor growth, angiogenesis, lymphangiogenesis, and immunosuppression. Although these two subpopulations have been extensively characterized, the role of M1- or M2-polarized macrophages in the progression and metastasis of lung cancer has not been clearly elucidated.

Here, we investigated the effect of M2-polarized macrophages on the metastatic behavior of Lewis lung carcinoma (LLC) cells in a co-culture Transwell system and found that M2 macrophages could promote the proliferation and migration of LLC cells and stimulate tumor-induced lymphangiogenesis by inducing VEGF-C (vascular endothelial growth factor-C) expression.

MATHERIAL AND METHODS

Cell lines

Mouse RAW264.7 macrophages and LLC cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS; both from Invitrogen, Carlsbad, CA, USA). The isolation and culture of lymphatic endothelial cells (LECs) were performed as described in our previous report (11). Briefly, female Balb/c mice were intraperitoneally injected with emulsified incomplete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA) to induce lymphangioma formation. After 30 days of induction, tumors in the peritoneal cavity were removed and mechanically disrupted, and LECs were isolated and resuspended in endothelial cell basal medium-2 (EBM-2; Cambrex BioScience, Wokingham, UK) supplemented with 20% FBS and 50 ng/ml endothelial cell growth supplement (Cambrex BioScience, Wokingham, UK), and these cells were grown at 37℃ in a humidified atmosphere of 5% CO_2. LECs were used in appropriate experiments or cultivated until the fourth passage.

Activation of RAW264.7 macrophages

As described in our previous study (12), M2- or M1-polaried macrophages were prepared by stimulating RAW264.7 macrophages with 10 IU/ml mouse recombinant IL-4 (Cytolab Ltd., Rehovot, Israel) or 100 IU/ml IFN-γ (Cytolab Ltd., Rehovot, Israel) plus l0 ng/ml LPS (Sigma-Aldrich, St Louis, MO, USA) overnight, respectively.

Cell proliferation assay

A Transwell system with a porous (0.4 μm pore size) polycarbonate membrane filter (Millipore Corp., Bedford, MA, USA) and 24-well plastic tissue culture plates were used for the macrophage-LLC co-cultures. LLC cells were first seeded into 24-well culture plates at a subconfluent density of 1×10^4 cells/well. Four hours later, different types of activated macrophages (1×10^4/well) were washed three times in PBS and then added to the upper chambers. The resultant four groups were as follows: i) blank control group, ii) M1 group,

iii) M2 group, and iv) RAW264.7 group. The proliferation of the LLC cells was determined by counting triplicate plates at the indicated times during a 6-day culture period using a Coulter counter. Cell viability for each experiment was determined by trypan blue staining. The results presented represent the mean values from three separate experiments.

Cell migration assay

The migration of LLC cells was assayed in Transwell cell culture chambers with a porous (8.0 μm pore size) polycarbonate membrane filter (Millipore Corp., Bedford, MA, USA), as described previously (13). Briefly, 1×10^4 LLC cells were seeded in the upper chamber. Following attachment, 1 ml of culture supernatant was added to the lower well. After 24 h of incubation, the membranes were removed and stained with hematoxylin and eosin (H&E), and the number of cells that had invaded the lower chamber was counted in three randomly selected fields under light microscopy.

Immunocytochemistry for VEGF-C expression

Cell smears were prepared from the co-cultures described above and were then immunostained with a mouse monoclonal VEGF-C antibody (1:100; Boster, Wuhan, CHINA) at 4℃ overnight. The cells were subsequently exposed to a biotinylated secondary antibody for 20 min, which was followed by treatment with streptavidin peroxidase. For color development, the slides were stained with 3,3'-diaminobenzidine (DAB) and then counterstained with H&E. A redbrown precipitate in the cytoplasm of the LLC cells indicated a positive reaction. The true color multi-function CMIAS pathological image-analyzing system was used for spectrodensitometry, and the integral optical density (IOD) represented the relative intensity of positive VEGF-C expression. Five magnified fields (200×) were selected randomly, and the mean values were calculated for statistical comparison.

NO production

As described previously, NO production was estimated according to the accumulation of NO_2^- in the medium after 24 h of macrophage activation using the Greiss reagent (9). Briefly, equal volumes of culture supernatant and Greiss reagent (100 μl) were mixed for 10 min at room temperature. The absorbance at 540 nm was measured using a Labsystems Multiscan Ascent assay plate reader. A graded solution of NO_2 was used to construct a standard curve, and the results presented represent the mean values from three separate samples.

In vitro capillary morphogenesis assay

LEC differentiation into capillary-like structures was determined using a two-dimensional Matrigel-based assay. Initially, ice-cold growth factor-reduced Matrigel was placed into each well of a 24-well tissue culture plate. The plates were maintained at 37℃ until the Matrigel had fully solidified. LECs were harvested in complete media, washed once, and then resuspended in serum-free EBM media. LECs were mixed with media collected from LLC cells, and they were subsequently treated with activated macrophage-conditioned medium for 24 h. After 24 h of incubation, the formation of tube-like structures was monitored by microscopic observation at 100× magnification, and more than 12 different fields in each well were photographed to measure the length of the tube-like structures, as described previously (14).
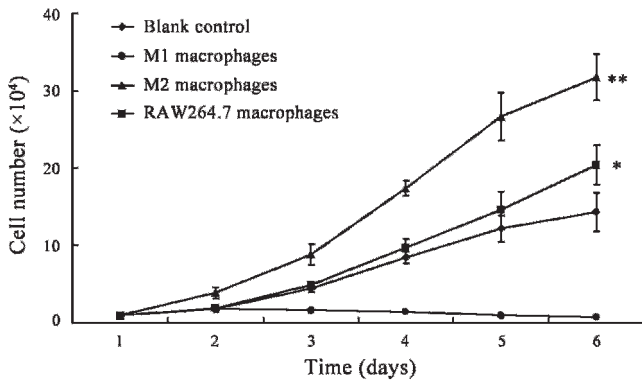
Figure 1 - Dynamic changes in LLC cell growth. Following culture of a blank control sample or co-culture with M1, M2, and RAW264.7 macrophages, the LLC cell number and viability were evaluated by trypan blue staining at the indicated times during a 6-day period. The data shown represent three experiments. * $p < 0.05$ and ** $p < 0.01$ compared to the blank control.

Statistical analysis

The measurement data are expressed as the means $\pm$ SD. Differences were compared using a one-way ANOVA analysis followed by Student's t-test. A p-value <0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

M2 macrophages induce the proliferation of LLC cells

To evaluate the effect of activated macrophages on the proliferation of LLC cells, cell viability was determined with trypan blue staining to create a growth curve. As shown in Figure 1, the addition of both M2 macrophages and RAW264.7 cells significantly increased the proliferation of LLC cells compared to the control ($p<0.05$). Furthermore, the proliferation velocity of LLC cells in co-culture with M2 macrophages was greater than that observed for the RAW264.7 co-culture group. However, the proliferation of LLC cells was inhibited following co-culture with M1 macrophages.

M2 macrophages induce migration of LLC cells

To determine the effect of activated macrophages on the migratory behavior of LLC cells, we utilized a Transwell system to evaluate cell migration. As shown in Figure 2, M2 macrophages and RAW264.7 cells significantly increased the migratory potential of LLC cells compared to the control condition ($p<0.01$), whereas M1 macrophages did not alter the migration of LLC cells. Furthermore, the migratory rate of LLC cells cultured with M2 macrophages was faster than that observed for the culture with RAW264.7 cells ($p<0.05$).

M2 macrophages increase VEGF-C expression in LLC cells

We next explored whether activated macrophages could induce the production of pro-lymphangiogenic stimuli in LLC cells, which would lead to the capillary morphogenesis of LECs. Following co-culture, the numbers of LLC cells co-cultured M2 macrophages or RAW264.7 cells were increased in comparison to the non-co-cultured group and the M1 macrophage co-culture group. In addition, changes in the morphology of LLC cells cultured with M2 macrophages or RAW264.7 cells were noted. Next, we sought to evaluate the expression of VEGF-C, a primary lymphatic vessel growth factor, using immunocytochemistry. With this technique, IOD values were used to represent the level of VEGF-C expression in LLC cells. As shown in Figure 3, the IODs for VEGF-C in the blank control, M1, M2 and RAW264.7
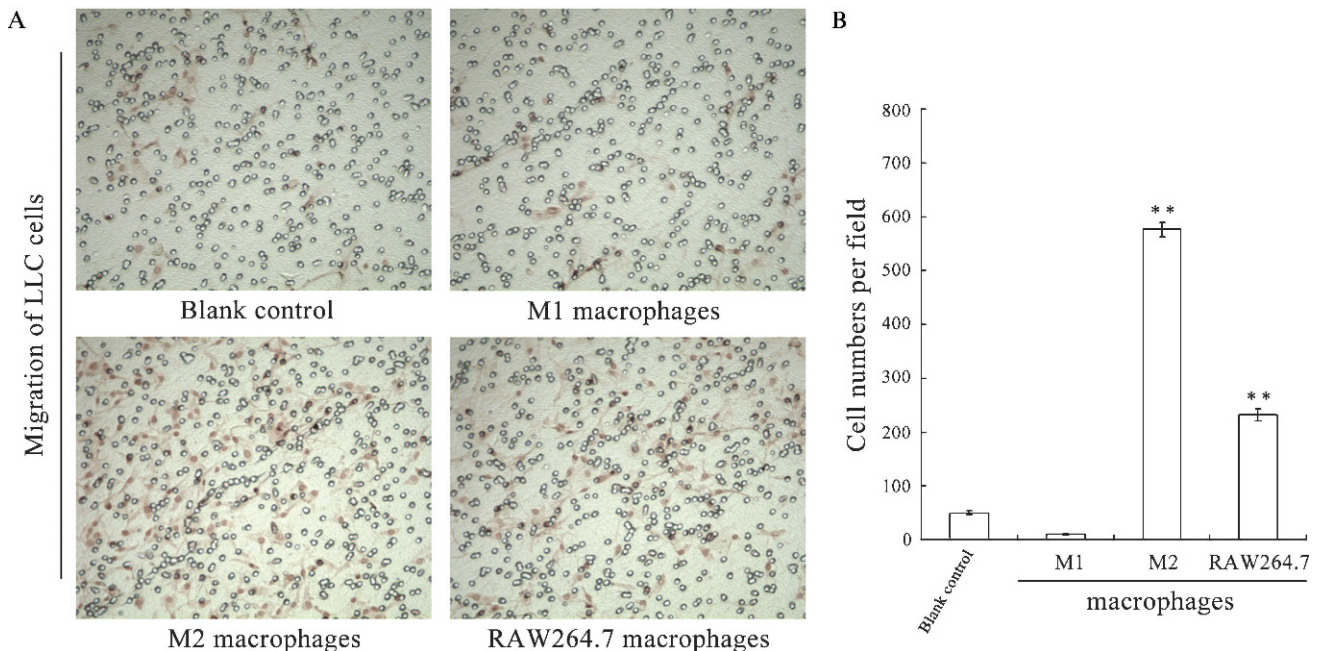

Figure 2 - Effect of activated macrophages on the migration of LLC cells. (A) Following the culture of a blank control or the addition of M1 macrophages, M2 macrophages or RAW264.7 macrophages for 24 h, invasive LLC cells on the outer surface of the upper chambers were stained with H&E (200×). (B) Comparison to the penetrated cells. The data shown represent three experiments. ** $p<0.01$ compared to the blank control.
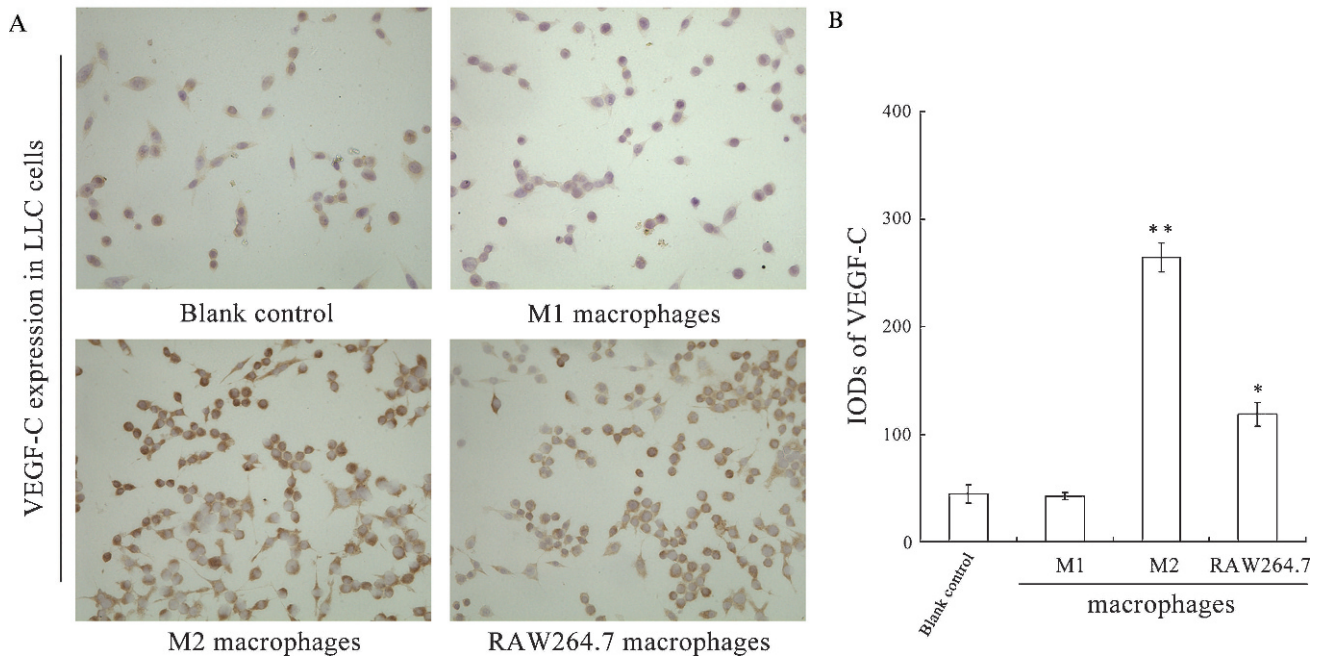
Figure 3 - VEGF-C expression by LLC cells induced following exposure to activated macrophages. (A) VEGF-C could be detected in the blank control and in the M1, M2, and RAW264.7 macrophage co-cultured groups. (B) Comparison of the IODs for VEGF-C. The data shown represent three experiments. * $p<0.05$ and ** $p<0.01$ compared to the blank control.

co-cultured groups were 44.53 ± 8.76, 42.19 ± 3.52, 264.19 ± 13.36, and 118.51 ± 10.82, respectively. In comparison to the control condition, co-culture with M2 and RAW264.7 macrophages increased the expression of VEGF-C by LLC cells ($p<0.05$); however, M2 macrophages had a more pronounced effect on the expression of VEGF-C than the RAW264.7 macrophages. No significant change in VEGF-C expression was observed in the control or M1 macrophage group.

M2 macrophages do not produce NO

We measured the accumulation of NO_2^- in culture supernatants using the Greiss assay. M1 macrophages produced relatively high levels of NO (234.12 ± 14.83 μmol/L), whereas M2 macrophages, RAW264.7 cells and the blank control culture failed to generate significant levels of NO.

M2 macrophages stimulate tumor-induced lymphangiogenesis

Using a two-dimensional Matrigel-based assay, we next evaluated the differentiation of LECs into capillary-like structures. As shown in Figure 4 after 24 h of culture, the network of tube-like structures in the M2 macrophage and the RAW264.7 co-culture groups was more extensive than that observed in the M1 macrophage or control group ($p<0.05$), and there were no significant differences between the two former and latter culture conditions.

DISCUSSION

Our data demonstrate that M2-polarized macrophages induced metastatic behavior in lung cancer cells by stimulating their proliferation and migration. In the past decade, various studies have suggested that TAMs adopt the M2-polarized phenotype (15-17); however, more recent reports have described the polarization of activated TAMs (i.e., M1 and M2) within the tumor microenvironment (18-21). Our

previous study demonstrated that M2-polarized TAMs in lung adenocarcinomas were associated with peritumoral lymphangiogenesis and a poor prognosis (22). Alternatively, in mouse Lewis lung adenocarcinoma, activated TAMs were shown to induce peritumoral lymphangiogenesis via the up-regulation of VEGF-C expression and the promotion of lymphangiogenesis-related behaviors in LECs (12). Moreover, recent studies have revealed that macrophages could enhance the invasiveness of cancer cells both *in vitro* and *in vivo* (23-26). It has also been shown that the presence of macrophages within tumors can be associated with a histologically more malignant phenotype that is characterized by an extensive stromal reaction, disorganized matrix deposition and neovascularization (27). Craig et al. (28) also found that the presence of macrophages increased tumor microvascular density *in vivo* and enhanced the rate of tumor growth. In support of these findings, the current study demonstrated that M2-polarized macrophages, but not M1 macrophages, could increase the proliferation and invasion of LLC cells. Moreover, following co-culture, non-activated RAW264.7 macrophages were polarized towards the M2 phenotype and LLC cells exhibited higher proliferative rates and a greater invasive potential. In a previous report, Hagemann et al. (18) found that ovarian cancer cells were able to polarize co-cultured macrophages towards a tumor-associated phenotype, which resulted in a TAM activation pattern similar to that found in ovarian tumors *in vivo*. Hence, our results suggested that RAW264.7 macrophages and LLC cells could mutually alter each other's behaviors.

Furthermore, our data indicated that M2-polarized macrophages could induce the expression of VEGF-C in LLC cells, although they lost the ability to produce NO. In a previous report, Chen et al. (29) confirmed that the expression of specific genes, such as IL-6, IL-8, VEGF, VEGF-C, matrix metalloproteinase (MMP)-9 and MMP-1, was upregulated in non-small cell lung cancer following co-culture with macrophages. In another study, Jedinak et al. (24) demonstrated that activated
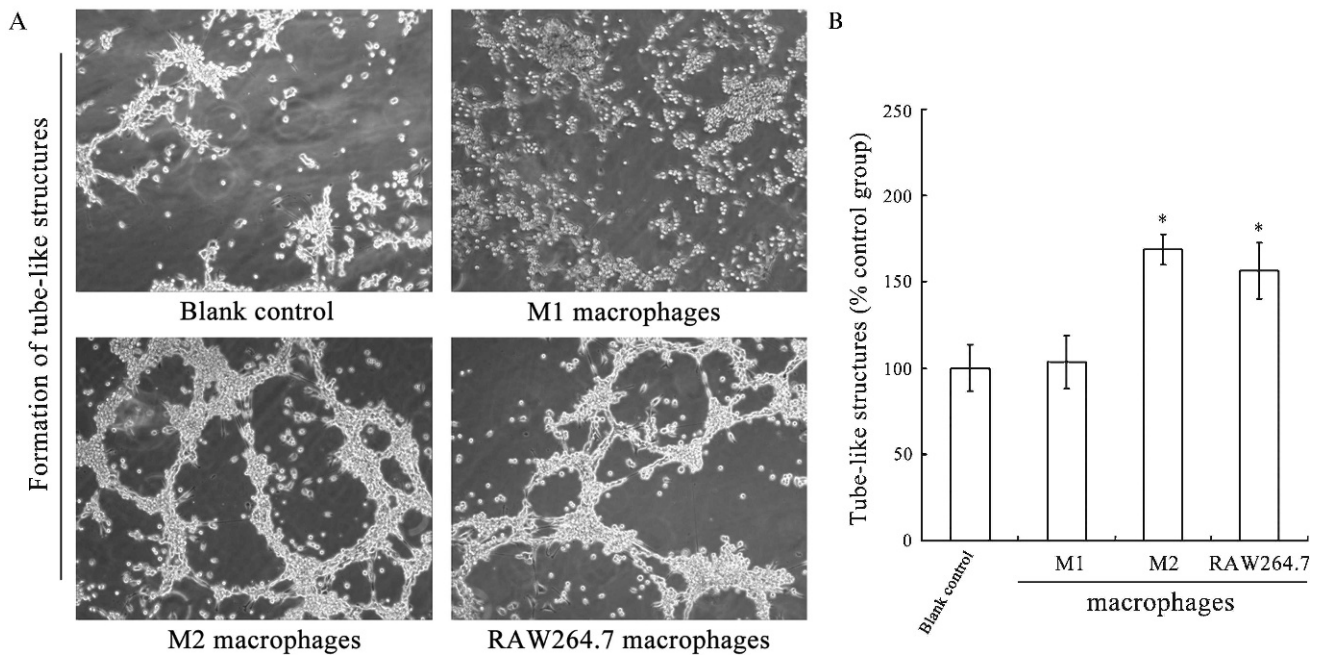
Figure 4 - Tumor-induced lymphangiogenesis stimulated by activated macrophages. (A) LECs were seeded in a 24-well plate pre-coated with Matrigel and maintained with media collected from LLC cells treated with activated macrophage-conditioned medium for 24 h. Capillary-like structures in the absence or presence of M1, M2 or RAW264.7 macrophages were determined 24 h later (200× magnification). (B) Comparison of the tube-like structures formed by LECs following exposure to macrophages with different activated phenotypes. The data shown represent three experiments. * $p<0.05$ compared to the blank control.

macrophage-conditioned medium markedly induced the proliferation and migration of human HCT116 colon cancer cells and increased the activation of NF-κB and the secretion of VEGF from colon cancer cells, which subsequently induced the capillary morphogenesis of human aortic endothelial cells. In this study, we demonstrated that both M2 macrophages and RAW 264.7 cells could increase the expression of VEGF-C by LLC cells, which may contribute to the changes observed related to their metastatic behavior. Moreover, in addition to VEGF-C, other factors secreted by LLC cells or M2 macrophages were also likely responsible for the observed behavioral changes of LLC cells, although these factors were not specifically examined in the present study. Furthermore, the high NO levels detected in the M1 macrophage and LLC cell co-culture supernatant were likely responsible for the inhibition of the metastatic behavior of LLC cells.

In addition, our data showed that M2-polarized macrophages stimulated tumor-induced lymphangiogenesis in part via the induction of VEGF-C expression. Lymphangiogenesis is considered the initial step and a necessary event in lymphatic and regional lymph node metastasis (30,31). Similar to angiogenesis, lymphangiogenesis consists of a complex multistep process involving endothelial proliferation, migration, and tube-like formation. In addition, these processes can be triggered by the binding of lymphangiogenic growth factors, such as VEGF-C and VEGF-D, to their receptors (including VEGFR-2 and VEGFR-3), which leads to lymphatic spread (32,33). Studies have also shown that macrophages support lymphangiogenesis in two ways: by transdifferentiation and direct incorporation into the endothelial layer or by stimulating the division of preexisting local LECs (34,35). Schoppmann et al. (36,37) reported that the peritumoral inflammatory reaction and VEGF-C-expressing

TAMs increase tumor lymphangiogenesis and lymphovascular invasion in cervical cancer and invasive breast cancer. In the current study, we demonstrated that following interaction with M2 macrophages or RAW 264.7 cells, LLC cells expressed higher levels of VEGF-C, and this may have led to increased tumor-induced lymphangiogenesis.

In conclusion, the interaction between M2 macrophages and lung adenocarcinoma cells induced the metastatic behavior of Lewis lung adenocarcinoma. Activated M2 macrophages have been shown to be involved in tumor progression and metastasis, which implies that they have the potential to become new targets for tumor therapy (38). Thus, the modulation of macrophage phenotype and function via the administration of immunoregulatory stimuli may provide therapeutic anti-cancer strategies. However, further study is necessary to investigate the detailed mechanisms of these interactions and to evaluate whether similar mechanisms are applicable *in vivo*.

AUTHOR CONTRIBUTIONS

Zhang B drafted the first manuscript and performed the experiments. Zhang Y performed the experiments, analyzed the data, prepared the

figures, and performed the statistical analysis. Yao G, Gao J, Yang B, Zhao Y, Rao Z and Gao J were responsible for the experiments. Zhang B and Zhang Y contributed equally to this work.

REFERENCES

1. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. Cancer Res. 2006;66(2):605-12, http://dx.doi.org/10.1158/0008-5472.CAN-05-4005.
2. Porta C, Subhra Kumar B, Larghi P, Rubino L, Mancino A, et al. Tumor promotion by tumor-associated macrophages. Adv Exp Med Biol. 2007;604:67-86, http://dx.doi.org/10.1007/978-0-387-69116-9_5.
3. Mantovani A. La mala educación of tumor-associated macrophages: diverse pathways and new players. Cancer Cell. 2010;17(2):111-2, http://dx.doi.org/10.1016/j.ccr.2010.01.019.
4. Ryder M, Ghossein RA, Ricarte-Filho JC, Knauf JA, Fagin JA. Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. Endocr Relat Cancer. 2008;15(4):1069-74, http://dx.doi.org/10.1677/ERC-08-0036.
5. Lee CH, Espinosa I, Vrijaldenhoven S, Subramanian S, Montgomery KD, Zhu S, et al. Prognostic significance of macrophage infiltration in leiomyosarcomas. Clin Cancer Res. 2008;14(5):1423-30, http://dx.doi.org/10.1158/1078-0432.CCR-07-1712.
6. Shieh YS, Hung YJ, Hsieh CB, Chen JS, Chou KC, Liu SY. Tumor-associated macrophage correlated with angiogenesis and progression of mucoepidermoid carcinoma of salivary glands. Ann Surg Oncol. 2009;16(3):751-60, http://dx.doi.org/10.1245/s10434-008-0259-6.
7. Zhang BC, Gao J, Wang J, Rao ZG, Wang BC, Gao JF. Tumor-associated macrophages infiltration is associated with peritumoral lymphangiogenesis and poor prognosis in lung adneocarcinoma. Med Oncol. 2011;28(4):1447-52, http://dx.doi.org/10.1007/s12032-010-9638-5.
8. Mosser DM. The many faces of macrophage activation. J Leukoc Biol. 2003;73(2):209-12, http://dx.doi.org/10.1189/jlb.0602325.
9. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol. 2006;80(6):1298-307, http://dx.doi.org/10.1189/jlb.0406249.
10. Raes G, Brys L, Dahal BK, Brandt J, Grooten J, Brombacher F, et al. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. J Leukoc Biol. 2005;77(3):321-7.
11. Wang J, Zhang B, Guo Y, Li G, Xie Q, Zhu B, et al. Artemisinin inhibits tumor lymphangiogenesis by suppression of vascular endothelial growth factor C. Pharmacology. 2008;82(2):148-55, http://dx.doi.org/10.1159/000148261.
12. Zhang B, Wang J, Gao J, Guo Y, Chen X, Wang B, et al. Alternatively activated RAW264.7 macrophages enhance tumor lymphangiogenesis in mouse lung adenocarcinoma. J Cell Biochem. 2009;107(1):134-43, http://dx.doi.org/10.1002/jcb.22110.
13. Jiang J, Grieb B, Thyagarajan A, Sliva D. Ganoderic acids suppress growth and invasive behavior of breast cancer cells by modulating AP-1 and NF-kappaB signaling. Int J Mol Med. 2008;21(5):577-84.
14. Wang J, Guo Y, Zhang BC, Chen ZT, Gao JF. Induction of apoptosis and inhibition of cell migration and tube-like formation by dihydroartemisinin in murine lymphatic endothelial cells. Pharmacology. 2007;80(4):207-18, http://dx.doi.org/10.1159/000104418.
15. Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer. 2004;40(11):1660-7, http://dx.doi.org/10.1016/j.ejca.2004.03.016.
16. Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006;42(6):717-27, http://dx.doi.org/10.1016/j.ejca.2006.01.003.
17. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci. 2008;13:453-61, http://dx.doi.org/10.2741/2692.
18. Hagemann T, Wilson J, Burke F, Kulbe H, Li NF, Plüddemann A, et al. Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. J Immunol. 2006;176(8):5023-32.
19. Kawamura K, Komohara Y, Takaishi K, Katabuchi H, Takeya M. Detection of M2 macrophages and colony-stimulating factor 1 expression in serous and mucinous ovarian epithelial tumors. Pathol Int. 2009;59(5):300-5, http://dx.doi.org/10.1111/j.1440-1827.2009.02369.x.
20. Komohara Y, Ohnishi K, Kuratsu J, Takeya M. Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. J Pathol. 2008;216(1):15-24, http://dx.doi.org/10.1002/path.2370.
21. Niino D, Komohara Y, Murayama T, Aoki R, Kimura Y, Hashikawa K, et al. Ratio of M2 macrophage expression is closely associated with poor prognosis for Angioimmunoblastic T-cell lymphoma (AITL). Pathol Int. 2010;60(4):278-83, http://dx.doi.org/10.1111/j.1440-1827.2010.02514.x.
22. Zhang B, Yao G, Zhang Y, Gao J, Yang B, Rao Z, et al. M2-polarized tumor-associated macrophages are associated with poor prognosis due to accelerated lymphangiogenesis in lung adenocarcinoma. Clinics. 2011;66(11):1879-86, http://dx.doi.org/10.1590/S1807-59322011001100006.
23. Hagemann T, Wilson J, Kulbe H, Li NF, Leinster DA, Charles K, et al. Macrophages induce invasiveness of epithelial cancer cells via NF-kB and JNK. J Immunol. 2005;175(2):1197-205.
24. Jedinak A, Dudhgaonkar S, Sliva D. Activated macrophages induce metastatic behavior of colon cancer cells. Immunobiology. 2010;215(3):242-9, http://dx.doi.org/10.1016/j.imbio.2009.03.004.
25. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res. 2004;64(19):7022-9, http://dx.doi.org/10.1158/0008-5472.CAN-04-1449.
26. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006;124(2):263-6, http://dx.doi.org/10.1016/j.cell.2006.01.007.
27. Oosterling SJ, van der Bij GJ, Meijer GA, Tuk CW, van Garderen E, van Rooijen N, et al. Macrophages direct tumour histology and clinical outcome in a colon cancer model. J Pathol. 2005;207(2):147-55, http://dx.doi.org/10.1002/path.1830.
28. Craig M, Ying C, Loberg RD. Co-inoculation of prostate cancer cells with U937 enhances tumor growth and angiogenesis in vivo. J Cell Biochem. 2008;103(1):1-8, http://dx.doi.org/10.1002/jcb.21379.
29. Chen JJ, Lin YC, Yao PL, Yuan A, Chen HY, Shun CT, et al. Tumor-associated macrophages: the double-edged sword in cancer progression. J Clin Oncol. 2005;23(5):953-64.
30. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. Nature. 2005;438(7070):946-53, http://dx.doi.org/10.1038/nature04480.
31. Stacker SA, Farnsworth RH, Karnezis T, Shayan R, Smith DP, Paavonen K, et al. Molecular pathways for lymphangiogenesis and their role in human disease. Novartis Found Symp. 2007;281:38-43; discussion 44-53, 208-9.
32. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, et al. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J. 2001;20(4):672-82, http://dx.doi.org/10.1093/emboj/20.4.672.
33. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, et al. Vascular endothelial growth factor-D promotes the metastatic spread of cancer via the lymphatics. Nature Med. 2001;7(2):186-91.
34. Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest. 2005;115(9):2363-72, http://dx.doi.org/10.1172/JCI23874.
35. Kerjaschki D. The crucial role of macrophages in lymphangiogenesis. J Clin Invest. 2005;115(9):2316-9, http://dx.doi.org/10.1172/JCI26354.
36. Schoppmann SF, Birner P, Stöckl J, Kalt R, Ullrich R, Caucig C, et al. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am J Pathol. 2002;161(3):947-56, http://dx.doi.org/10.1016/S0002-9440(10)64255-1.
37. Schoppmann SF, Fenzl A, Nagy K, Unger S, Bayer G, Geleff S, et al. VEGF-C expressing tumor-associated macrophages in lymph node positive breast cancer: Impact on lymphangiogenesis and survival. Surgery. 2006;139(6):839-46, http://dx.doi.org/10.1016/j.surg.2005.12.008.
38. Colombo MP, Mantovani A. Targeting myelomonocytic cells to revert inflammation-dependent cancer promotion. Cancer Res. 2005;65(20):9113-6, http://dx.doi.org/10.1158/0008-5472.CAN-05-2714.