Received: 25 February 2019 | Revised: 2 June 2019 | Accepted: 4 June 2019

DOI: 10.1111/cas.14091

ORIGINAL ARTICLE

Cancer Science WILEY

Vascular endothelial growth factor (VEGF) impairs the motility and immune function of human mature dendritic cells through the VEGF receptor 2-RhoA-cofilin1 pathway

Jinhua Long[1,2,3] | Zuquan Hu[1,2,4] | Hui Xue[2] | Yun Wang[1,2] | Jin Chen[1,2] | Fuzhou Tang[2] | Jing Zhou[2] | Lina Liu[2] | Wei Qiu[2] | Shichao Zhang[2] | Yan Ouyang[2] | Yuannong Ye[2] | Guoqiang Xu[1] | Long Li[5] | Zhu Zeng[1,2,4]

[1]Department of Immunology, School of Basic Medical Sciences, Guizhou Medical University, Guiyang, China

[2]Immune Cells and Antibody Engineering Research Center of Guizhou Province, Key Laboratory of Biology and Medical Engineering, School of Biology and Engineering, Guizhou Medical University, Guiyang, China

[3]Department of Head and Neck, Affiliated Tumor Hospital, Guizhou Medical University, Guiyang, China

[4]Key Laboratory of Environmental Pollution Monitoring and Disease Control, Ministry of Education, Guizhou Medical University, Guiyang, China

[5]Department of Nephrology, The Third Affiliated Hospital of Guizhou Medical University, Duyun, China

Correspondence
Zhu Zeng, School of Basic Medical Sciences/ School of Biology and Engineering, Guizhou Medical University, Guiyang, China.
Email: zengzhu@gmc.edu.cn

Long Li, Department of Nephrology, The Third Affiliated Hospital of Guizhou Medical University, Duyun, China.
Email: gzyxyll@medmail.com.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 31771014, 31660258, 31860262 and 11762006; China Postdoctoral Science Foundation, Grant/Award Number: 2015M582747XB; Key Project of the Ministry of Education of China, Grant/Award Number: 210196; Science and Technology Foundation of Guizhou Province, Grant/Award Number: 2018-1412, 2019-1266, 2019-2787, 2016-5676 and 2017-5718; Science and Technology Innovative Talent Team of Guizhou Province, Grant/Award Number: 2015-4021; 2011 Collaborative Innovation Program of Guizhou Province, Grant/Award Number: 2015-04; Cell and Gene Engineering Innovative Research Groups of Guizhou Province, Grant/Award Number: KY-2016-031

Abstract

Dendritic cells (DCs) are potent and specialized antigen presenting cells, which play a crucial role in initiating and amplifying both the innate and adaptive immune responses against cancer. Tumor cells can escape from immune attack by secreting suppressive cytokines that solely or cooperatively impair the immune function of DCs. However, the underlying mechanisms are not fully defined. Vascular endothelial growth factor (VEGF) has been identified as a major cytokine in the tumor microenvironment. To elucidate the effects of VEGF on the motility and immune function of mature DCs (mDCs), the cells were treated with 50 ng/mL VEGF and investigated by proteomics and molecular biological technologies. The results showed that VEGF can impair the migration capacity and immune function of mDCs through the RhoA-cofilin1 pathway mediated by the VEGF receptor 2, suggesting impaired motility of mDCs by VEGF is one of the aspects of immune escape mechanisms of tumors. It is clinically important to understand the biological behavior of DCs and the immune escape mechanisms of tumor as well as how to improve the efficiency of antitumor therapy based on DCs.

KEYWORDS
immune function, mature dendritic cell, motility, signaling pathway, vascular endothelial growth factor

Abbreviations: BA, blocking Ab; BP, blocking peptide; CCR, C-C chemokine receptor; COF1, cofilin1; DC, dendritic cell; F-actin, filamentary actin; G-actin, globular actin; imDC, immature DC; ISC, immune stimulatory capability; mDC, mature DC; MLR, mixed leukocyte reaction; NF-κB, nuclear factor-κB; P-COF1, phosphorylated cofilin1; qRT-PCR, quantitative RT-PCR; TMCs, transmigration capability; TOF, time-of-flight; VEGFR, VEGF receptor; VEGF, vascular endothelial growth factor.

Jinhua Long and Zuquan Hu equally contributed to this work.

1 | INTRODUCTION

Dendritic cells are the most potent and specialized antigen-presenting cells that play a central role in host-antitumor immunity.[1] Functionally, DCs have 2 differentiation stages: imDCs and mDCs. The imDCs are present in non-lymphoid tissues. Following the capture of antigens, they travel through blood or lymph to secondary lymphoid organs, and gradually differentiate into mDCs that upregulate the expressions of peptide-MHC complexes and accessory molecules (eg, CD11c, CD80, CD83, CD86, and CCR7 et al) on their surfaces, which are necessary for naive T-cell activation, leading to immune response or tolerance.[2,3] The motility of DCs is important for migration of imDCs in peripheral tissues and physical interaction between mDCs and naïve T cells in secondary lymph nodes.[4] Dendritic cells can conduct all the elements of the immune orchestra. Information on the interaction between mDCs and naïve T cells can be developed for novel clinical therapy against immune-related diseases.[5] Dendritic cell-based immunotherapies against cancers have achieved some promising successes, but there are still many challenges,[1,6] in which the impaired motility and immune function of mDCs by tumor microenvironment-derived suppressive cytokines, including VEGF, transformed growth factor-β_1, and interleukin-10, are not well characterized.[5,7] Villablanca et al[8] found that the tumor-derived factors can inhibit the immune function of mDCs through CCR7. Herber et al[9] reported that DCs in the tumor-bearing host upregulate the capability of triglyceride uptake through scavenger receptor A, leading to lipid accumulation in cells and impaired motility and immune function. Although our previous studies showed that the biophysical properties and motility of human mDCs deteriorated by VEGF through cytoskeleton remodelling,[10] its action target is still elusive.

Vascular endothelial growth factor is excreted by most cancer cells to stimulate the proliferation of endothelial cells and function as an angiogenic factor in vivo.[11] The infiltration and density of DCs are directly correlated with positive prognosis. Moreover, DC density is negatively associated with VEGF level in vivo.[12] Vascular endothelial growth factor can inhibit the functional maturation of imDCs[13,14] and impair the differentiation of DCs.[13,15] As a mAb of VEGF, bevacizumab has achieved great successes in the clinical field of tumors, but many problems still need to be solved, such as toxic side-effects, inaccurate curative effect, and inaccurate targeting.[16,17] Several groups have established that the blockade of the VEGF signaling pathway by various strategies can partially recover the function of DCs in vivo and in vitro.[18] The VEGF receptor-1 and VEGFR2 play different roles in DC differentiation.[19] Mimura et al[20] confirmed that VEGF inhibits the function of human mDCs mediated by VEGFR2. The impairment of DC differentiation and maturation by VEGF is an important event when the tumor burden alters the function of DCs, but the underlying molecular targets are not well understood. Therefore, VEGF-associated suppressive effects on DCs are critical to DC-based immunotherapy against cancer. In order to investigate the effects of VEGF on the motility and immune function of mDCs, mDCs treated with VEGF were studied by proteomic and molecular biological technologies. The results showed that VEGF can impair the motility and immune function of mDCs through the RhoA-COF1 pathway through VEGFR2, indicating that the impairment of mDC motility by VEGF is one of the immune escape mechanisms of tumors. Clinically, this result provides the clue for the key importance of blocking the VEGF signaling pathway in the cancer microenvironment with the aim of improving the effectiveness of DC-based immunotherapy against cancer.

2 | MATERIALS AND METHODS

2.1 | Isolation of monocytes and generation of DCs

Dendritic cells were generated from fresh PBMCs of healthy human subjects as described by Steinman[21] with minor modifications according to our previous protocol.[10] All donors gave informed consent and the study protocol was approved by the ethics committee of Guizhou Medical University (Guiyang, China). Ficoll-Paque gradient centrifugation was deployed to enrich $CD14^+$ monocytes and cocktail immunomagnetic beads (Dynal, ThermoFisher Scientific) was used for further purification. The PBMCs were cultured in RPMI-1640 medium supplemented with 15% FBS (Gibco), 1% penicillin/streptomycin, 150 ng/mL recombinant human granulocyte-macrophage colony stimulating factor, and 100 ng/mL recombinant human interleukin-4 (Peprotech). On the seventh day, recombinant human tumor necrosis factor-α (Peprotech) was added to the final concentration of 10 ng/mL. After 72 h culture, the phenotypes of DCs were analyzed using flow cytometer (BD FACScan) by staining the cell surface markers of CD11c, CD40, CD80, CD83, CD86, CCR7, and HLA-DR. Trypan blue dye was used to measure cell viability.

2.2 | Culture of HUVECs and T cells

Human umbilical vein endothelial cells were cultured in terms of our previous protocol.[22-25] T cells were separated from the PBMCs by RosetteSep human T cell enrichment cocktail (StemCell) in accordance with the manufacturer's recommendations.

2.3 | Treatments of mDCs

Mature DCs were harvested and treated with or without 50 ng/mL recombinant human VEGF-165 (rhVEGF-165; R&D Systems) for 24 hours at 37°C according to the procedures described by Mimura et al.[20] The working concentrations were determined based on previous reports[9,10,15,22] and our preliminary experiments. As an inhibitor for RhoA signaling and BP for P-COF1, 10 μmol/L Y27632 (Sigma) and 1 μg/mL Ser3-P-COF1 BP (ECM Biosciences), as well as the BAs of goat anti-human VEGFR1 mAb (10 μg/mL) and mouse anti-human VEGFR2 mAb (50 ng/mL),[20] were respectively applied to treat mDCs for 30 minutes

TABLE 1 Gene-specific primers in quantitative RT-PCR

Names of gene	Primers	PCR product (bp)	Tm (°C)
18s RNA			
Forward	5′-aaggtgaaggtcggagtcaagg -3′	300	56
Reverse	5′-tgctaagcagttggtggtgcag -3′		
Cofilin1			
Forward	5′-ctttgtgagcccccttctgg -3′	200	55
Reverse	5′-atcaaaagcagtttgggaagg -3′		

Tm, melting temperature.

before administration of rhVEGF-165. Untreated mDCs and mDCs treated with 50 ng/mL mouse anti-human IgG (Sigma) served as blank and negative controls, respectively.

2.4 | Analyses of 2-D gel-based proteomics

To minimize individual differences and obtain sufficient quantity of proteins for 2-D gel, the protein extracts of 7 preparations of mDCs from seven healthy individuals were combined after cell characterization to form a pool of protein extracts. The 2-D gels were obtained according to our previous protocol.[25] The resulting peptides from 2-D gel were extracted with TFA/acetonitrile/water, and the peptide mixtures were analyzed by MALDI TOF (Shimadzu) using α-cyano-4-hydroxy-cinnamic acid as a matrix on a plate with delayed extraction or Q-TOF Ultima Global (Waters). Identification of the proteins using these mass fingerprinting data was undertaken using the Mascot software (Matrix Science).

2.5 | Measurements of mRNA levels by qRT-PCR

Total RNA was reverse-transcribed using the Omniscript RT Kit (Qiagen). Quantitative RT-PCR was carried out by using a Real Master Mix (SYBR Green) kit (Tiangen). Genes, including the control gene 18S RNA, were amplified in parallel in a DNA Engine Opticon continuous fluorescence detection system (MJ Research) in terms of Table 1. The mRNA expression levels were calculated from the cycle threshold value and normalized to that of 18S RNA.

2.6 | Measurements of protein expression levels by western blot analysis

Mature DCs were lysed and electrophoresed on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 5% BSA in 0.1% Tween-20 in PBS, the membranes were probed with primary Abs. Anti-COF1 (Sigma), anti-P-COF1 (Sigma), anti-human VEGFR1/Flt-1 (R&D Systems), and anti-human VEGFR2/KDR (R&D Systems) Abs were diluted in blocking buffer and reacted with the blots overnight at 4°C. The bound primary Abs were probed by a 1:2000 diluted HRP-conjugated IgG Ab and visualized by the ECL system (Amersham). The gray values of proteins were measured by ImageJ (version 1.45, National Institutes of Health). The expression levels of proteins were normalized to those of the corresponding total proteins.

2.7 | Measurements of Rho GTPase activity by pull-down assay

Affinity purification assays of CDC42, RhoA, and Rac activation are based on the fact that these proteins act as molecular switches, cycling between inactive GDP-bound and active GTP-bound states.[26] The expression levels of CDC42, RhoA, and Rac were measured by the CDC42/RhoA/Rac activation assay kits (Upstate Biotechnology) according to the manufacturer's protocols. The secondary Abs were HRP-conjugated IgG Ab. The immunoblot was processed and treated with chemiluminescent reagents (Pierce), and the bands were visualized in the ECL system.

2.8 | Measurement of TMCs in Transwell chambers

Human umbilical vein endothelial cell monolayers were seeded onto the upper compartment surface of the microporous membrane of a Transwell chamber (5 μm pores; Corning). Cells (10^6) were added onto the HUVEC monolayers and incubated at 37°C for 12 hours. As the chemokine for mDCs, 0.6 μg/mL CCL21 (R&D Systems) was added to the lower compartment. Cells collected from the lower compartment were counted with FACScan. The ratio of the cell counts in the lower compartment to that added to the upper compartment was calculated to represent the cell TMCs.

2.9 | Mixed leukocyte reaction assay

The immune stimulatory capabilities of mDCs were determined by the primary allogeneic MLR assay as described in our previous protocol,[22-25] using human T lymphocytes as responder cells.

2.10 | Localizations of P-COF1 and COF1 by immunofluorescence

Cells were collected and fixed as described.[22] Mature DCs were then incubated with 1 μg/mL goat anti-human COF1 Ab labelled by Alexa Fluor-488 and rabbit anti-human P-COF1 (Ser3) Ab labelled by Alexa Fluor-350. Then the F-actin was stained using Alexa Fluor-594 phalloidin. Subsequently, 5 μL methanolic stock solution was diluted into

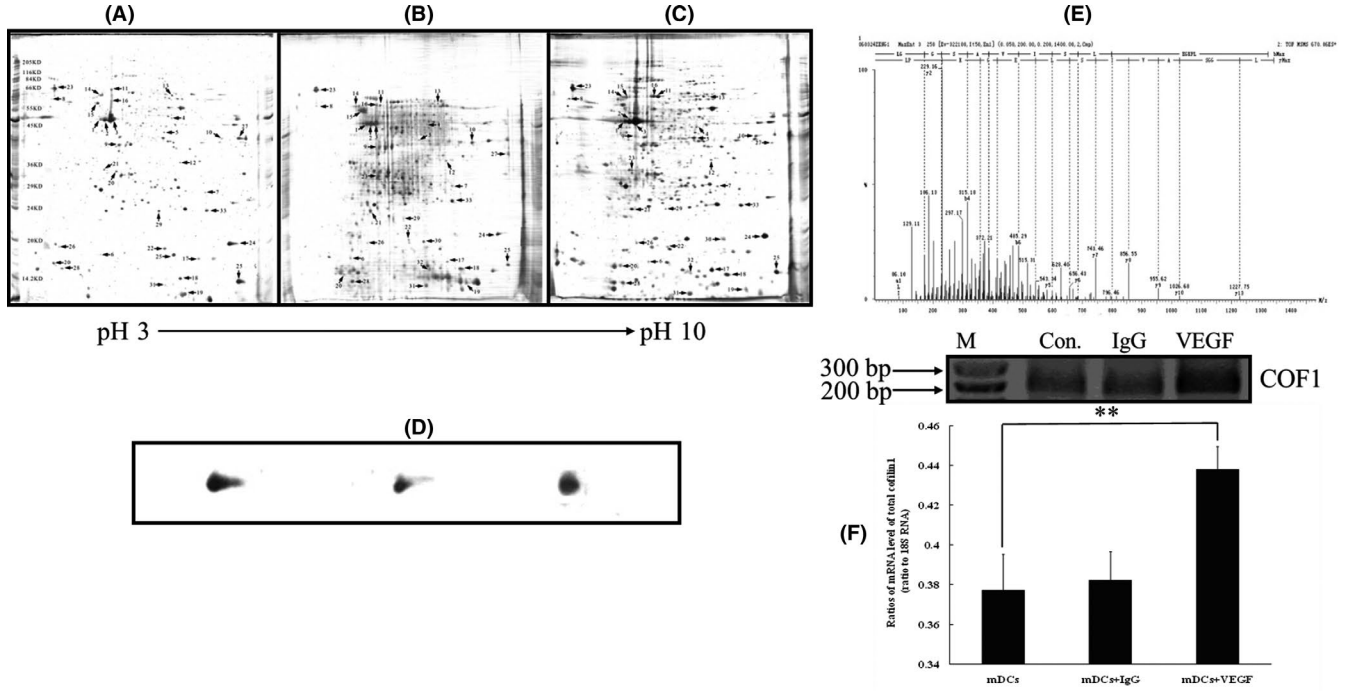

FIGURE 1 Silver-stained representative images of 2-D gel-based proteomics and validation by quantitative RT-PCR. A-C, The soluble proteins separated by 2-D were extracted from 5×10^6 mature dendritic cells (mDCs) alone (A), in the presence of 50 ng/mL IgG (B), and in the presence of 50 ng/mL recombinant human vascular endothelial growth factor (rhVEGF)-165 (C). A total of 33 protein spots of interest were numbered (arrows); they were successfully identified by MALDI TOF (Shimadzu) or Q-TOF Ultima Global (Waters). These results are representative of 3 independent experiments. D, No. 24 protein spot was enlarged and identified as cofilin1 (COF1). E, Peptide mass fingerprinting of COF1. *X*-axis indicates the mass-to-charge ratio (m/z), *Y*-axis indicates the ionic strength. F, Gene expression levels of *COF1* were calculated from the cycle threshold value and normalized to that of 18S RNA. Representative *COF1* mRNA in mDCs image stained with ethidium bromide (mean ± SD, n = 3), $^{**}P < .01$. Con, control; M, DNA molecular weight standards

200 μL PBS solution for each coverslip for staining, and the stained solution was placed on the coverslip for 20 minutes at room temperature. The coverslips were washed 3 times with PBS and then mounted on a glass slide. One drop of 3% agarose was added to the resulting cells. The coverslips were imaged by confocal laser scanning microscopy (Leica), and 3-D images were reconstructed.

3 | RESULTS

3.1 | Analyses of proteomics and RT-PCR validation

The effects of VEGF on mDCs were investigated by 2-D-based proteomics, with mDCs + IgG as control (Figure 1A-C). Numerous changes in the relative abundance of a particular protein were reproducibly detected. From the spots differentially expressed between control mDCs and VEGF-treated mDCs, 33 protein spots were successfully identified (Table 2), in which No. 24 protein was confirmed as COF1 by Q-TOF Ultima Global (Figure 1E), a cytoskeleton-binding protein that showed interesting expression changes. As shown in Figure 1D, the "lost" tail appeared in the No. 24 protein spot of mDCs after treatment with VEGF. The spots of the same protein with different pI could be related to phosphorylation or acetylation, and were visualized in 2-D as a "train of spots". [27] Therefore, it could be inferred that the COF1 in mDCs treated with VEGF has an abnormal phosphorylation modification. As shown in Figure 2B, the ratios of P-COF1 to total COF1 were increased ($P < .01$), suggesting that the P-COF1 levels in mDCs were upregulated by VEGF.

3.2 | Expression levels of Rho GTPase

The upstream signaling of COF1 is associated with members of the Rho GTPase family, including Rac, CDC42, and RhoA.[28] The mDCs under different conditioned media expressed VEGFR1 and VEGFR2 (data not shown). The expression levels of Rac, CDC42, and RhoA were analyzed by pull-down assay and western blotting. The results showed that VEGF caused an increase in RhoA, but no changes in Rac or CDC42 (data not shown). To investigate the role of RhoA in VEGF signaling, mDCs were treated with Y27632, an inhibitor of Rho-associated coiled-coil containing protein kinase. As shown in Figure 2A, the expressions of RhoA-GTPase in mDCs treated with VEGF were upregulated ($P < .01$), and this effect disappeared after treatment with Y27632 plus VEGF, suggesting that RhoA is downstream of VEGF.

3.3 | Expression levels of P-COF1 and total COF1

Cofilin1 is an essential protein responsible for high turnover rates of actin filaments in vivo, which can induce a twist in the filament,

TABLE 2 Identities of differentially expressed proteins between mature dendritic cells (mDCs) and mDCs + vascular endothelial growth factor (VEGF)

Spots number	Protein name	Relative volume mDCs : (mDCs + VEGF)	M_r/pI 2-D	M_r/pI Databank	Accession number SwissProt
1	Actin, cytoplasmic	1:2	48.2/5.5	41.7/5.3	P02570
2	Actin, cytoplasmic	1:3	48.8/5.4	41.7/5.3	P02570
3	Actin, cytoplasmic	1:2	47.4/5.3	41.7/5.3	P02570
4	Actin, cytoplasmic	1:2	42.3/6.2	41.7/5.3	P02570
5	Actin, cytoplasmic	1:3	37.2/6.0	41.7/5.3	P02570
6	Actin, cytoplasmic	1:2	15.6/4.7	42.0/5.2	P02570
7	ARP2/3 22 kDa	1:2	22.1/7.8	17.1/7.3	O15511
8	Calreticulin	5:1	54.8/3.7	48.1/4.3	P27797
9	Chloride intracellular channel (RNCC)	1:2	36.8/5.7	26.9/5.1	O00299
10	F-actin capping protein β subunit	1:4	37.6/6.1	31.3/5.4	P47756
11	Glucose-regulated protein 78 kDa (Grp78)	2.5:1	75.9/4.6	72.3/5.1	P11021
12	Glutathione S-transferase P	1:3.5	31.2/6.2	23.3/5.4	P09211
13	Heat shock cognate 71 kDa protein	1:5	53.5/7.1	70.9/5.4	P11142
14	Lymphocyte-specific protein 1	3:1	61.2/4.9	31.7/4.7	P33241
15	Lymphocyte-specific protein 1	3.5:1	58.1/5.1	31.7/4.7	P33241
16	MHCI	3:1	10.2/4.5	–	P30480
17	Migration inhibitory factor-related protein	1:4	16.5/5.5	13.2/5.7	P06702
18	Migration inhibitory factor-related protein	1:5	13.6/5.4	13.2/5.7	P06702
19	Myosin light chain	1:3	14.3/3.4	16.9/4.6	P16475
20	Rho GDP-dissociation inhibitor 1	1:18	31.3/4.8	23.2/5.0	P52565
21	Rho GDP-dissociation inhibitor 2	1:3	33.6/5.1	23.0/5.1	P52566
22	Rho GDP-dissociation inhibitor 2	1:7	15.7/5.2	23.0/5.1	P52566
23	Vimentin	1:1	74.9/3.9	53.7/5.1	P08670
24	Cofilin1	1:3.5	15.7/9.1	18.3/8.26	P23528
25	Profilin	2:1	10.8/9.2	14.9/8.48	P07737
26	Calmodulin	1:3	17.5/3.78	16.7/4.09	P62158
27	Thioredoxin peroxidase 2	1:3	38.5/8.8	41.2/9.1	Q63716
28	Thioredoxin	1:2	9.6/3.9	11.6/4.8	P10599
29	Superoxide dismutase [Cu-Zn]	1:5	23.9/5.8	21.9/7.6	P00441
30	Gelectin-1	1:3	21.3/6.2	14.7/5.3	P09382
31	S100A6	1:5	9.2/5.9	–	P06703
32	ACTG1 protein	-:2	7.5/4.7	18.4/5.25	BC009848
33	Vimentin	1:2	36.7/9.1	32.8/8.9	P08670

–, Not detectable.

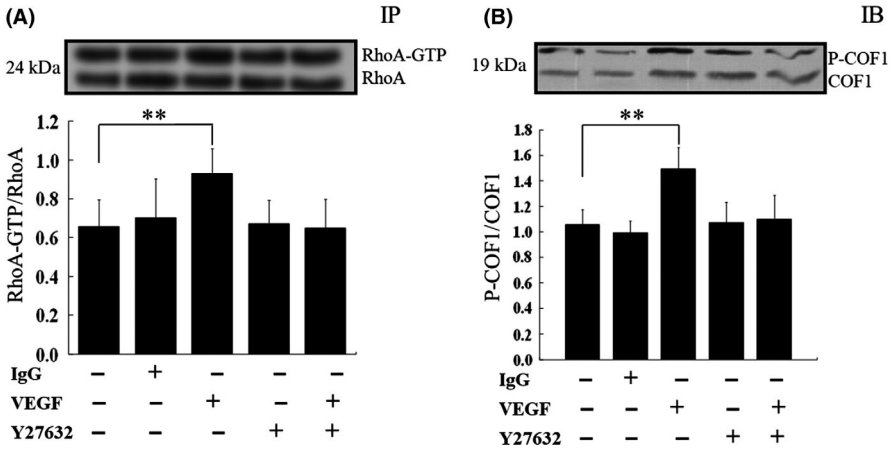

FIGURE 2 The RhoA-COF1 pathway is a downstream signal of vascular endothelial growth factor (VEGF) in mature dendritic cells (mDCs). mDCs were harvested and treated with 50 ng/mL rhVEGF-165 for 24 h. RhoA specific inhibitor Y27632 was applied to mDCs for 30 min before treatment with VEGF-165. A, RhoA is a downstream signal of VEGF in mDCs. Representative western blotting image (mean ± SD, n = 4), $^{**}P < .01$. B, Expression levels of phosphorylated (P-)COF1 in mDCs are enhanced by VEGF through RhoA signaling. Representative western blotting images (mean ± SD, n = 5), $^{**}P < .01$

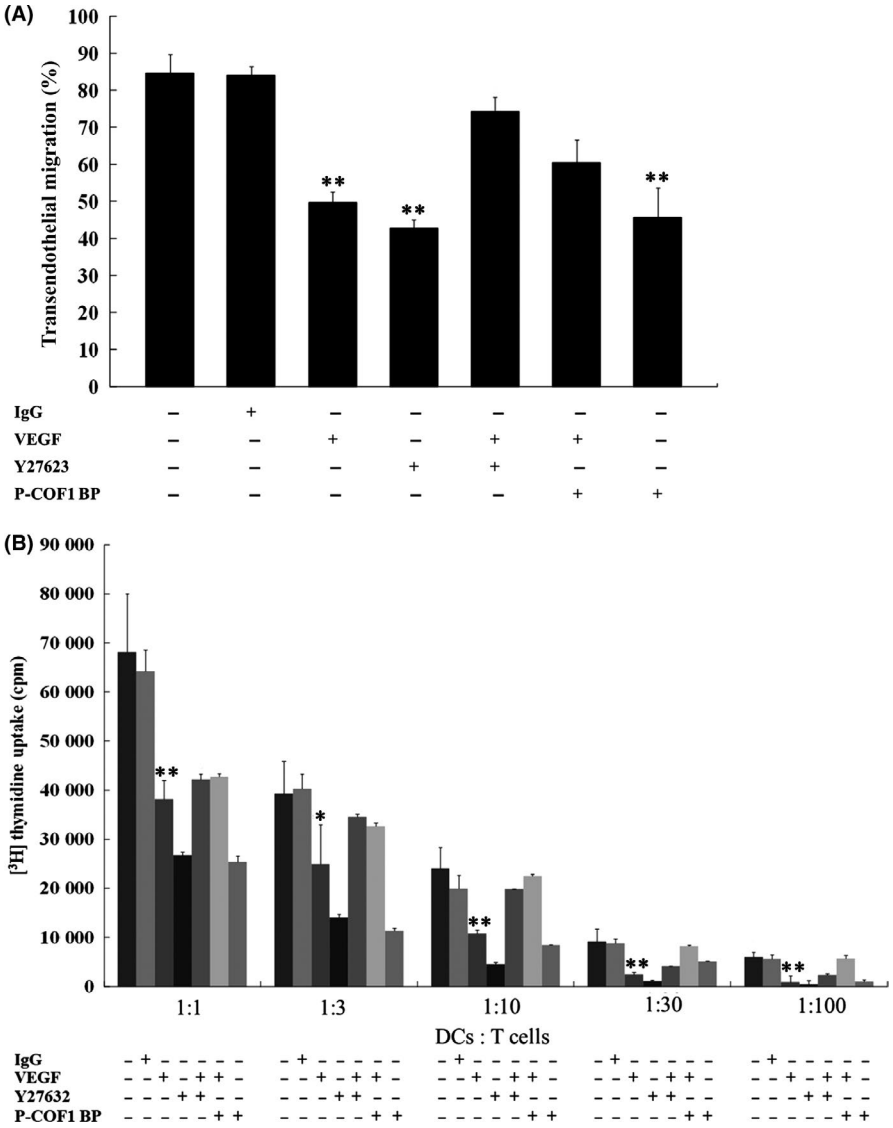

FIGURE 3 Motility and immune regulation function of mature dendritic cells (mDCs) impaired by vascular endothelial growth factor (VEGF) through RhoA-COF1 signaling. A, Transmigration capabilities of mDCs are impaired by VEGF through Rho-COF1 signaling. mDCs were treated with 50 ng/mL rhVEGF-165 for 24 h. RhoA specific inhibitor Y27632 was applied to mDCs for 30 min before treatment with VEGF-165. mDCs were treated with phosphorylated (P-)COF1 blocking peptide (BP) to assess the role of phosphorylation levels of COF1 in mDC migration. In Transwell assay, the ratio of the cell counts in the lower compartment to that added to the upper compartment represents the transendothelial migration percentage of cells, which reflects cell transmigration capabilities (TMCs) (mean ± SD, n = 4). Compared with control, $^{**}P < .01$. B, Immune stimulatory capabilities of mDCs were inhibited by VEGF through RhoA-COF1 signaling. mDCs were added in graded doses (10^3-10^5 cells/well) to T cells (1×10^5), and proliferation of T cells was measured by the uptake of ^{3}H-thymidine. Summation of results (mean ± SD, n = 4). *X*-axis (mDCs / T cells) represents the ratio of cell numbers of mDCs to T cells. Compared with control, $^{*}P < .05$, $^{**}P < .01$

accelerate the release of Pi from ADP-Pi subunits, and sever F-actin into G-actin; moreover, the severing activity is greatly reduced by phosphorylation.[29] As shown in Figure 1F, the mRNA expression level of total *COF1* was upregulated by VEGF. Measurement of the phosphorylation levels of COF1 in mDCs by western blotting (Figure 2B) showed that the expression levels of P-COF1 were upregulated by VEGF ($P < .01$), and that the level recovered to normal when pretreated with Y27632, indicating that the expression levels of P-COF1 were enhanced by VEGF through RhoA signaling.

3.4 | Transendothelial migration capabilities

The TMCs of mDCs, which are very important for their antigen presentation, primarily depend on the reorganization of F-actin cytoskeleton.[22-25] It was hypothesized that the upregulation of P-COF1 caused by VEGF could reorganize cytoskeletal structure and impair the motility of mDCs. Therefore, the cell TMCs were investigated by using the Transwell chamber. As shown in Figure 3A, the TMCs of mDCs were markedly impaired by VEGF ($P < .01$). To further investigate the role of RhoA and P-COF1 in transmigration, mDCs were treated with Y27632 and P-COF1 BP, respectively, and their TMCs were also decreased. These results support the notion that the TMCs of mDCs are impaired by VEGF and RhoA-COF1 signaling is involved in regulating the migration of mDCs.

3.5 | Immune stimulatory capabilities

The motilities of mDCs are closely related to their ISCs.[2-4] The results of MLR (Figure 3B) showed that the stimulatory capabilities of mDCs were reduced by VEGF at all ratios of mDCs to T cells from 1:1 to 1:100. Simultaneously, Y27632 or P-COF1 BP could also decrease the ISCs of mDCs, which might due to the disruption of their proper immune function. These results indicated that RhoA-COF1 signaling is an important pathway in exerting normal immune functions and could be involved in the inhibition effects of VEGF.

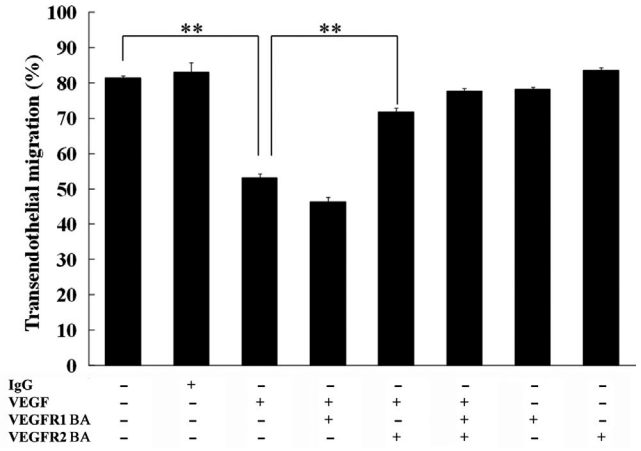

FIGURE 5 The transmigration capabilities (TMCs) of mature dendritic cells (mDCs) decreased by vascular endothelial growth factor (VEGF) through VEGF receptor 2 (VEGFR2). mDCs were treated with 50 ng/mL rhVEGF-165 for 24 h. The blocking Abs (BAs) of VEGFR1 and VEGFR2 were applied to mDCs for 30 min before treatment with VEGF-165. In Transwell assay, the ratio of the cell counts in the lower compartment to that added to the upper compartment represents the cell TMCs. Summation of results on TMCs of mDCs (mean ± SD, n = 4), $^{**}P < .01$

3.6 | Involvement of VEGF receptor in RhoA-COF1 signaling

Dendritic cells at different differentiation stages can express VEGFR1 and VEGFR2 at various levels.[19,20] To identify which VEGFR is involved in RhoA-COF1 signaling, the blocking Abs of VEGFR1 and VEGFR2 were applied to treat mDCs. The results showed that VEGFR2 blocking Ab plus VEGF, in comparison with VEGF alone, decreased the expressions of RhoA-GTPase (Figure 4A) and P-COF1 (Figure 4B) in mDCs ($P < .01$) and improved the cell TMCs (Figure 5) ($P < .01$), suggesting that the motilities of mDCs were impaired by VEGF through VEGFR2.

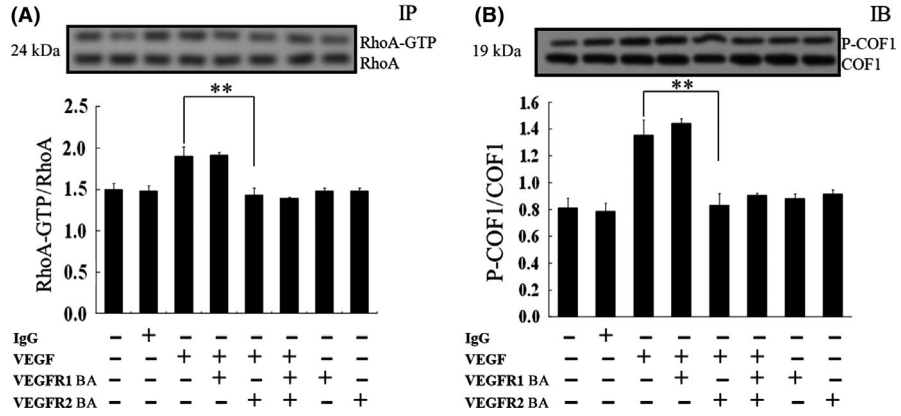

FIGURE 4 The RhoA-COF1 signaling pathway afftected by vascular endothelial growth factor (VEGF) through VEGF receptor 2 (VEGFR2). Mature dendritic cells (mDCs) were treated with 50 ng/mL rhVEGF-165 for 24 h. Blocking Abs (BAs) of VEGFR1 and VEGFR2 were applied to mDCs for 30 min before treatment with rhVEGF-165. A, VEGF affected the expression of RhoA-GTPase in mDCs through VEGFR2. Representative western blotting images (mean ± SD, n = 4), $^{**}P < .01$. B, VEGF increased the expression of phosphorylated (P-)COF1 in mDCs through VEGFR2. Representative western blotting images (mean ± SD, n = 5), $^{**}P < .01$

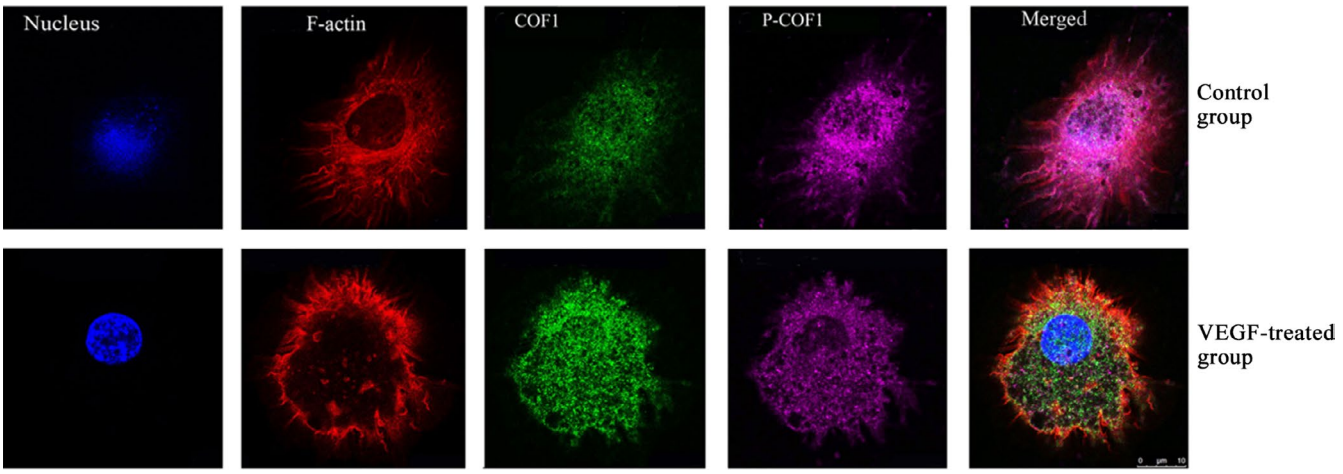

FIGURE 6 Localization of cofilin1 (COF1) and phosphorylated (P-)COF1 in mature dendritic cells (mDCs) changed by vascular endothelial growth factor (VEGF). mDCs were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 in PBS. Cells were blocked in 5% BSA/PBS and incubated with goat anti-human COF1 Ab labelled by Alexa Fluor-488 and rabbit anti-human P-COF1 (Ser3) Ab labelled by Alexa Fluor-350. Cells were stained for F-actin using Alexa Fluor-594 phalloidin. COF1 and P-COF1 were colocalized throughout the cytoplasm of mDCs and translocated to the plasma membrane of mDCs after treatment with VEGF

3.7 | Localization of P-COF1 and COF1 in mDCs

The regulatory functions of COF1 are related to their spatial distribution in the cytoplasm.[30] The localization of COF1 (green), P-COF1 (magenta), and F-actin (red) in mDCs treated with or without VEGF were identified by immunofluorescence staining and confocal laser scanning microscopy. As shown in Figure 6, COF1 and P-COF1 colocalized throughout the cytoplasm of mDCs (about 85%). Following treatment with VEGF, they translocated toward to the plasma membrane of mDCs (about 70%), indicating that the localizations of COF1 and P-COF1 in mDCs were altered by VEGF.

4 | DISCUSSION

The impairments of DCs by tumor-derived suppressive cytokines are key challenges for DC-based immunotherapies against cancers.[1-3] The defective function of DCs in tumor-bearing hosts has been confirmed by many groups.[1,5-7] Moreover, it has been established that the functional maturation and differentiation of imDCs are inhibited and vitiated by VEGF.[31] The present study focuses on the effects of VEGF on the motility and immune stimulatory capability of mDCs, as well as the potential molecular mechanism.

In order to explore the mechanism of VEGF-induced impairment of mDC function at the protein level, the protein expression profiles were investigated by mass spectrography-based proteomics technology. A total of 33 proteins with differential expression were successfully identified (Table 2), which were associated with the cytoskeleton, migration, antigen presentation, and metabolism. Among them, No. 24 protein COF1, a cytoskeleton-binding protein, attracted our attention because of its upregulated expression level and potential modification (Figures 1A-D). The results of qRT-PCR confirmed that the *COF1* gene in mDCs was also upregulated by VEGF (Figure 1F). Our previous studies found that VEGF impairs the motility and immune function of mDCs through derangement of biophysical characteristics and cytoskeleton reorganization,[10] but the potential molecular mechanisms are still elusive. Therefore, we hypothesized that the VEGF-induced abnormal expression of COF1 could affect the motility and immune function of mDCs.

Cofilin1, a family of related proteins with similar biochemical activities called the actin depolymerizing factor/COF family,[29,30] are ubiquitous among eukaryotes and essential proteins responsible for high turnover rates of actin filaments in vivo, which can increase both the number of free barbed ends for polymerization and the rate of actin depolymerization (hence replenishing G-actin in the cell).[32] Cofilin can induce a twist in the filament, accelerate the release of Pi from ADP-Pi subunits, and sever actin filaments into G-actin. Their severing activity is greatly reduced by phosphorylation of upstream signaling molecules, including Rho GTPase.[29,33] Therefore, we investigated the expression changes of Rho GTPase, including RhoA, Rac, and CDC42, by pull-down assay and western blotting. As shown in Figure 2A, the levels of RhoA-GTPase were upregulated by VEGF, and this change was abrogated by pretreatment with Y27632. These results indicated that the levels of RhoA GTPase in mDCs were regulated by VEGF. Vascular endothelial growth factor did not cause any change in Rac and CDC42 (data not shown). To explore whether the phosphorylation levels of COF1 were regulated by VEGF through RhoA signaling, the expression levels of P-COF1 and total COF1 were measured. The results (Figure 2B) showed that the phosphorylation levels of COF1 in mDCs were upregulated by VEGF, confirming the existence of the VEGF-RhoA-COF1 signaling pathway in mDCs. Verdijk et al[34] found that cofilin is dephosphorylated during DC maturation. Therefore, the elevated phosphorylation levels of COF1 in DCs induced by

VEGF could lead to the impaired motility and immune function of mDCs. To assess this possibility, transendothelial migration and MLR experiments were carried out, as shown in Figure 3. The migration and ISCs of mDCs were regulated by VEGF and the RhoA-COF1 pathway might be involved in the functional impairment of mDCs. In addition, the migration and immune function of mDCs were inhibited by Y27632 and P-COF1 BP, which could be due to the reduced actin polymerization and disappearance of dendrites.[26] Vascular endothelial growth factor signaling is also transduced by way of several other intracellular signaling pathways, including Erk, p38MAPK, or the serine/threonine protein kinase Akt, leading to increased cell proliferation, survival, permeability, and migration of endothelial cells.[35] It was reported that VEGF can enhance the phosphorylation of Erk1/2, but not those of p38MAPK or Akt in mDCs.[36] Moreover, our results and those from other groups showed that VEGF can impair the immune function through the NF-κB pathway.[13,37] From these results, it could be inferred that the molecular targets of VEGF to mDCs were COF1, Erk1 and 2, and NF-κB, all of which are related to the cytoskeleton, motility, and gene transcription.

Vascular endothelial growth factor acts through a series of tyrosine kinase receptors, including VEGFR1, 2, and 3 and neuropilin 1 and 2 and its binding sites have been identified on vascular endothelial cells, monocytes, mDCs, and other cell types.[38] Among the VEGFRs, mDCs can express VEGFR1 and VEGFR2.[19] As shown in Figures 4 and 5, the motility of mDCs was impaired by VEGF through the VEGFR2-RhoA-COF1 pathway. Several groups have shown that VEGFR1 is the major mediator of VEGF effects on the NF-κB pathway in hematopoietic stem cells and that VEGF affects the early stage of myeloid/DC differentiation.[13,19] Clauss et al[39] showed that VEGFR1 is biologically active in monocytes/macrophages and that VEGF stimulates the migration and chemotaxis of human monocytes. However, it has also been reported that VEGFR2 is the major mediator of mitogenic and angiogenic signals, and that VEGFR1 does not mediate an effective mitogenic signal of VEGF in endothelial cells.[40,41] Fan et al[42] established that VEGF-inducible phosphorylation of profilin1 (a COF1 functional coupling actin-binding protein that promotes actin polymerization) at Tyr-129 is critical for endothelial cell migration and angiogenesis through VEGFR2. The present study supports the notion that VEGF can inhibit the function of mDCs through VEGFR2[20] and shows that the downstream signaling of VEGFR2 is the RhoA-COF1 pathway.

The performance of biological functions by cytoskeleton-binding proteins often depends on their localization in the cytoplasm. The images of immunofluorescence microscopy for P-COF1, COF1, and F-actin (Figure 6) showed that most P-COF1 and COF1 in mDCs were translocated from the cytoplasm to the plasma membrane by VEGF. Ghosh et al[43] reported that COF1 plays an important role in polymerizing actin, generating protrusions, and determining the direction of cell migration. Breitsprecher et al[44] found that COF cooperates with fascin to disassemble filopodial actin filaments. Eiseler et al[45] found that the expression of constitutively active protein kinase D1

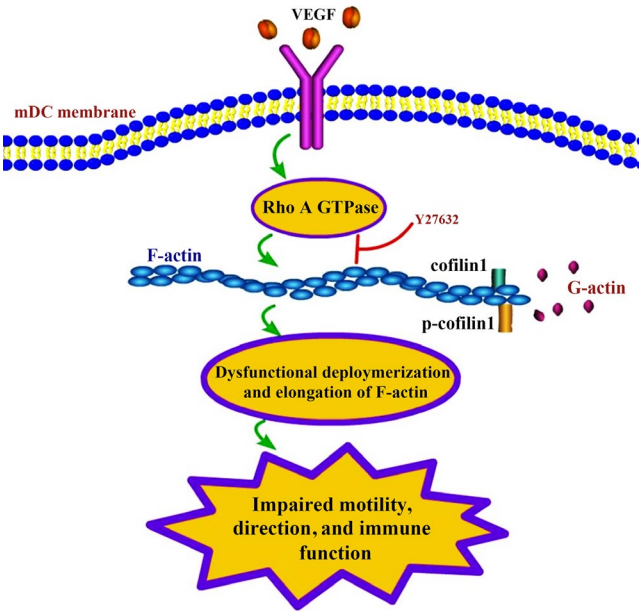

FIGURE 7 Schematic signaling diagram summarizing the effect of vascular endothelial growth factor (VEGF) on the motility and immune function of mature dendritic cells (mDCs). First, tumor-derived VEGF binds to VEGF receptor 2 on the membrane of mDCs, leading to upregulation of RhoA GTPase. Second, increased RhoA-GTPase upregulates the expression of phosphorylated (P-) cofilin1 to cause dysfunction of de novo filamentary actin (F-actin). Finally, dysfunctional F-actin impairs the motility, direction, and immune function of mDCs. G-actin, globular actin

in invasive tumor cells enhances the phosphorylation of COF, which effectively blocks the formation of free actin-filament barbed ends, directing cell migration. It could be inferred that abnormal expression and localization of COF1 in mDCs caused by VEGF might induce dysfunctional elongation of F-actin, leading to impaired motility and misled directions of mDCs in vivo. This might be one of the important aspects of the tumor immune escape mechanism. Interestingly, in recent years, some scholars have found that the expression of COF1 and P-COF1 in breast cancer tissues is closely related to the prognosis of breast cancer patients,[46,47] meaning that abnormal expression of COF1 might be a potential prognostic factor in cancer.

In summary, the present study showed that VEGF can impair the motility and immune function of mDCs through the RhoA-COF1 pathway mediated by VEGFR2. First, as shown in Figure 7, VEGF binds to VEGFR2 on the mDC membrane, leading to upregulation of RhoA-GTPase. Second, the increased RhoA-GTPase upregulates the expression of P-COF1 to cause dysfunction of de novo F-actin. Finally, the dysfunctional F-actin impairs the motility, direction, and immune function of mDCs. This notion was supported by some clinical pathological data. This might be one of the key aspects of the immune escape mechanism of tumors and provide the clue for the signal pathway of VEGF that should be appropriately blocked before undertaking DC-based antitumor immunotherapy. Clinically, it is crucial for further elucidation of the biological behavior of DCs and improvement of the efficiency of DC-based therapy against cancer.

ACKNOWLEDGMENTS

We thank Dr. Shu Chien, Dr. Dayu Teng, Mrs. Suli Yuan, Mr. Jerry Norwich, and Mr. Phu Minh Nguyen of the University of California San Diego for their technical expertise and help. This study was jointly funded by the National Natural Science Foundation of China (31771014, 31660258, 31860262, and 11762006), the China Postdoctoral Science Foundation (2015M582747XB), the Key Project of the Ministry of Education of China (210196), the Science and Technology Foundation of Guizhou Province (2018-1412, 2019-1266, 2019-2787, 2016-5676, and 2017-5718), the Science and Technology Innovative Talent Team of Guizhou Province (2015-4021), the 2011 Collaborative Innovation Program of Guizhou Province (2015-04), and the Cell and Gene Engineering Innovative Research Groups of Guizhou Province (KY-2016-031).

DISCLOSURE

Authors declare no conflicts of interest for this article.

ORCID

Shichao Zhang https://orcid.org/0000-0002-8760-3482
Zhu Zeng https://orcid.org/0000-0003-1989-5142

REFERENCES

1. Steinman RM. Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol*. 2012;30:1-22.
2. Steinman RM. Dendritic cells in vivo: a key target for a new vaccine science. *Immunity*. 2008;29:319-324.
3. Eisenbarth SC. Dendritic cell subsets in T cell programming: location dictates function. *Nat Rev Immunol*. 2019;19:89-103.
4. Worbs T, Hammerschmidt SI, Förster R. Dendritic cell migration in health and disease. *Nat Rev Immunol*. 2017;17:30-48.
5. Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature*. 2007;449:419-426.
6. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331:1565-1570.
7. Palucka K, Banchereau J. Cancer immunotherapy *via* dendritic cells. *Nat Rev Cancer*. 2012;12:265-277.
8. Villablanca EJ, Raccosta L, Zhou D, et al. Tumor-mediated liver X receptor-alpha activation inhibits CC chemokine receptor-7 expression on dendritic cells and dampens antitumor responses. *Nat Med*. 2010;16:98-105.
9. Herber DL, Cao W, Nefedova Y, et al. Lipid accumulation and dendritic cell dysfunction in cancer. *Nat Med*. 2010;16:880-886.
10. Hu ZQ, Xue H, Long JH, et al. Biophysical properties and motility of human mature dendritic cells deteriorated by vascular endothelial growth factor through cytoskeleton remodeling. *Int J Mol Sci*. 2016;17:1756.
11. Wu JB, Tang YL, Liang XH. Targeting VEGF pathway to normalize the vasculature: an emerging insight in cancer therapy. *Onco Targets Ther*. 2018;11:6901-6909.
12. van Willigen WW, Bloemendal M, Gerritsen WR, et al. Dendritic cell cancer therapy: vaccinating the right patient at the right time. *Front Immunol*. 2018;9:2265.
13. Gabrilovich D, Ishida T, Oyama T, et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood*. 1998;92:4150-4166.
14. Ohm JE, Gabrilovich DI, Sempowski GD, et al. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood*. 2003;101:4878-4886.
15. Harada K, Ihara F, Takami M, et al. Soluble factors derived from neuroblastoma cell lines suppress dendritic cell differentiation and activation. *Cancer Sci*. 2019;110:888-902.
16. Assoun S, Brosseau S, Steinmetz C, et al. Bevacizumab in advanced lung cancer: state of the art. *Future Oncol*. 2017;13:2515-2535.
17. Schiff D, Wen PY. The siren song of bevacizumab: swan song or clarion call? *Neuro Oncol*. 2018;20:147-148.
18. Sitohy B, Nagy JA, Dvorak HF. Anti-VEGF/VEGFR therapy for cancer: reassessing the target. *Cancer Res*. 2012;72:1909-1914.
19. Dikov MM, Ohm JE, Ray N, et al. Differential roles of vascular endothelial growth factor receptors 1 and 2 in dendritic cell differentiation. *J Immunol*. 2005;174:215-222.
20. Mimura K, Kono K, Takahashi A, et al. Vascular endothelial growth factor inhibits the function of human mature dendritic cells mediated by VEGF receptor-2. *Cancer Immunol Immunother*. 2007;56:761-770.
21. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*. 1991;9:271-296.
22. Zeng Z, Yao W, Xu X, et al. Hepatocellular carcinoma cells deteriorate the biophysical properties of dendritic cells. *Cell Biochem Biophys*. 2009;55:33-43.
23. Zeng Z, Xu X, Zhang Y, et al. Tumor-derived factors impaired motility and immune functions of dendritic cells through derangement of biophysical characteristics and reorganization of cytoskeleton. *Cell Motil Cytoskeleton*. 2007;64:186-198.
24. Zheng Q, Long J, Jia B, et al. Transforming growth factor-β1 deteriorates microrheological characteristics and motility of mature dendritic cells in concentration-dependent fashion. *Clin Hemorheol Microcirc*. 2013;56:25-40.
25. Xu X, Zeng Z, Yao W, et al. Biomechanical alterations of dendritic cells by co-culturing with K562 CML cells and their potential role in immune escape. *J Biomech*. 2010;43:2339-2347.
26. Kobayashi M, Azuma E, Ido M, et al. A pivotal role of Rho GTPase in the regulation of morphology and function of dendritic cells. *J Immunol*. 2001;167:3585-3591.
27. Ferret-Bernard S, Curwen RS, Mountford AP. Proteomic profiling reveals that Th2-inducing dendritic cells stimulated with helminth antigens have a 'limited maturation' phenotype. *Proteomics*. 2008;8:980-993.
28. Narumiya S, Thumkeo D. Rho signaling research: history, current status and future directions. *FEBS Lett*. 2018;592:1763-1776.
29. Lappalainen P. Actin-binding proteins: the long road to understanding the dynamic landscape of cellular actin networks. *Mol Biol Cell*. 2016;27:2519-2522.
30. Kanellos G, Frame MC. Cellular functions of the ADF/cofilin family at a glance. *J Cell Sci*. 2016;129:3211-3218.
31. Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer*. 2008;8:579-591.
32. Nagai S, Moreno O, Smith CA, et al. Role of the cofilin activity cycle in astrocytoma migration and invasion. *Genes Cancer*. 2011;2:859-869.
33. Katsuno H, Toriyama M, Hosokawa Y, et al. Actin migration driven by directional assembly and disassembly of membrane-anchored actin filaments. *Cell Rep*. 2015;12:648-660.
34. Verdijk P, van Veelen PA, de Ru AH, et al. Morphological changes during dendritic cell maturation correlate with cofilin activation and translocation to the cell membrane. *Eur J Immunol*. 2004;34:156-164.

35. Caporarello N, Lupo G, Olivieri M, et al. Classical VEGF, Notch and Ang signalling in cancer angiogenesis, alternative approaches and future directions (Review). *Mol Med Rep*. 2017;16:4393-4402.
36. Sugiyama M, Kakeji Y, Tsujitani S, et al. Antagonism of VEGF by genetically engineered dendritic cells is essential to induce antitumor immunity against malignant ascites. *Mol Cancer Ther*. 2011;10:540-549.
37. Zeng Z, Long J, Chen L. The effect of hepatocellular carcinoma cell microenvironment on the functional status of dendritic cells at different differentiation stages. *J Chongqing Med Uni*. 2010;35:1883-1888.
38. Zirlik K, Duyster J. Anti-angiogenics: current situation and future perspectives. *Oncol Res Treat*. 2018;41:166-171.
39. Clauss M. Molecular biology of the VEGF and the VEGF receptor family. *Semin Thromb Hemost*. 2000;26:561-569.
40. Kowanetz M, Ferrara N. Vascular endothelial growth factor signaling pathways: therapeutic perspective. *Clin Cancer Res*. 2006;12:5018-5022.
41. Huang Y, Chen X, Dikov MM, et al. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. *Blood*. 2007;110:624-631.
42. Fan Y, Arif A, Gong Y, et al. Stimulus-dependent phosphorylation of profilin-1 in angiogenesis. *Nat Cell Biol*. 2012;14:1046-1056.
43. Ghosh M, Song X, Mouneimne G, et al. Cofilin promotes actin polymerization and defines the direction of cell motility. *Science*. 2004;304:743-746.
44. Breitsprecher D, Koestler SA, Chizhov I, et al. Cofilin cooperates with fascin to disassemble filopodial actin filaments. *J Cell Sci*. 2011;124:3305-3318.
45. Eiseler T, Doppler H, Yan IK, et al. Protein kinase D1 regulates cofilin-mediated F-actin reorganization and cell motility through slingshot. *Nat Cell Biol*. 2009;11:545-556.
46. Maimaiti Y, Liu Z, Tan J, et al. Dephosphorylated cofilin expression is associated with poor prognosis in cases of human breast cancer: a tissue microarray analysis. *Onco Targets Ther*. 2016;9:6461-6466.
47. Maimaiti Y, Tan J, Liu Z, et al. Overexpression of cofilin correlates with poor survival in breast cancer: a tissue microarray analysis. *Oncol Lett*. 2017;14:2288-2294.

How to cite this article: Long J, Hu Z, Xue H, et al. Vascular endothelial growth factor (VEGF) impairs the motility and immune function of human mature dendritic cells through the VEGF receptor 2-RhoA-cofilin1 pathway. *Cancer Sci*. 2019; 110: 2357–2367. https://doi.org/10.1111/cas.14091