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Knockdown of Delta-like 3 restricts lipopolysaccharide-induced inflammation, migration and invasion of A2058 melanoma cells via blocking Twist1-mediated epithelial-mesenchymal transition

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ABSTRACT

Aims: To investigate the effects and mechanisms of DLL3 in inflammation-mediated A2058 melanoma cell invasion and metastasis.

Materials and methods: Melanoma A2058 cells was stimulated with lipopolysaccharide (LPS), with or without transfection of DLL3 siRNA, or DLL3 overexpression vector, or Twist1 siRNA. Cell migration and invasion were detected by wound healing and transwell invasion assay. The production of inflammatory factors TNF-α and IL-6 was measured by ELISA. The expression of Notch signaling-related molecules was detected by PCR and western blot. The protein expression of MMP1, MMP9, VEGF, DLL3, and EMT-related molecules was tested by western blot.

Key findings: LPS treatment increased migration and invasion of A2058 cells, accompanied by increased expression of TNF-α and IL-6. DLL3 was both upregulated in the LPS- or TNF-α-stimulated A2058 cells, and DLL3 knockdown inhibited LPS-induced inflammation, migration and invasion of A2058 cells, accompanied by downregulation of MMP1, MMP9 and VEGF. Besides, DLL3 knockdown inhibits the expression of Twist1, a key EMT regulating factor, as well as the EMT hallmarks slug, N-cadherin and vimentin. Moreover, Twist1 silence inhibited EMT, and limited LPS-induced migration and invasion of A2058 cells, with decreased expression of MMP1, MMP9 and VEGF and reduced production of TNF-α and IL-6 in LPS-stimulated A2058 cells.

Significance: Knockdown of DLL3 restricts LPS-induced inflammation, migration and invasion of A2058 melanoma cells via blocking Twist1-mediated EMT. Therefore, targeting DLL3 may be a promising therapeutic strategy against inflammation-aggravated melanoma progression.

1. Introduction

Melanoma is a type of malignant neoplasm arising from melanocytes. It is commonly seen in skin, mucosa and eye choroid. Recently, the increased inflammation is reported to be the one of the key reasons of skin cancer including melanoma [1]. However, current understanding for the relationship and the regulatory mechanism between inflammation and melanoma is still limited.

Chronic inflammatory conditions are highly beneficial for tumor development, and malignant cells express numerous pro-inflammatory mediators conduce to tumor microenvironment, which supports tumor progression and facilitates cancer cells to evade host immune surveillance [2]. As a common inflammatory cytokine, Interleukin (IL)-6 stimulates oral cancer initiation by silencing tumor suppressor or DNA repair genes via induction of DNA methylation [3]. Furthermore, TNF-α- or IL-6 can induce invasion and migration of mouse melanoma cell B16F10, and these effects can be inhibited by paeonol, a natural anti-inflammatory phenolic ingredient, suggesting that targeting inflammation may a potential strategy against the metastasis of melanoma cells [4].

Notch signaling is verified to have a crosstalk with inflammation in carcinogenesis [5]. It is also involves in the regulation of tumor microenvironment and metastasis via epithelial-mesenchymal transition (EMT), the basis of metastasis [6,7]. Furthermore, Notch 1, a key

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receptor of Notch signaling, can be activated by inflammation in colon cancer via matrix metalloproteinases9 (MMP9) [8], a key marker of EMT and regulates the metastasis in melanoma [9,10]. Delta-like 3 (DLL3), an important member of in Notch ligands family [11], is studied frequently in tumor progression. DLL3 knockdown impedes melanoma proliferation and migration by activating mitogen-activated protein kinase (MAPK) signaling [12]. Silence DLL3 by methylation inhibits the growth of hepatocellular carcinoma cells [13]. Moreover, some studies have reported that pro-inflammatory cytokines can change the mRNA level of DLL3 in cancer cells [5,14,15]. However, the exact relationship and function between inflammation and DLL3 in the development of melanoma is unclear.

In present study, we found that LPS, an important inflammatory inducer, induced migration and invasion of A2058 melanoma cells. Importantly, the expression of DLL3 was up-regulated in LPS, or tumor necrosis factor α (TNF-α) stimulated A2058 melanoma cells. Inhibition of DLL3 impeded LPS-induced inflammatory response and cell migration and invasion by inhibiting EMT process in A2058 melanoma cells. Our findings suggest that DLL3 promotes A2058 melanoma cell migration and invasion via EMT in inflammatory microenvironment, implying a new target for the treatment of melanoma.

2. Material and methods

2.1. Cell culture and treatments

The human melanoma cell line A2058 was obtained from American Type Culture Collection (Manassas, VA, USA), and grown in high-glucose DMEM (Gibco BRL, Grand Island, NY, USA,) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO2 atmosphere. To induce inflammation, A2058 cells were treated with 1 μg/ml of LPS (Sigma, St. Louis, MO, USA), or tumor necrosis factor-α (TNF-α), with or without IL-37 (1 μg/ml, Sigma) for indicated times.

2.2. Cell transfection

For inhibition of DLL3 and Twist1, DLL3 siRNA (si-DLL3) and Twist1 siRNA (si-Twist1) were synthesized by Invitrogen. A2058 cells in logarithmic phase were collected (1×10^5 cells/well) and cultured for 12 h. Then, the cells were transfected with the specific siRNA using DharmaFECT 2 Transfection Reagent (GE Healthcare) following the Manufacturer's instructions.

For overexpression of DLL3, the cDNA of DLL3 gene was inserted into pLent-Puro-CMV lentiviral vectors (pPC-LV-DLL3) (Hanbio Biotechnology Co., Ltd., Shanghai, China) to made recombinant vector. A2058 cells were seeded in the 6-wells (1×10^5cells/well) and cultured for 12 h. The cells were then transfected with pPC-LV-DLL3 or empty vector pLent-Puro-CMV (pPC) (1 μg/ml) using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA). The transfected A2058 cells were grown in 5% CO_2 incubator at 37 °C for 48 h before LPS to induce inflammation.

2.3. Wound healing test

For the migration assay, cells were seeded in 24-well plate at 1×10^5 cells/well and incubated overnight until the cells reached 80% confluence. Cells were then treated with 1 μg/ml of LPS, followed by scraping by a sterile pipette tip to create a straight scratch. After rinsing off the destroyed cells with PBS, the plate was cultured for another 24 h. Cell migration was observed using an Olympus IX 70 microscope and imaged at 0 h and 24 h with a digital camera (Leica DFC300FX).

2.4. Transwell invasion assay

For the invasion assay, the upper chamber of Transwell inserts was coated with Matrigel (BD Biosciences, Bedford, MA, USA) at a dilution ratio of 1: 8 and air-dried at room temperature. After 48 h of transfection, 2×10^4 cells were cultured in serum-free DMEM in the Matrigel-precoated upper chamber, and the lower chamber was filled with DMEM containing 10% FBS. Besides, 1 μg/ml of LPS was added to both the upper and the lower chambers. After 24 h incubation, the invaded cells were fixed with methanol and stained with 0.5% crystal violet (Sigma). The number of invaded cells were observed and counted under a DMLB2 light microscope (Leica, Germany) at a magnification of x200 in 5 randomly fields in each well.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cells were collected and the cell suspensions were centrifuged at 3500*g* for 15 min. The supernatant was then collected and stored at −80 °C for inflammatory cytokines analysis. The levels of interleukin (IL)-6, and tumor necrosis factor (TNF)-α were analyzed by ELISA kits (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's instructions.

2.6. Western blot

The proteins, extracted from cultured cells, were separated through SDS-PAGE and then were transferred onto Polyvinylidene Fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were blocked in PBST (PBS with 0.1% Tween 20) containing 5% nonfat milk for 2 h at room temperature, and then incubated with the primary antibodies against MMP-9 (ab228402, 1:1000; Abcam, Cambridge, MA, USA), MMP-1 (ab38929, 1:1000; Abcam), VEGF (ab69479, 1:500; Abcam), Jagged1 (1:500; Abcam), Jagged2 (ab109627, 1:1000; Abcam), DLL1 (ab84620, 1:500; Abcam), DLL3 (ab103102, 1:2000; Abcam), DLL4(ab7280, 1:1000; Abcam), Twist1 (ab49254, 1:1000; Abcam), slug (ab183760, 1:1000; Abcam), E-cadherin (ab40772, 1:1000; Abcam), N-cadherin (ab18203, 1:1000; Abcam), vimentin (ab137321, 1:1000; Abcam), β-actin (ab16769, 1:1000; Abcam) and the corresponding HRP-conjugated secondary antibodies. Membranes were extensively washed several times with PBST. Proteins were detected using a ChemiDoc XRS imaging system and Quantity One analysis software (Bio-Rad, San Francisco, California, USA). β-actin was used as an endogenous reference.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from A2058 cells was isolated using Trizol reagent (Invitrogen). RNA was transcribed into cDNAs using the Primer-Script one step RT-PCR kit (Takara Biotechnology, Dalian, China). qRT-PCR was performed using the SYBR-green detection system (Roche Molecular Systems Inc., Branchburg, NJ, USA). GADPH expression level was as standardization for each gene expression. The condition of RT-PCR was 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, and at last 1 min at 55 °C. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression fold change of mRNAs. The primer sequences were as follows: Jagged1, forward 5′-GCTTCCACTGGCACTGGTAGTTTC-3′ and reverse 5′-TGCTGACATCAAATCCCCCCTC-3′; Jagged2, forward 5′-GTCGTCATTCCCTTTCAGTTCG-3′ and reverse 5′-AGTTCTCATCACA GCGTACTCG-3′; DLL1, forward 5′-CCTACTGCACAGAGCCGATCT-3′ and reverse: 5′-GCAGGTGGCTCCATTCTTGC-3′; DLL3, forward 5′-AGT TGCACTTCTCCTACCGCG-3′ and reverse 5′-ACGGCATTCATCAGGCTC TTC-3′; DLL4, forward 5′-GTGAACTGCACATCAGCGATTG-3′ and reverse 5′-GTTGCAGACGAAGTTGTTTGGG-3′; GAPDH, forward 5′-GTTG TCTCCTGCGACTTCA-3′ and reverse 5′-TGGTCCAGGGTTTCTTACTC-3′.

2.8. Statistical analysis

All statistic data are presented as the mean ± standard deviation (SD). P value < 0.05 was considered significant. Statistical

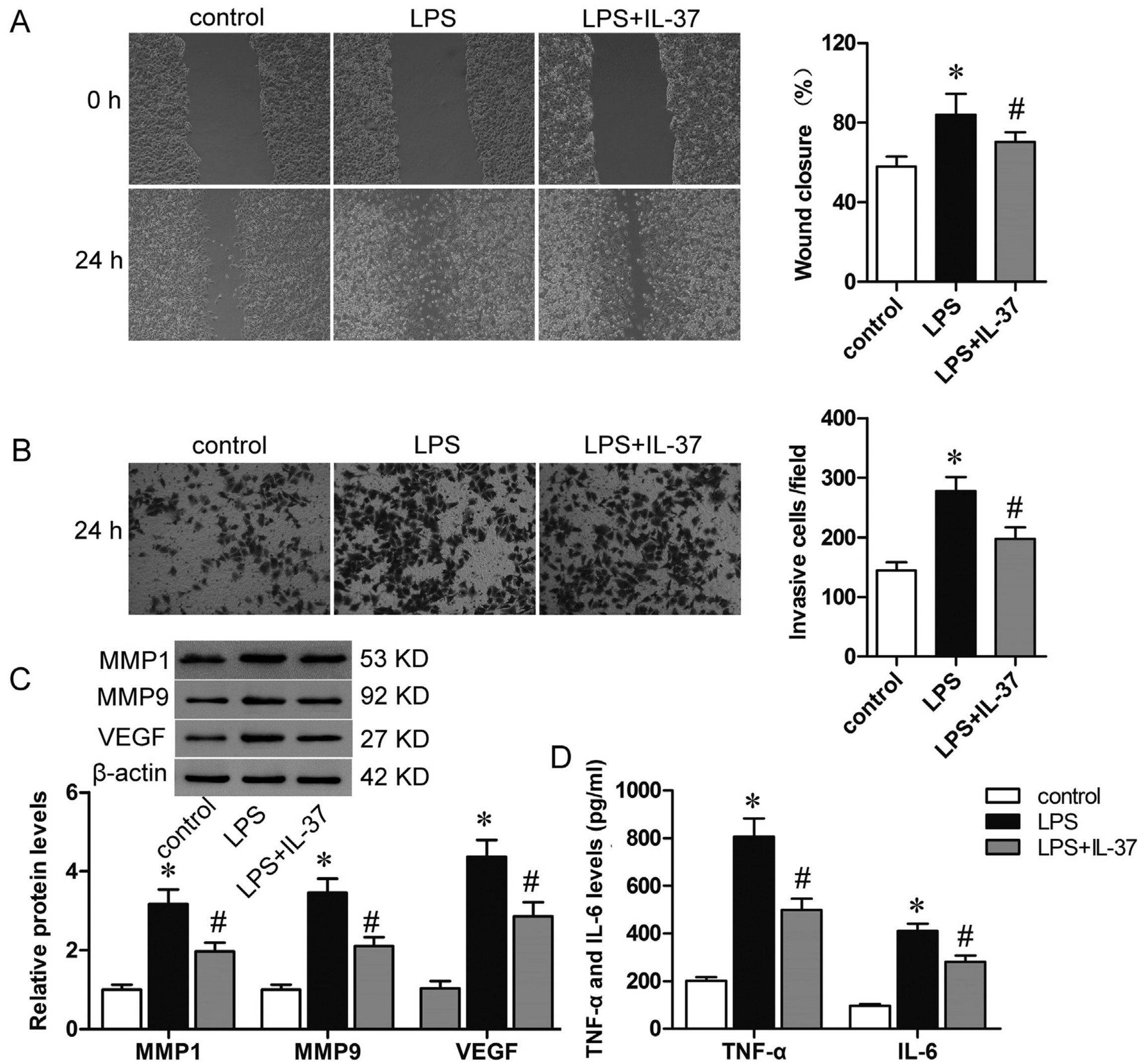

Fig. 1. The effects of LPS-induced inflammation on migration and invasion of A2058 melanoma cells. A2058 cells were treated with 1 μg/ml of LPS for 24 h in the absence or presence of 1 μg/ml of IL-37. (A) Cell migration was evaluated by a scratch wound healing assay. Bar graph quantified the migration area as a percentage of the original wound size. Magnification times: 20×. (B) Cell invasion was assessed using a transwell invasion assay. Bar graph showing the average numbers of invading cells per microscopic field. Magnification times: 20×. (C) The protein levels of MMP1, MMP9 and VEGF were detected by western blot. β-actin was used as the internal control. (D) The secretion levels of TNF-α and IL-6 in the supernatant of A2058 cells were detected by ELISA kits. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. LPS.

significance was determined using one factor analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Statistical analyses of the data were performed by using SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. LPS-induced inflammation promotes migration and invasion of A2058 melanoma cells

Increased inflammation is one of the key inducement of melanoma, and metastasis of cancer is closely associated with inflammation [1,16,17]. To explore whether inflammation affects the metastasis of melanoma, A2058 cells were exposed to LPS, an inflammation inducer. As a result, LPS stimulation significantly accelerated both wound closure and invasion of A2058 cells (Fig. 1A–B). After LPS stimulation, the protein levels of MMP-9 and VEGF were significantly up-regulated in A2058 cells (Fig. 1C). Simultaneously, the secretion of inflammatory cytokines IL-6 and TNF-α was obviously increased in A2058 cells after LPS stimulation (Fig. 1D). However, addition of IL-37, an important anti-inflammatory modulator, significantly abolished LPS-mediated wound closure and invasion (Fig. 1A–B), decreased LPS-induced up-regulation of MMP1, MMP9 and VEGF (Fig. 1C), reduced LPS-induced production of IL-6 and TNF-α in A2058 cells (Fig. 1D). These results suggest that the metastasis of melanoma cells is positively associated with inflammation.

3.2. Inflammation up-regulates the expression of DLL3 in A2058 melanoma cells

Notch signaling reportedly involved in inflammation-mediated malignant processes [5,14,15]. To investigate whether inflammation plays a role in Notch ligands-dependent manner in melanomas, the expression of Jagged1, Jagged2, DLL1, DLL3, and DLL4 were tested in A2058 cells exposed to LPS or TNF-α, another key inflammatory factor and metastasis promotion factor [18]. Intriguingly, the mRNA levels of DLL3 were obviously increased, while the levels of Jagged1, Jagged2, DLL1 and DLL4 have no significant changes in both LPS- and TNF-α-treated A2058 cells (Fig. 2A). Similarly, unlike with no significant changes in protein expression of other notch ligands, the protein levels

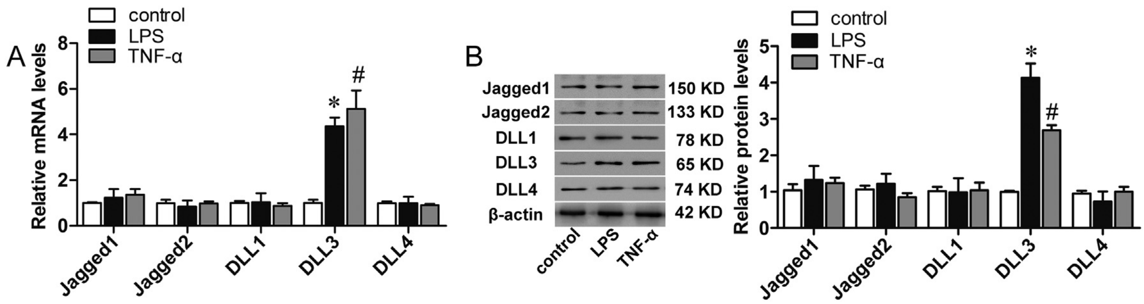

Fig. 2. The expression levels of Notch ligands in LPS- or TNF-α-stimulated A2058 melanoma cells. A2058 cells were treated with 1 μg/ml of LPS or TNF-α for 24 h. (A) The mRNA levels of Jagged1, Jagged2, DLL1, DLL3, and DLL4 in A2058 cells were determined by qRT-PCR. (B) The protein levels of Jagged1, Jagged2, DLL1, DLL3, and DLL4 in A2058 cells were assessed by western blot. Data are expressed as the mean ± SD. *$P < 0.05$ vs. control; #$P < 0.05$ vs. LPS.

of DLL3 were significantly up-regulated in A2058 cells stimulated with either LPS or TNF-α (Fig. 2B). These findings indicate that DLL3 may be involved in inflammation-mediated melanoma progression.

3.3. Knockdown of DLL3 inhibits LPS-induced inflammation, migration and invasion of A2058 melanoma cells

To evaluate the role of DLL3 in inflammation-induced melanoma migration and invasion, DLL3 was stably down-regulated transfection with its specific siRNA in A2058 cells (Fig. 3A–B). Resultly, knockdown of DLL3 significantly reduced LPS-induced migration and invasion of melanoma cells (Fig. 3C–D), decreased LPS- induced upregulation of MMP1, MMP9 and VEGF protein levels in A2058 cells (Fig. 3E). Besides, DLL3 knockdown also attenuated LPS-triggered secretion IL-6 and TNF-α in A2058 cells (Fig. 3F). These results suggest that DLL3 knockdown impedes LPS-mediated inflammatory responses, thereby inhibiting migration and invasion of melanoma cells.

3.4. DLL3 regulates EMT process in LPS-stimulated A2058 melanoma cells

EMT is critical for the progression of tumor invasion and metastasis [6,7]. As expected, LPS significantly increased the expression of Twist1 (Fig. 4A), a key EMT-inducing factor. Besides, LPS also obviously up-regulated the expression levels of slug, N-cadherin and vimentin, and down-regulated the expression levels of E-cadherin in A2058 cells (Fig. 4A). However, LPS-induced up-regulation of Twist1, slug, N-cadherin, and vimentin and down-regulation of E-cadherin were markedly attenuated by DLL3 knockdown (Fig. 4A). To further investigate the impact of DLL3 on EMT in melanoma cells, DLL3 was overexpressed in A2058 cells (Fig. 4B). Overexpression of DLL3 significantly up-regulated the protein expression of slug, N-cadherin, vimentin and Twist1, and down-regulated E-cadherin in A2058 cells (Fig. 4B). These results indicate that DLL3 may be positively regulating LPS-mediated EMT in melanoma cells.

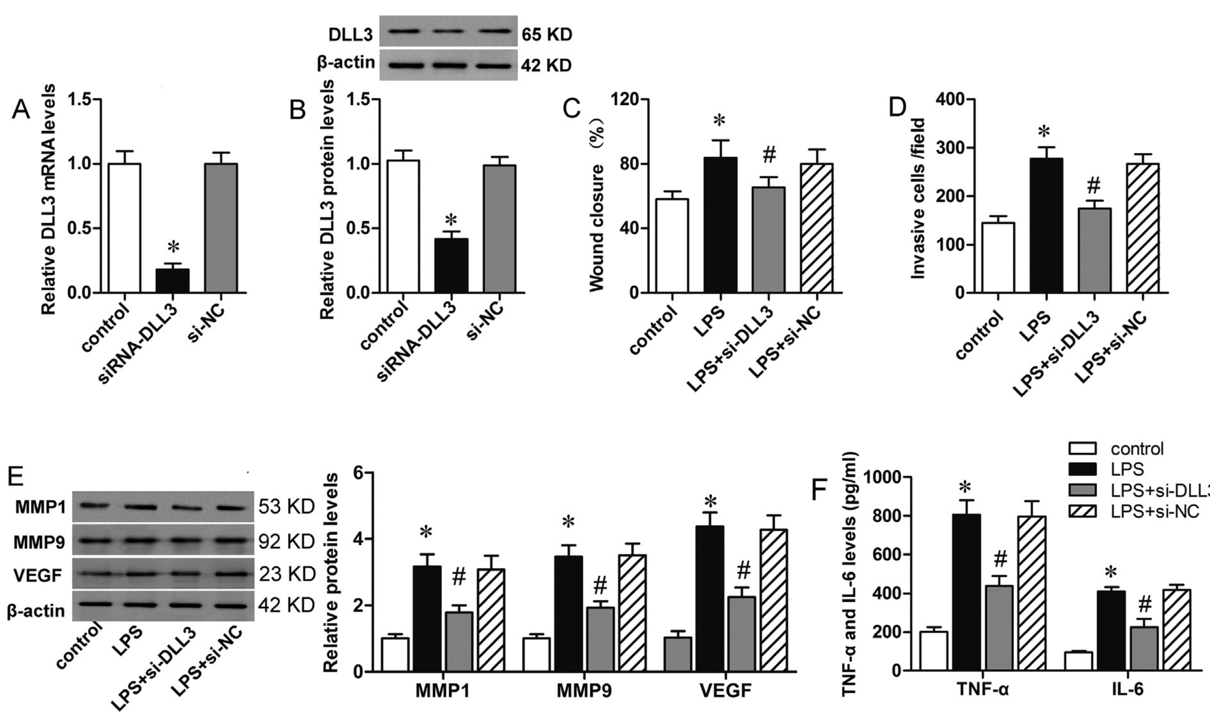

Fig. 3. The effects of DLL3 on LPS-induced inflammation, migration and invasion of A2058 melanoma cells. A2058 cells were transfected with the specific siRNA of DLL3 (si-DLL3) or its negative control si-NC. (A) The mRNA and (B) protein levels of DLL3 were determined by qRT-PCR and western blot. These transfected or non-transfected cells were then treated with 1 μg/ml of LPS for 24 h. (C) Cell migration was detected by wound healing test and quantified as a percentage of original wound size. (D) Cell invasion was detected by transwell invasion assay and quantified as cell numbers. (E) The protein levels of MMP1, MMP9 and VEGF were detected by western blot. (F) The secretion of TNF-α and IL-6 were detected by ELISA kits. Data are expressed as the mean ± SD. *$P < 0.05$ vs. control; #$P < 0.05$ vs. LPS.

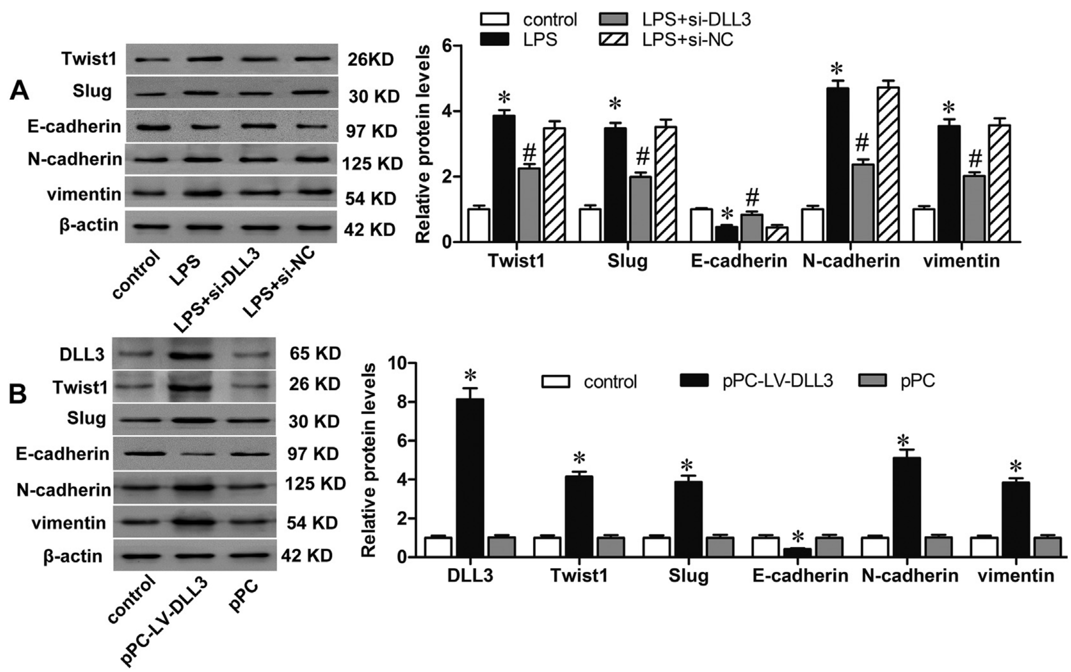

Fig. 4. The effects of DLL3 on LPS-mediated EMT in A2058 melanoma cells. A2058 cells transfected with si-DLL3 or si-NC were exposed to LPS for 24 h. (A) The protein expression levels of Twist1, slug, E-cadherin, N-cadherin and vimentin were examined by Western blot.
A2058 cells were transfected with pLent-Puro-CMV lentiviral vectors (pPC-LV-DLL3) or pPC, a pLent-Puro-CMV empty plasmid. (B) The protein expression levels of DLL3, Twist1, slug, E-cadherin, N-cadherin and vimentin were examined by Western blot. Data are expressed as the mean ± SD. $*P < 0.05$ vs. control; $\#P < 0.05$ vs. LPS.

3.5. Knockdown of Twist1 inhibits LPS-induced inflammation, EMT, migration and invasion of A2058 melanoma cells

Since Twist1 is a key inducer of EMT process [19], the siRNA of Twist1 (si-Twist1) were transfected into A2058 cells to confirm the role of EMT in LPS-induced invasion and migration of melanoma cells. The significant down-regulation of Twist1, slug, N-cadherin, vimentin and up-regulation of E-cadherin was observed in A2058 cells after si-Twist1 transfection (Fig. 5A). Moreover, cell migration and invasion induced by LPS were impeded by si-Twist1 (Fig. 5B–C), accompanied by down-regulation of MMP1, MMP9 and VEGF (Fig. 5D). Besides, the production of inflammatory factors TNF-α and IL-6 were also attenuated by si-Twist1 (Fig. 5E). These results suggest that blocking twist-mediated EMT restricts LPS-induced melanoma invasion and migration.

4. Discussion

Malignant cells secrete pro-inflammatory mediators to promote the progression of tumor microenvironment [2]. A2058 is a highly metastatic melanoma cell line commonly used to study melanoma [20]. In present study, LPS treatment resulted in stronger migration and invasion ability of A2058 cells, accompanied by increase of pro-inflammatory factors IL-6 and TNF-α, suggesting the close connection between metastasis and inflammation in melanoma.

Further results confirmed that induction of inflammatory response can induce metastasis in melanoma. LPS, a common inducer of inflammation, facilitated the migration and invasion of the melanoma cells in present results. Abundant researches have proved that LPS induces invasion and migration in different types of cancer cells [21,22], as well as the migration of melanoma [23]. Moreover, the pro-inflammatory cytokine TNF-α also induced melanoma cell invasion and migration. Similarly, TNF-α- or IL-6- induce invasion and migration of mouse melanoma cell B16F10 [4]. Additionally, the expression of markers metastasis markers MMP1, MMP9 and VEGF in A2058 cells were both significantly upregulated by LPS. However, IL-37, an identified anti-inflammatory factor, abolished LPS-induced migration and invasion of melanoma cells, reduced LPS-induced up-regulation of MMP1, MMP9 and VEGF in A2058 cells, suggesting that impeding inflammation in can suppress melanoma metastasis. Intriguingly, anti-metastasis role of IL-37 in many kinds of tumors like colon cancer and renal cell carcinoma has been widely demonstrated [24,25]. These studies confirmed that inflammation is one of the predisposing factors for migration and invasion in melanoma cells.

Notch signaling regulates tumorigenesis and malignant transformation, and interacts with inflammatory signals, thereby participating in tumorigenicity and tumor progression [6,26]. In present study, the expression levels of Notch ligands, such as Jagged1, Jagged2, DLL1, DLL3, and DLL4 in LPS- or TNF-α-stimulated A2058 cells were examined, and only DLL3 was upregulated following these inflammatory stimulation, implying that DLL3 may be participated in the migration and invasion of melanoma cells induced by inflammatory environment. Similarly, DLL3 levels were also upregulated by TNF-α in fibroblast-like synoviocytes [15]. Although the mRNA levels of DLL3 in murine Lewis lung carcinoma were inhibited by TNF-α, overexpression of DLL3 promoted the lung carcinoma growth [14]. DLL3 has been widely studied in multiple carcinomas, especially in small cell lung cancer [14,27,28]. DLL3 down-regulation by methylation induced human hepatocellular carcinoma apoptosis [29]. In pituitary adenomas, DLL3 was downregulated accompanying the inhibition of migration and invasion [30,31]. These evidences promoted us to explore whether DLL3 can modulate melanoma metastasis in an inflammatory environment. As expected, DLL3 knockdown abolished the high migration and invasion induced by LPS. Besides, the expression levels of MMP1, MMP9 and VEGF induced by LPS were both significantly down-regulated after DLL3 silencing. These results confirmed that DLL3 can be regulated by inflammation environment, thereby and promoting melanoma metastasis under inflammatory environment.

EMT refers to the loss of epithelial cell features and the acquisition of a mesenchymal cell phenotype, which is necessary for tumor cells to acquire migration and invasion capabilities [6,7]. It is generally

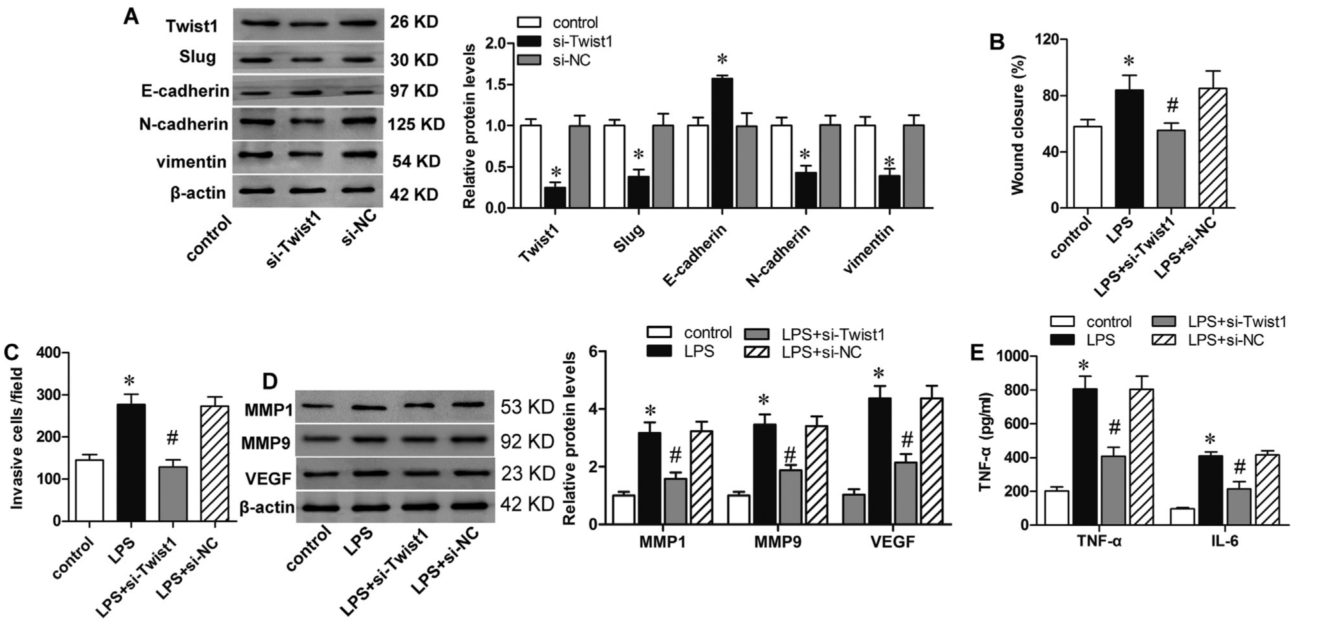

Fig. 5. Inhibition of EMT by silencing Twist1 affects LPS-induced inflammation, EMT, migration and invasion of A2058 melanoma cells. A2058 cells were transfected with si-Twist1 or si-NC and then exposed to 1 μg/ml of LPS for 24 h. (A) The protein expression levels of Twist1, slug, E-cadherin, N-cadherin and vimentin in A2058 cells were examined by Western blot. (B) Cell migration in LPS-stimulated A2058 cells was detected by wound healing test. (C) Cell invasion in LPS-stimulated A2058 cells was detected by transwell invasion assay. (D) The protein levels of MMP1, MMP9 and VEGF in LPS-stimulated A2058 cells were detected by western blot. (E) The secretion of TNF-α and IL-6 in LPS-stimulated A2058 cells were detected by ELISA kits. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs. control; $\#P < 0.05$ vs. LPS.

thought that EMT can be induced by tumor inflammatory microenvironment [32]. In this study, the expression of EMT inducer Twist1, and EMT markers slug, N-cadherin and vimentin were all increased by LPS-induced inflammation environment in A2058 cells. Similarly, previous research confirmed that EMT can be induced by LPS in mouse melanoma model [23], suggesting that LPS can induce EMT of melanoma. Moreover, LPS-induced EMT is critical for inflammation-initiated metastasis [23]. In present study, DLL3 overexpression in melanoma cells increased the expression of EMT markers, while knockdown of DLL3 inhibited LPS-induced Twist1-mediated EMT. Similarly, the increase of DLL3 induces EMT characteristics in an EMT-suffering NOTCH4+ DLL3- melanoma [33]. Importantly, inhibition of EMT by Twist1 knockdown limited LPS-induced migration and invasion of melanoma cells, with decreased expression of slug, Twist1, N-cadherin and vimentin and increased expression of E-cadherin, reduced expression of metastasis markers MMP1, MMP9 and VEGF, and reduced secretion of inflammatory cytokines IL-6 and TNF-α. These evidences indicated that DLL3 plays a positive role in inflammation-dependent migration and invasion via promoting EMT in melanoma cells.

Taken together, DLL3 knockdown inhibits inflammatory stimulation-induced **A2058** melanoma cell invasion and migration via blocking Twist1-mediated EMT, suggesting that DLL3 may be involved in inflammatory microenvironment-aggravated aggressive process of melanoma, supporting a new target to inflammation-aggravated melanoma progression under tumor microenvironment.

Conflict of interests

The authors declare that they have no competing interests.

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