Decreased VEGFA alleviates the symptoms of LPS-induced sepsis in a mouse model by inhibiting glycolysis and thereby regulating the polarization of macrophages

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ABSTRACT

The immune imbalance caused by excessive inflammatory reactions is the primary cause of sepsis. Macrophages with M1 and M2 polarization states are important immune cells that regulate the balance of the inflammatory response in sepsis. Encouraging the conversion of macrophages from the M1 to the M2 type is an important strategy for relieving sepsis. Here, we demonstrated the upregulation of vascular endothelial growth factor A (VEGFA) in a mouse model of sepsis. Then, siRNA technology was applied to inhibit the expression of VEGFA in macrophages. Flow cytometry and RT-qPCR results showed that low expression of VEGFA inhibited LPS-induced M1 polarization of macrophages. Decreased VEGFA was also proven to lower TNF-α, IL-1β, and IL-6 secretion by LPS-induced macrophages. In addition, the effects of knocking down VEGFA on the energy metabolism pattern of macrophages were investigated by glycolysis pressure tests and mitochondrial pressure tests, and VEGFA knockdown reversed the induction of glycolysis in macrophages by LPS. The mitochondrial content and ATP content results also confirmed this finding. After the tail vein of septic mice was injected with macrophages transfected with si-VEGFA, the liver and kidney damage and the pathological conditions of the lung were alleviated. The secretion of TNF-α and IL-6 was decreased, while IL-10 was increased in their serum. Immunohistochemical staining revealed decreased expression of CD86 and increased expression of CD206 in the si-VEGFA group. This study demonstrates that decreased VEGFA inhibits glycolysis and thus inhibits LPS-induced M1 polarization of macrophages, ultimately relieving sepsis.

Key words: Sepsis; vascular endothelial growth factor A; macrophage polarization; glycolysis.

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Data availability: The data used to support the findings of this study are available from the corresponding author upon request.

Introduction

Sepsis is a systemic inflammatory syndrome caused by an infection that can eventually result in multiple organ failures and can even be life-threatening.[1] Currently, the incidence of sepsis is approximately 0.3%, and its mortality rate is 20-40%.[2] Sepsis is characterized by a high inflammatory response in the early phase, while in the later phase, the high inflammatory response will cause an imbalance of the immune system and finally result in multiple organ dysfunction.[3] Studies have indicated that inhibition of high inflammation in the early phase of sepsis can reduce the damage to immune cells, which is an important means to prevent and reduce the immune imbalance in sepsis.[4] Therefore, it is urgent to seek effective strategies to alleviate the early inflammatory response in the treatment of sepsis.

Macrophages are important actors in controlling the immune response of pathogens invading in the early phase of sepsis.[5] Driven by the microenvironment *in vivo*, macrophages can be activated into the M1 type, which promotes inflammation, or the M2 type, which suppresses inflammation.[6] It has been found that the polarization of macrophages and the progression of sepsis are strongly linked.[5] Therefore, the regulation of polarization may have a significant effect in the treatment of sepsis, especially in the early phase, and promoting M2 polarization will help alleviate the inflammatory response and avoid further deterioration of sepsis. Macrophages with the M1 or M2 phenotype have different energy metabolic pathways: M1-type macrophages obtain energy mainly from aerobic glycolysis, while M2-type macrophages obtain energy mainly through the oxidative phosphorylation of mitochondria.[7] Recent studies have shown that inhibition of aerobic glycolysis in macrophages can effectively inhibit the activation of macrophages and their transition to the M1-type, thus inhibiting the inflammatory response.[8]

Vascular endothelial growth factor A (VEGFA) is the most important vascular endothelial growth factor and is mainly produced by endothelial cells, mononuclear macrophages, and fibroblasts.[9] The main function of VEGFA is to selectively enhance the mitosis of vascular endothelial cells, stimulate endothelial cell proliferation, and promote angiogenesis.[9] In addition to participating in angiogenesis, VEGFA is also found to be closely related to aerobic glycolysis.[10] A large number of studies have shown that the solid tumor is an anoxic microenvironment, and hypoxia can induce the expression of hypoxia-inducible factor (HIF-1α) and then upregulate the expression of VEGFA and other factors, which can help tumor cells survive in the anoxic microenvironment by changing the metabolic mode to anaerobic glycolysis and promoting angiogenesis to increase oxygen supply.[11,12] Studies have shown that the activation of macrophages induced by LPS leads to increased VEGFA expression.[13] In addition, inhibition of HIF-1α/VEGFA can block the M2 polarization of macrophages.[14] Therefore, we hypothesize that VEGFA can regulate glycolysis in macrophages to inhibit M1 polarization, thereby alleviating sepsis.

Here, we intended to investigate the effect of VEGFA on the polarization and expression of proinflammatory cytokines in LPS-treated macrophages and to clarify whether VEGFA can alleviate sepsis by inhibiting the M1 polarization of macrophages by inhibiting glycolysis. This project may provide new strategies for the treatment of sepsis.

Materials and Methods

Establishment of animal model

Male C57BL/6 mice (25-30 g) were obtained from the Experimental Animal Center of Nanchang University. The animal research scheme of this project was authorized by the Experimental Animal Ethics Committee of Pingxiang People's Hospital. Under anesthesia, the mouse model of sepsis was constructed by cecal ligation and puncture (CLP).[15] Briefly, the abdominal hair of the mice was shaved, and the exposed skin was disinfected. Then, a small incision was cut along the midabdominal line, and the cecum was exposed and ligated at 3/5 sites. Next, the puncture hole was created by two forward and backward insertions at approximately 0.5 cm above the blind end. Finally, the cecum was repositioned in the abdominal cavity, and the wound was sutured.

Evaluation of VEGFA levels in the CLP model

Twenty CLP mice were euthanized 24 h after modeling, and the serum of normal mice (n=10) and CLP mice was collected. The level of VEGFA in serum was assessed using a Mouse VEGFA ELISA Kit (Biorbyt, Cambridge, UK) following the manual. In addition, the lung tissues were removed, and the relative gene expression of VEGFA was detected by RT–qPCR. Lung tissues were washed with saline and ground in liquid nitrogen. A Total RNA Purification Kit (AmyJet Scientific Inc., Wuhan, China) was applied for RNA extraction. Then, a cDNA synthesis kit (Yeasen Biotechnology Co., Ltd, Shanghai, China) was employed to prepare the reverse transcription reaction system and obtain cDNA. Subsequently, cDNA and specific primers for VEGFA (primer sequences are listed in Table 1) were mixed with SYBR Green Master Mix (Yeasen), and a fluorescence quantitative PCR instrument (SLAN-96S, HONGSHI, China) was used for qPCR and real-time fluorescence detection. The procedure for PCR was as follows: 95°C for 2 min, 95°C for 15 s, 60°C for 30 s, and 40 cycles of amplification.

Table 1. List of primers.

Gene	Primers	
VEGFA	Forward	5'-GCCTAGCACACTGCATCATGACG-3'
	Reverse	5'-ACGTACGCATTATCATCGTAATCA-3'
iNOS	Forward	5'-CAGTTACAGACTACTCGTAGAGTC-3'
	Reverse	5'-GAATGCTACAGACTAGAGCTATG-3'
CD86	Forward	5'-CGCTCGTAGCTGCCATCGATTC-3'
	Reverse	5'-GAATCAGACGTTGAAGATCAGA-3'
Arg-1	Forward	5'-CTGACGATTCGTTAGACTCAA-3'
	Reverse	5'-GACATGTCCATTGACATCAGC-3'
IL-10	Forward	5'-GCTCATCGAGCCTAGTCATTG -3'
	Reverse	5'-TACAGCGTAGATCAACTTGAC -3'
TNF-α	Forward	5'-ACTAAGAGCCATACCAGCTTC-3'
	Reverse	5'-CGATTCGTAGCGATCGTCGGA-3'
IL-1β	Forward	5'-GACTTGACAGCATTCAACTGTC-3'
	Reverse	5'-TCGCTACTAGCAATGACGCCTG-3'
IL-6	Forward	5'-CAGAGTGACGATGCGTACATACT -3'
	Reverse	5'-ATCTAGTAAGTCCGACGATTGA -3'
HIF-1α	Forward	5'-ACCTAGATTCTACGTGCCATAGC -3'
	Reverse	5'-CGAGCTGCAGATCGTCATCGTC -3'
SLC2α10	Forward	5'-GAGATGCAGAAGCTTACCTAGC-3'
	Reverse	5'-CTGAGTCTAGCCACGTTAGAC-3'
GAPDH	Forward	5'-CGGATTGACGGAACTAGACACGTG-3'
	Reverse	5'-GACTTCAGCTGAACTACGGATC -3'

Culture of macrophages

Mouse macrophage RAW264.7 cells were provided by the American Type Culture Collection and cultured in DMEM (Gibco, Waltham, MA, USA) containing penicillin–streptomycin (LMAI Bio, Shanghai, China) and 10% fetal bovine serum (Corning Inc., Corning, NY, USA). The cells were then placed in an incubator (PY-30, Crystal Industries, Addison, TX, USA) containing 5% CO_2 at 37°C.

Construction of macrophages with VEGFA knockdown

To knockdown the *VEGFA* gene, we customized si-NC and si-VEGFA in Dharmacon Inc. (Lafayette, CO, USA), and the RAW264.7 cell model knocking down VEGFA was then constructed: 2×10^5 macrophages were spread in 24-well culture plates, and transfection was carried out when the confluence reached 80%. Six pmol of si-NC or si-VEGFA was diluted into 50 µL Opti-MEM medium (Gibco), and 2 µL of Rfect siRNA/miRNA Transfection Reagent (Baidai Biotechnology Co., Ltd., Changzhou, China) was diluted into 50 µL Opti-MEM medium and incubated for 5 min. The diluted plasmid and transfection reagent were then mixed and incubated at 25°C for 20 min, and the mixture was injected into the plate at 100 µL/well. The plates were placed in the incubator and cultured for 48 h.

Flow cytometry for the detection of macrophage phenotype

si-NC- or si-VEGFA-transfected macrophages were inoculated into 6-well plates until they were attached, and then they were exposed to 100 ng/mL LPS (Absin Bioscience Inc., Shanghai, China) for 6 h to induce macrophage polarization. Afterward, the cells were rinsed clean and digested with trypsin (Boster Biological Technology, Ltd., Wuhan, China), and then the density was adjusted with PBS to 1×10^7 cells/mL. Next, antibodies against F4/80-PE (ab237335, 1:100 dilution; Abcam, Cambridge, UK), CD86-APC (ab218757, 1:1000 dilution; Abcam), or CD206-APC (141707, 1:50 dilution; BioLegend, San Diego, CA, USA) were added and incubated with the cells in the dark for 30 min. After rinsing and resuspending the cells with PBS, the proportion of M1 or M2 macrophages was analyzed by flow cytometry (CytoFLEX, Beckman Coulter, Brea, CA, USA).

RT-qPCR detection of M1/M2 macrophage markers and cytokines

After the transfected RAW264.7 cells were induced by LPS, the reagents were added to extract RNA, and the RT-qPCR experiment was performed according to the steps in paragraph "Evaluation of VEGFA levels in the CLP model" to detect the gene expression of the markers of M1/M2 polarization: iNOS, CD86, Arg-1, IL-10, and the cytokines TNF-α, IL-1β, and IL-6. The primer sequences are listed in Table 1.

Detection of secreted cytokines by ELISA

The levels of TNF-α, IL-1β and IL-6 in the supernatant of RAW264.7 cells in the si-negative control (si-NC), si-VEGFA, si-NC +LPS, and si-VEGFA +LPS groups were detected by using corresponding ELISA kits (Biorbyt). The experiment was performed according to the instructions of the manual, and the colorimetric method was used for detection.

Detection of the energy metabolism of macrophages

The Seahorse XF Cell Energy Metabolism Detection System (Agilent Technologies, Inc., Santa Clara, CA, USA) was applied to perform both glycolysis and mitochondrial stress tests[16]. For the glycolysis stress test, the directions of the Seahorse XF glycolysis stress test kit were followed. In short, the probe plate was hydrated one day in advance, and RAW264.7 cells of si-NC, si-VEGFA, si-NC+LPS, and si-VEGFA+LPS groups were seeded into Seahorse XF24 at 1×10^4 cells/well for 16 h. Then, the DMEM was replaced with glycolysis medium (supplemented with 10 µM L-glutamine) and cultured in an incubator without CO_2 at 37°C for 1 h. Then, glucose, oligomycin, and 2-deoxyglucose were added into the A/B/C wells of the hydrated probe plate, and the plate was put into the instrument for calibration. Next, the cell plate was placed in for on-board detection. After running, the Wave 2.3 Software was applied for data analysis. The mitochondrial stress test was also carried out using a kit purchased from Agilent Technologies, Inc. The processes were the same as those in the glycolysis stress test, except that the DMEM was substituted with mitochondrial aerobic oxidation medium (supplemented with 10 µM L-glutamine, 10 µM sodium pyruvate, and 10 µM glucose), and oligomycin, carbonyl cyanide-4-trifluoromethoxyaniline (FCCP), and an antimycin A/rotenone mixture were added to the A/B/C wells of the hydrated probe plate. The copy number of mitochondrial DNA (mtDNA) was detected by RT–qPCR to evaluate the mitochondrial content. The DNA of RAW264.7 cells was obtained with a DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). Then, the mtDNA content in RAW264.7 cells in the si-NC, si-VEGFA, si-NC+LPS, and si-VEGFA+LPS groups was calculated by RT–qPCR using the copy number of the mitochondria-encoded gene cytochrome C oxidase II (COX II) as the mtDNA copy number and GAPDH as the internal reference. Primer information is shown in Table 1. The ATP content in the four groups of RAW264.7 cells was determined by an ATP assay kit (Abnova, Walnut, CA,USA) following the directions of the manual. In addition, the expression levels of HIF-1α and SLC2a10, which are associated with aerobic glycolysis, were quantitatively analyzed by RT–qPCR.

Animal experiment

Fifteen CLP sepsis model mice were randomly divided into three groups: the model group, si-NC group, and si-VEGFA group. Si-NC- or si-VEGFA-transfected RAW264.7 cells were injected into mice through the tail vein. Each mouse was injected with 0.2 mL of cell suspension at a concentration of 5×10^6 cells/mL. The model group was injected with an equivalent volume of saline. Another 5 normal C57BL/6 male mice were injected with the same amount of saline as a control. Six hours after the treatment, the mice were killed by ether anesthesia, and venous blood was collected. Aspartate transaminase (AST), alanine aminotransferase (ALT), and serum creatinine (Scr) were detected by a biochemical analyzer (VetScanVS2; Abaxis, Union City, CA, US). The contents of TNF-α, IL-6, and IL-10 in the serum were assessed using a mouse TNF-α/IL-6/IL-10 ELISA kit (Mlbio, Shnaghai, China).

Histological analysis of mice

The lung tissues were removed and fixed with 10% formalin for 24 h. The fixed tissues were dehydrated with gradient alcohol and transparent with xylene, then embedded with paraffin and sliced. The slices were then stained with hematoxylin and eosin (H&E) (Biosharp, Hefei, China) to evaluate the lung damage of the mice. Additionally, immunohistochemical (IHC) staining for CD86 and CD206 was performed on the sections. Lung tissue sections were reacted with antibodies anti-CD86 (1:200 dilution, #PA5-88284; Invitrogen, Waltham, MA, USA) or anti-CD206 (1:100 dilution, #PA5-101657; Invitrogen) overnight at 4°C, followed by incubation with HRP-labeled goat anti-rabbit secondary antibody (1:5000 dilution, #31460; Invitrogen) at room temperature for 30 min. DAB chromogenic agent (Keygen, China) was used for color development, and then the nuclei were counterstained with hematoxylin (Biosharp), and the slices were observed and recorded by a microscope (IX73, Olympus, Tokyo, Japan). Lung tissues from healthy mice were used as a negative control. The positive cells showed brown-yellow granules on the cell surface, and the percentage of positive cells and the

intensity of positive staining were used to assess the positive level of immunohistochemical staining.

Statistical analysis

GraphPad Prism 7.0 was used for data processing. All *in vitro* experiments were repeated three times, and the final results are expressed as the mean ± standard deviation. Differences between two or more groups were analyzed by Student's *t*-test or ANOVA; $p<0.05$ indicates a statistically significant difference.

Results

VEGFA is highly expressed in the septic mouse model

To validate whether VEGFA is related to sepsis, we examined the VEGFA content in the CLP sepsis mouse model. In the serum (Figure 1A) and lung tissues (Figure 1B) of the CLP model mice, the level of VEGFA was dramatically higher than that of healthy mice. This means that the high level of VEGFA may be associated with the pathogenesis and progression of sepsis.

Low VEGFA expression inhibits LPS-induced M1 polarization of macrophages

Next, we explored the effect of VEGFA on the polarization of macrophages treated with LPS. First, siRNA technology was used to knock down the expression of VEGFA, and the efficiency of si-VEGFA was about 40% (Figure S1). The ratios of M1 macrophages (F4/80+, CD86+) and M2 macrophages (F4/80+, CD206+) were determined after the transfected cells were induced by LPS. The results showed that the percentage of activated macrophages that were not treated with LPS was very low (<10%), while the cells were strongly activated and polarized by LPS. The ratio of F4/80+ and CD86+ cells in the si-VEGFA + LPS group was lower than that in the si-NC + LPS group (17.84% *vs* 29.36%), while the ratio of F4/80+ and CD206+ cells was significantly higher (31.04% *vs* 15.54%) (Figure 2A). This indicated that the low

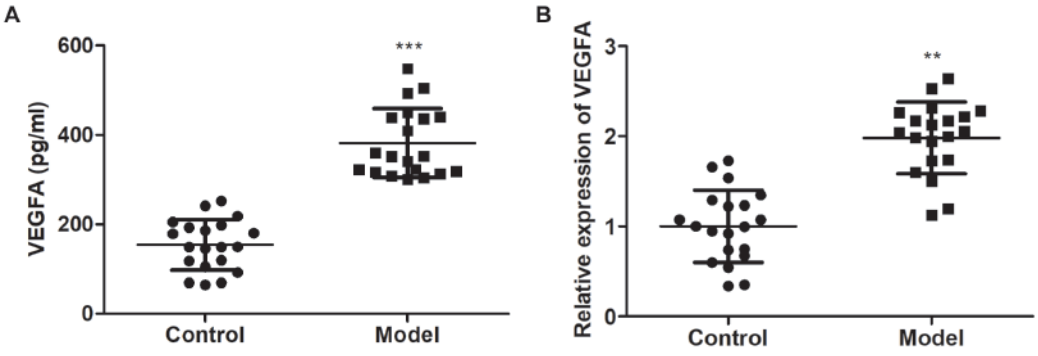

Figure 1. VEGFA is high expressed in sepsis mice model. A) Higher level of VEGFA in the serum of sepsis mice model was determined by ELISA; ***p<0.001. B) Higher expression of VEGFA in the lung tissues of sepsis mice model was confirmed by RT-qPCR; **p<0.01.

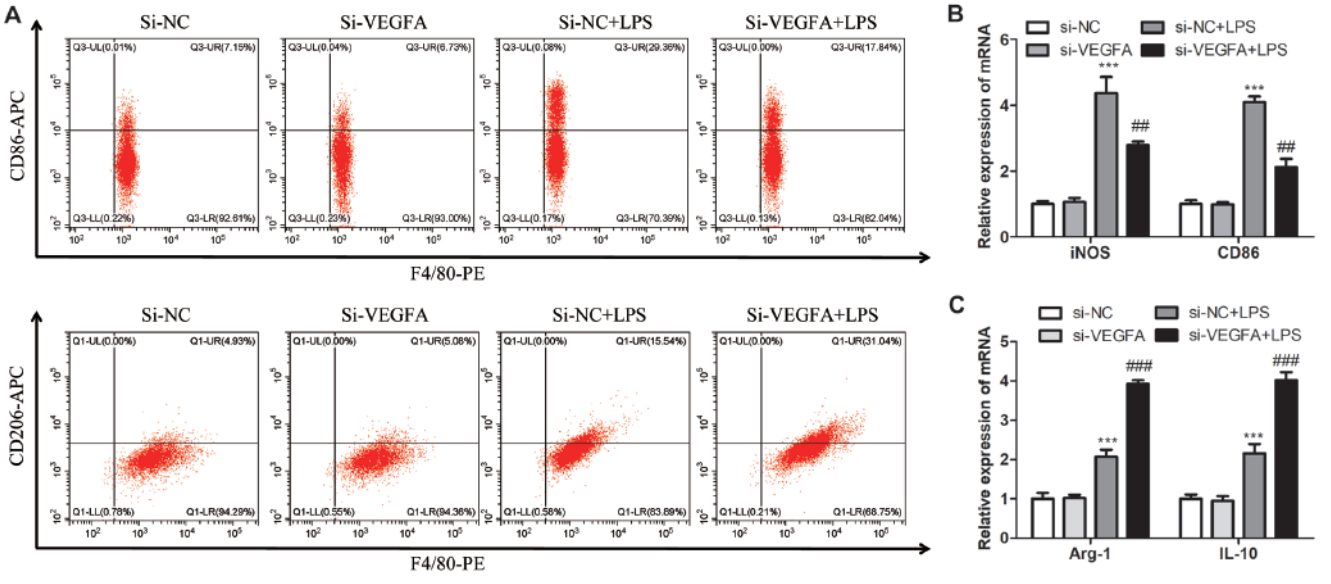

Figure 2. Low expression of VEGFA inhibits LPS-induced M1 polarization of macrophages. A) The proportion of M1 macrophages (F4/80 + CD86 +) and M2 macrophages (F4/80 + CD206 +) in si-NC, si-VEGFA, si-NC +LPS and si-VEGFA +LPS groups was determined by flow cytometry; decreased VEGFA reduced the proportion of LPS-induced M1 macrophages; RT-qPCR was performed to evaluated the relative expression of M1 macrophages markers iNOS, CD86 (B) and M2 macrophages markers Arg-1, IL-10 (C); ***p<0.001 *vs* si-NC group, ##p<0.01, ###p<0.001 *vs* si-NC + LPS group.

level of VEGFA reduced the number of LPS-induced M1 macrophages. In addition, we found that knockdown of VEGFA downregulated the levels of the M1 macrophage markers iNOS and CD86 compared with those in the si-NC + LPS group (Figure 2B) but upregulated the levels of the M2 macrophage markers Arg-1 and IL-10 (Figure 2C). The above results confirmed that low VEGFA expression inhibited LPS-induced M1 polarization of macrophages.

Low expression of VEGFA inhibits the level of proinflammatory cytokines in LPS-induced macrophages

Sepsis is characterized by a high level of inflammatory response. Therefore, we intended to further explore the efficacy of VEGFA knockdown on the secretion of inflammatory cytokines from LPS-treated macrophages. RT-qPCR (Figure 3A) and ELISA experiments (Figure 3B) were performed, and their results were consistent. The expression and secretion of the proinflammatory cytokines IL-1β, IL-6, and TNF-α were induced by LPS, whereas they were markedly reduced due to the knockdown of VEGFA.

Low VEGFA expression inhibits glycolysis

The energy metabolism of M1-polarized macrophages occurs mainly through aerobic glycolysis, while that of M2-polarized macrophages occurs mainly through the mitochondrial oxidative phosphorylation pathway. To clarify whether the regulation of VEGFA knockdown on macrophage polarization is achieved by regulating energy metabolism, we performed a series of assays. Through the glycolysis stress test, we found that LPS induced glycolysis in macrophages, including increased glycolysis, glycolysis capacity, and glycolysis reserve, which was reversed by the knockdown of VEGFA (Figure 4A). Next, we performed a mitochondrial aerobic oxidative stress test, and the results showed that LPS treatment inhibited the mitochondrial aerobic oxidation pathway in RAW264.7 cells, including reductions in basal respiration, respiratory capacity, and respiratory reserve. This inhibition was also reversed by the knockdown of VEGFA (Figure 4B). Furthermore, we found that the copy number of mitochondrial mtDNA and the total ATP content were significantly reduced in RAW264.7 cells treated with LPS, and both the number of mitochondria and ATP content were restored by the knockdown of VEGFA (Figure 4 C,D). Collectively, these results suggested that low VEGFA expression promoted a transition in macrophage energy metabolism from aerobic glycolysis to mitochondrial aerobic oxidation.

Low expression of VEGFA attenuates the symptoms of septic mice and promotes M2 polarization of macrophages

Furthermore, we intended to investigate whether overexpression of VEGFA contributed to the amelioration of sepsis in mice. The levels of ALT, AST, and Scr were higher in the serum of mice in the sepsis model group and the si-NC group, suggesting liver and kidney injury, which was significantly improved after knocking down VEGFA (Figure 5A). The serum levels of the cytokines TNF-α and IL-6, which promote inflammation, were enhanced in the model and si-NC groups, whereas the level of IL-10, which inhibits inflammation, was reduced and reversed by knockdown of VEGFA (Figure 5B). In addition, the si-VEGFA group exhibited a lower degree of lung tissue pathological damage caused by sepsis than the model group and the si-NC group (Figure 5C). Moreover, to determine the effect of knocking down VEGFA on the polarization of macrophages in mouse tissues, we evaluated the level of the M1/M2 macrophage markers CD86/CD206 in lung tissues by IHC. As shown in Figure 5D, compared with the model and si-NC groups, the degree of CD86 staining in the lung tissues of the si-VEGFA group was weakened, while the degree of CD206 staining was enhanced. This suggested that the low expression of VEGFA contributed to the polarization transition of macrophages to the M2 phenotype in the tissues of septic mice. Overall, the results of animal experiments confirmed that the low expression of VEGFA alleviated the symptoms of septic mice and promoted M2 polarization of macrophages.

Discussion

VEGFA has been proven to be an important participator in the progression of various cancers and cardiovascular diseases.[17,18] Studies have also pointed out that VEGFA is correlated with various diseases involving endothelial barrier damage, such as sepsis.[19] Several researchers have found that the VEGFA level is elevated in sepsis patients, and its level is related to the progression of sepsis.[17,20] Here, we also confirmed that VEGFA is highly expressed in the CLP sepsis mouse model. However, the mechanism through which VEGFA participates in the pathogenesis and progression of sepsis has not been clarified.

In the current research, we found that knockdown of VEGFA could inhibit M1 polarization of macrophages induced by LPS,

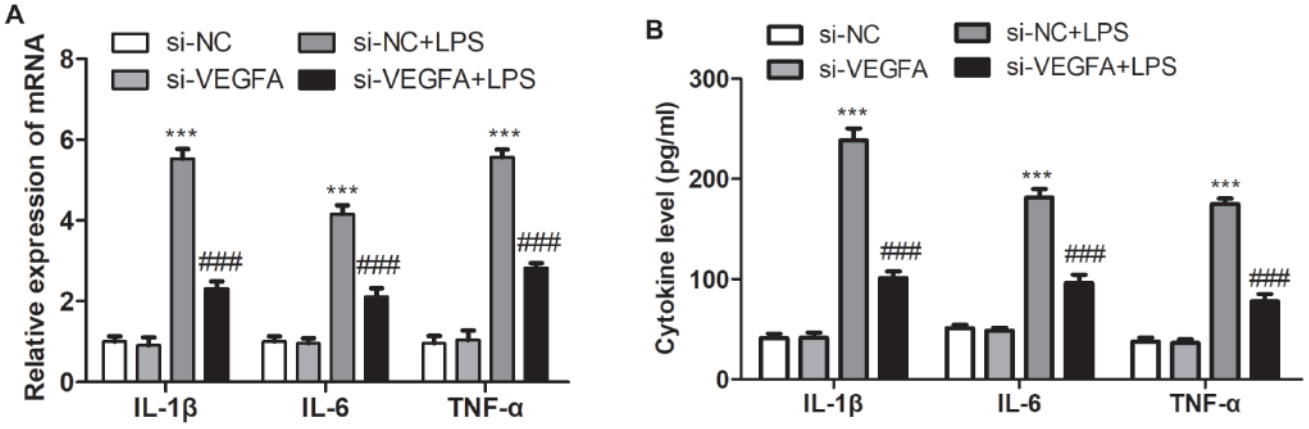

Figure 3. Low expression of VEGFA inhibits the expression and secretion of pro-inflammatory cytokines in LPS-induced macrophages. Relative expression (A) and secretion (B) of IL-1β, IL-6 and TNF-α was examined by RT-qPCR and ELISA, respectively. Low expression of VEGFA reduced the expression and secretion of these cytokines; ***p<0.001 *vs* si-NC group, ###p<0.001 *vs* si-NC + LPS group.

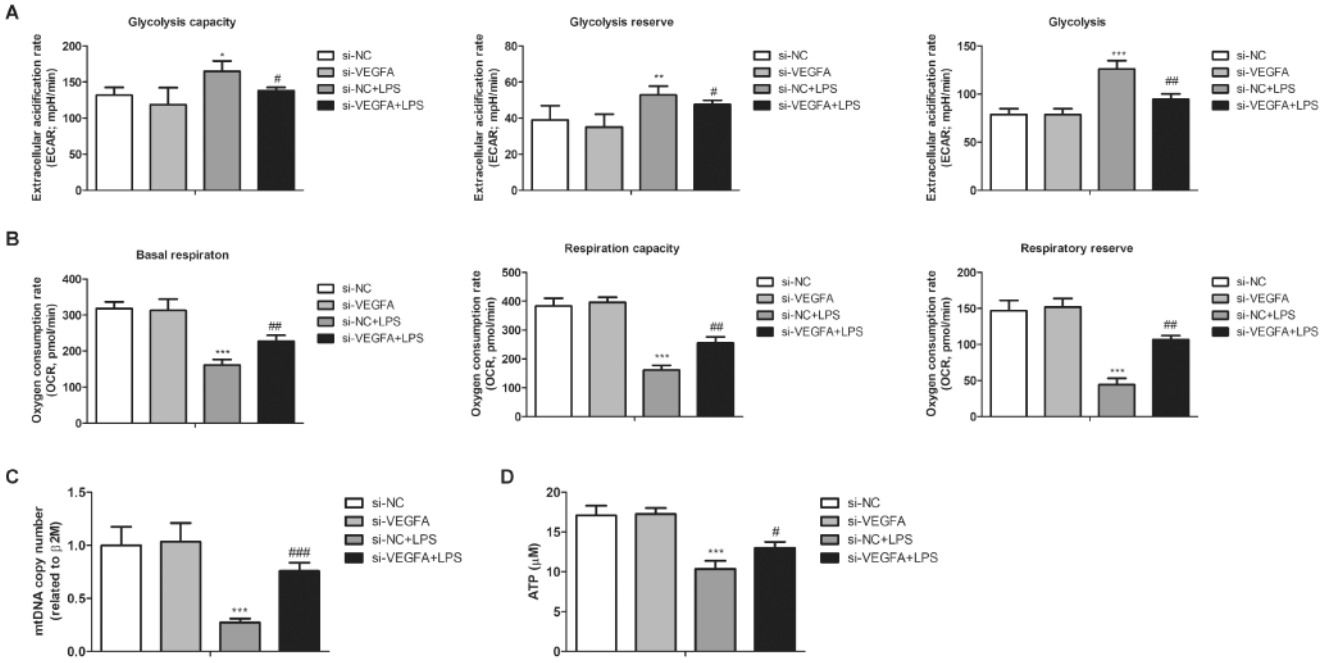

Figure 4. Low expression of VEGFA inhibits the glycolysis induced by LPS. A) Three groups of glycolysis stress test parameters (glycolysis capacity, glycolysis reserve, glycolysis) confirmed that glycolysis in macrophages induced by LPS was reversed by knocking down VEGFA. B) Three groups of mitochondrial stress test parameters (basal respiration, respiration capacity, respiratory reserve) confirmed the enhanced oxidative phosphorylation in macrophages due to the down-regulation of VEGFA. Knockdown of VEGFA increased the number of mitochondria (C) and ATP content (D) in LPS-treated macrophages; *p<0.05, **p<0.01, ***p<0.001 *vs* si-NC group, #p<0.05, ##p<0.01, ### p<0.001 *vs* si-NC + LPS group.

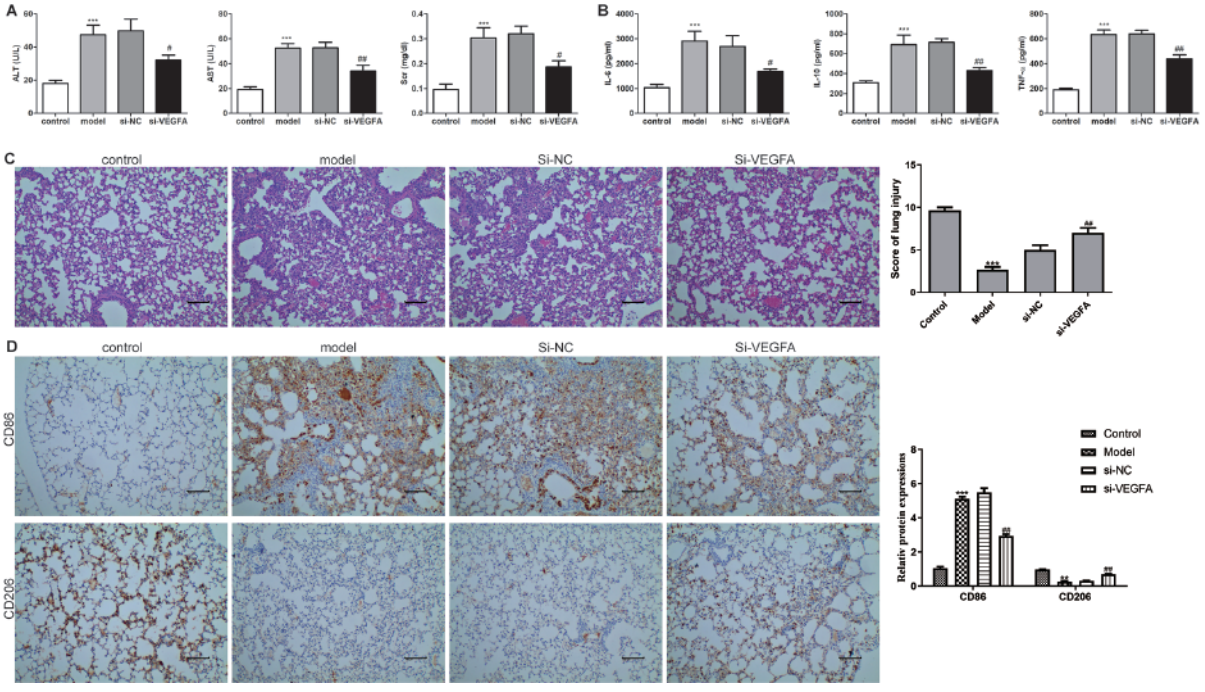

Figure 5. Low expression of VEGFA attenuates the symptoms of sepsis mice and promotes M2 polarization of macrophages. A) Both liver and kidney injury indices were decreased in septic mice injected with si-VEGFA compared with the si-NC group. B) ELISA assay confirmed that the serum levels of TNF-α and IL-6 were decreased in si-VEGFA group, while IL-10 was increased; ***p<0.001 *vs* control group, #p<0.05, ##p<0.01 *vs* si-NC and model group. C) H&E staining and the score results showed that lung injury in si-VEGFA group was obviously alleviated compared with model and si-NC group. D) Immunohistochemical staining showed that CD206 was up-regulated and CD86 expression was down-regulated in si-VEGFA group. The relative expressions of proteins were quantified on the right. Scale bar: 100 μm.

which is manifested by downregulating the expression of the M1 polarization markers iNOS and CD86 and upregulating the expression of the M2 polarization markers Arg-1 and IL-10. In addition, our study demonstrated that knockdown of VEGFA inhibits the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 expressed and secreted by LPS-induced macrophages. Linde *et al*. also found that VEGFA helps establish an anti-inflammatory microenvironment in skin cancer and induces M2 polarization of macrophages with the assistance of IL-4 and IL-10.[21] Wheeler *et al*. found that the addition of VEGF to THP-1 cell medium significantly enhanced the migration of macrophages and induced the transformation from the M1 to the M2 phenotype, suggesting that VEGF plays an important role in macrophage recruitment and M2 polarization induction.[22] Many studies have confirmed that the number of M1 macrophages in the peripheral blood of sepsis patients has markedly increased.[23,24] Combined with the discovery of the high expression of VEGFA in sepsis, we speculate that VEGFA may control the inflammatory response in sepsis by regulating the polarization of macrophages.

At present, it is unclear how VEGFA regulates macrophage polarization. It has been found that metabolic changes are the core to macrophage polarization transformation after infection and tissue injury.[25] LPS-induced proinflammatory M1-type macrophages are characterized by increased aerobic glycolysis and decreased oxidative phosphorylation.[26] In addition, VEGFA has been reported to contribute to glycolysis in cancers. Wan *et al.* found that silencing VEGFA in human umbilical vein endothelial cells significantly inhibited cellular glycolysis and angiogenesis.[10] Zheng *et al*. also found that circMYOF promotes glycolysis by targeting VEGFA and its downstream PI3K/AKT pathway in pancreatic cancer.[27] Therefore, we hypothesized that VEGFA may adjust the polarization state of macrophages by regulating the process of glycolysis. In the current research, we confirmed that the promotion of glycolysis in macrophages by LPS was reversed after transfection with si-VEGFA, while the mitochondrial oxidative phosphorylation process was promoted. VEGFA regulates the polarization state of macrophages by regulating their energy metabolism pathway. Regarding the mechanism through which VEGFA regulates the energy metabolism pathway, there was no further investigation in this study. At present, the mechanism by which VEGFA regulates glycometabolism is not fully understood. Shi *et al.* demonstrated that VEGF promotes glycolysis in pancreatic cancer by upregulating hypoxia-inducible factor 1α.[28] However, related mechanistic research is still lacking.

This research innovatively proposed that a low level of VEGFA promotes the M2 polarization of macrophages by inhibiting glycolysis, thus relieving the inflammation of sepsis and alleviating the symptoms of sepsis. There are still certain limitations: for example, the effect of low levels of VEGFA on glycolysis in lung tissue has not been verified in a sepsis mouse model; moreover, the detailed molecular mechanism of VEGFA involved in the regulation of macrophage glycolysis and polarization in sepsis still needs to be further studied in future work.

In conclusion, this manuscript confirmed the increased expression of VEGFA in sepsis. Knockdown of VEGFA inhibited LPS-induced M1 polarization and the secretion of proinflammatory cytokines in macrophages. Furthermore, we proved that the low expression of VEGFA inhibited the induction of glycolysis in macrophages by LPS. In conclusion, we verified that low expression of VEGFA inhibited glycolysis and thus inhibited the M1 polarization of macrophages induced by LPS, ultimately relieving sepsis.

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