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Slit2/Robo4 signaling pathway modulates endothelial hyper-permeability in a two-event *in vitro* model of transfusion-related acute lung injury

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ABSTRACT

Transfusion-related acute lung injury (TRALI) remains the leading cause of transfusion-related mortality. Endothelium semipermeable barrier function plays a critical role in the pathophysiology of transfusion-related acute lung injury (TRALI). Recently, Roundabout protein 4 (Robo4), interaction with its ligand Slit 2, was appreciated as a modulator of endothelial permeability and integrity. However, not much is known about the role of Slit2/Robo4 signaling pathway in the pathophysiology of TRALI. In this study, the TRALI model was performed by the "two-event" model of polymorphonuclear neutrophils (PMN)-mediated pulmonary microvascular endothelial cells (PMVECs) damage. We investigated the expression of Slit2/Robo4 and VE-cadherin and examined the pulmonary endothelial hyper-permeability in TRALI model. We found that the expression of Slit2/Robo4 and VE-cadherin were significantly decreased in a time-dependent manner, whereas the PMVECs permeability was gradually increased over time in TRALI model. Moreover, the treatment with Slit2-N, an active fragment of Slit2, increased the expression of Slit2/Robo4 and VE-cadherin to protect PMVECs from PMN-mediated pulmonary endothelial hyper-permeability. These results indicate that targeting Slit2/Robo4 signaling pathway may modulate the permeability as well as protect the integrity of endothelial barrier. In addition, Slit2-N appears to be a promising candidate for developing novel therapies against TRALI.

1. Background

Transfusion-related acute lung injury (TRALI) is a clinical syndrome characterized by noncardiogenic pulmonary edema and hyoxemia [1], occurring within 6 h of blood component transfusion [2]. Despite advances in preventive strategies and supportive treatment, TRALI continues to be the leading cause of fatalities from transfusion. Although the mechanism of TRALI has not been completely delineated, the 'two-event' model is thought to best explain its pathophysiology. This model assumed that the 'first hit' induced by surgery, infection or hematologic malignancy primes and attracts recipient's neutrophils to the pulmonary vasculature. This is followed by a 'second event' from the blood transfusion, containing human leucocyte antigen (HLA) or human neutrophil antigen (HNA) antibodies or biologically active lipids. The 'second hit' induced activation of the neutrophils, endothelial injury, capillary leakage and pulmonary edema, resulting in TRALI [3]. Nevertheless, studies showed that patients who expressed HLA or HNA antigen underwent transfusions from HLA or HNA antibodies donors did not cause TRALI, even some patients having already suffered the 'first hit' [4–6]. Thus, these studies indicated that the mechanisms of TRALI were still not fully known.

Endothelial cells serve as a vital protective barrier with its critical role in insulating host tissue from harmful environments and the communication between inflammatory components, coagulation pathways and the microcirculation [7–9]. The disruption of this barrier induces a direct increase in vascular permeability with capillary leakage and interstitial oedema, which play critical role in the development of TRALI [10].

The Slit2/Robo4 (Roundabout 4) signaling pathway is reported to be responsible for regulating the endothelial permeability [7,11–15], although the Slits/Robos were evolutionarily conserved proteins which had a well-recognized role in axon guidance in the nervous system [16,17]. Slit2, expressing in blood and endothelial cells, belongs to a family of three glycoproteins and always binds to the Robo receptors to mediate various signal transductions [18–21], such as leukocyte migration and vascular injury. Among the four Robo proteins, Robo4 is predominantly expressed in endothelial cells and is regarded as a new specific protein for endothelial cell [22,23]. Recently, accumulating

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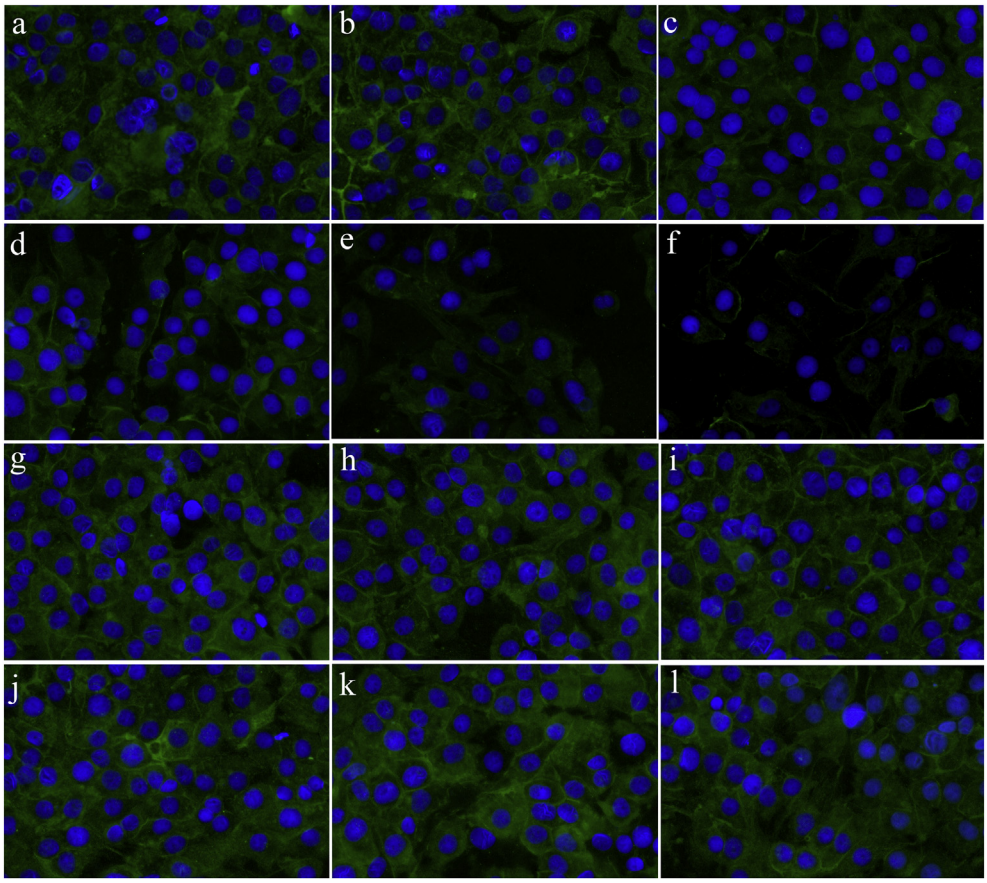

Fig. 1. Immunofluorescent analysis of Robo4 expression in PMVECs. Time-dependent changes of Robo4 expression in TRALI. After PMVECs were exposed to 2 μg/ml LPS for 6 h, the HNA-3a$^+$ PMNs (1×10^6) were added into them and incubated for 30 min. After that, the co-culture was incubated with plasma from the donors with antibodies to HNA-3a$^+$ for 0 h (a), 0.5 h (b), 1 h (c), 2 h (d), 4 h (e) and 6 h (f). g–k were the control groups, g was LPS (2 μg/ml) group, h was LPS (2 μg/ml) + PMNs (HNA-3a$^+$) group, i was LPS (2 μg/ml) + plasma (HNA-3a$^+$) group, j was LPS (2 μg/ml) + plasma (HNA-3a$^-$) group, k was plasma (HNA-3a$^+$) group, l was LPS (2 μg/ml) + PMNs (HNA-3a$^+$) + plasma (HNA-3a$^-$) group. All control groups incubation time was same as the model of TRALI. Original magnification, 400×; Green = Robo4, Blue = DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

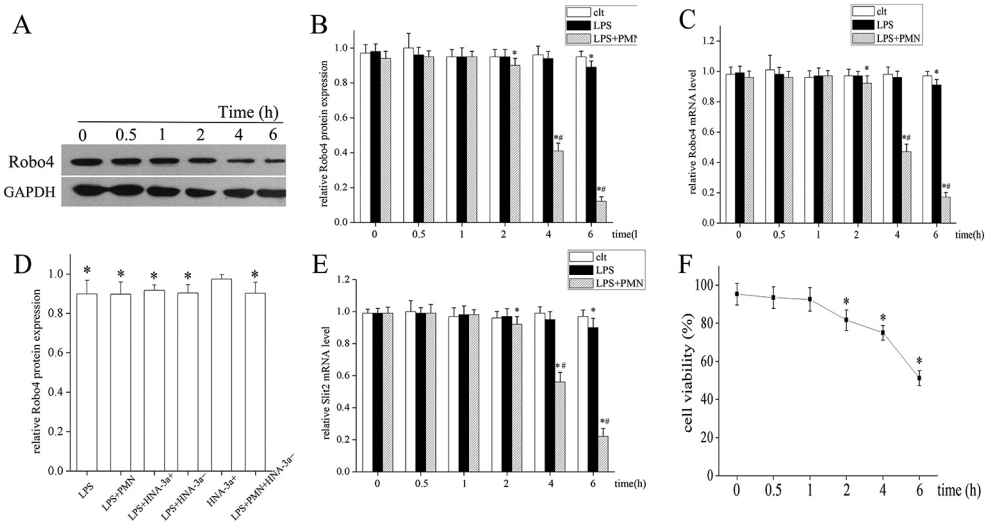

Fig. 2. The expression of Robo4 and Slit2 in TRALI model. A and B Western blot analysis of Robo4 expression (*P < 0.05 compared to control group (0 h), #P < 0.01 compared with LPS group). C RT-PCR analysis of Robo4 expression (*P < 0.05 compared to control group (0 h), #P < 0.01 compared with LPS group). D Western blot analysis of Robo4 expression in control groups (*P < 0.05 compared to HNA-3a$^+$ group). E RT-PCR analysis of Slit2 expression (compared with control group *P < 0.05, compared with LPS group #P < 0.01). Data indicate the means ± SEM of 6 times independent experiments.

studies reveal a role for Slit2/Robo4 in modulating vascular endothelial functions, such as maintaining the endothelial integrity by regulating the VE-cadherin protein [24,25], which is responsible to constitute the adherens junctions of endothelial cells to maintain the endothelium semipermeable barrier function [12,26–29]. Remarkably, little is known about the role of Slit2/Robo4 signaling pathway in the pathophysiology of TRALI. Therefore, we sought to investigate the role of Slit2/Robo4 in PMN-mediated PMVECs damage to mimic the two-hit model of TRALI *in vitro*. We found that the Slit2/Robo4 was significantly decreased and PMVECs permeability was gradually increased over time in TRALI model. We speculate that Slit2/Robo4 signaling may be attractive targets for the treatment of TRALI aimed at controlling the endothelial hyper-permeability.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, USA) N-terminal Slit2 (Slit2-N) protein was obtained from Abcam (Cambridge, USA). Slit2, Robo4 and VE-cadherin polyclonal antibodies were obtained from Abcam (Cambridge, USA). Alexa Fluor 488 and 594-conjugated anti-rabbit IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). GAPDH antibodies were

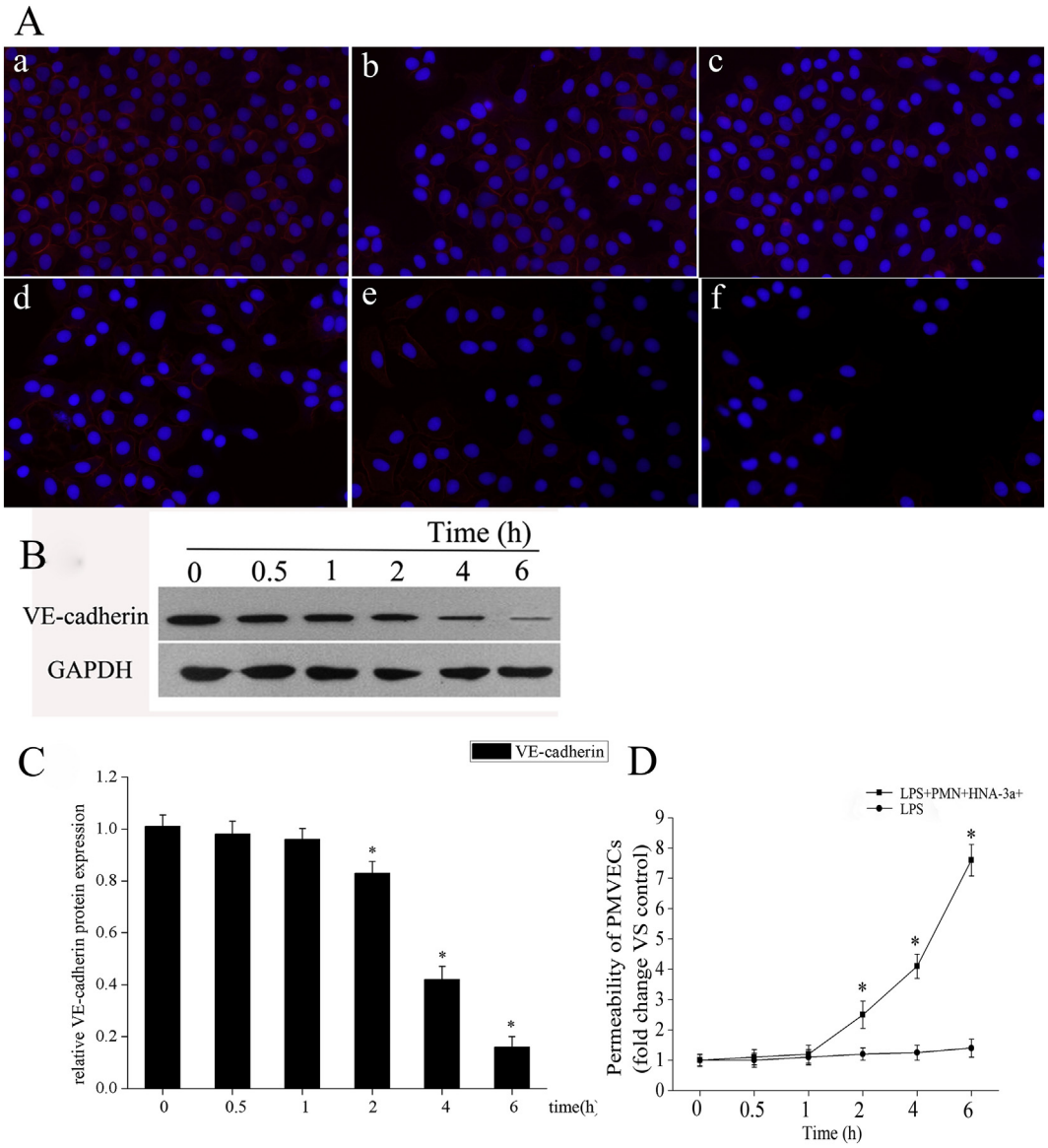

Fig. 3. Time-dependent changes of VE-cadherin expression and the permeability of PMVECs in TRALI. PMVECs were incubated with 2 μg/ml LPS for 6 h, then HNA-3a$^+$ PMNs (1×10^6) were added, incubated for 30 min; and the co-culture was incubated with plasma from the donors with antibodies to HNA-3a$^+$. The co-culture was cultured for 0 h (a), 0.5 h (b), 1 h (c), 2 h (d), 4 h (e) and 6 h (f). A. Immunofluorescent analysis of VE-cadherin expression in PMVECs. Original magnification, 400×; Red = VE-cadherin, Blue = DAPI. B and C Western blot analysis of VE-cadherin expression. D. Permeability Assay showed that the PMVECs permeability was increased in a time-dependent manner. Data indicate the means ± SEM of 6 times independent experiments. *$P < 0.05$ compared to control group (0 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Fetal bovine serum, trypsin and Dulbecco's modified Eargle's medium (DMEM) were purchased from Life Technologies (Grand Island, USA). BCA protein assay kits were from Sigma-Aldrich (St. Louis, USA). Other chemicals not listed here were of the highest grade commercially available.

2.2. Study participants and sample processing

The written informed consent was obtained from donors who had antibodies to HNA-3a, which was approved by the local research ethics committee (Research Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University). PMNs were isolated from whole blood drawn from donors as described by Christopher C et al. [30].

2.3. A two-event in vitro model of PMN-mediated pulmonary endothelial damage

The model of TRALI *in vitro* was performed by the two-hit model of PMN-mediated PMVECs damage as described by Christopher C et al. [30]. PMVECs were incubated with 2 μg/ml LPS for 6 h at 37 °C in 5% CO_2, then the HNA-3a$^+$ PMNs (1×10^6) were added, incubated for 30 min; and the co-culture was incubated with plasma from the donors with antibodies to HNA-3a$^+$ for 0.5, 1, 2, 4 and 6 h. The control groups included LPS (2 μg/ml), LPS (2 μg/ml) + PMNs (HNA-3a$^+$), LPS (2 μg/ml) + plasma (HNA-3a$^+$), LPS (2 μg/ml) + plasma (HNA-3a$^-$), plasma (HNA-3a$^+$), LPS (2 μg/ml) + PMNs (HNA-3a$^+$) + plasma (HNA-3a$^-$). All control groups incubation time was same as the model of TRALI.

2.4. Animals

Adult male Sprague–Dawley rats, weighing 250–300 g, were

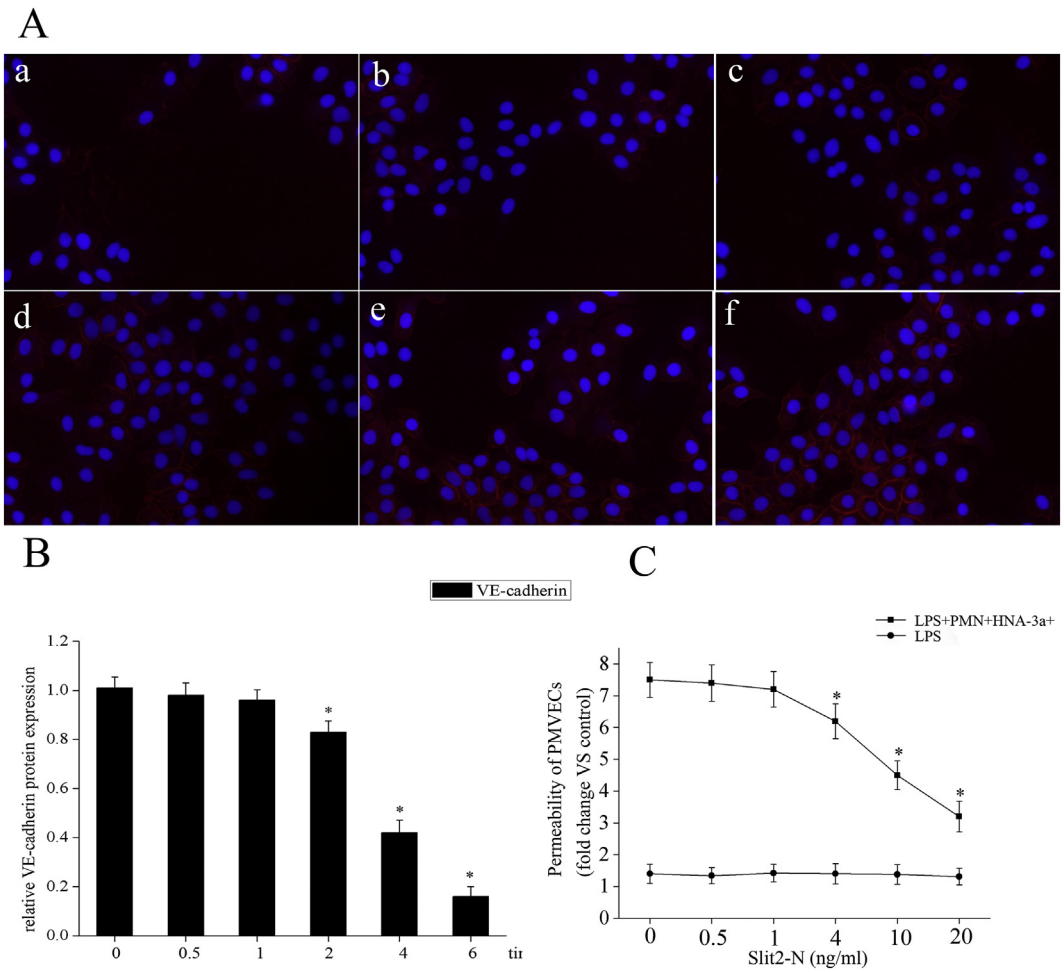

Fig. 4. The expression of VE-cadherin in TRALI with Slit2-N treatment and the effect of Slit2-N for permeability A. Immunofluorescent analysis of VE-cadherin expression in PMVECs (400×). The co-culture was cultured for 6 h, with Slit2-N 0 ng/ml (a) 0.5 ng/ml (b), 1 ng/ml (c), 4 ng/ml (d), 10 ng/ml (e), 20 ng/ml (f) for 24 h. Original magnification, 400×; Red = VE-cadherin, Blue = DAPI. (B) Western blot analysis of VE-cadherin expression. C. Transendothelial Permeability Assay showed that Slit2-N decreased the PMVECs permeability in a dose-dependent manner. Data indicate the means ± SEM of 6 times independent experiments. *$P < 0.05$ compared to control group (0 h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

purchased from Shanghai Experimental Animal Center of China. Rats were housed in plastic cages, provided water and food ad libitum, using a 12 h light/dark cycle, and temperature was maintained at 24 ± 2 °C. The use of animals in this study was approved by the Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

2.5. Primary rats PMVECs isolation, culture, and treatment

Primary PMVECs from Sprague-Dawley rats were isolated as described by Chen et al. with a slight modification [31]. Briefly, the fresh lungs tissues were minced to < 1 mm^3 tissue-blocks after discarding the visceral pleura. The tissue-blocks were place into a culture plate and cultured in a 5% CO_2, 95% air atmosphere in DMEM containing 20% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After 60 h when PMVECs had grown out from the tissues, the remaining tissue was removed by aspiration, to allow cells to reach confluence. Confluent PMVECs were then passaged with 0.25% trypsin treatment. The PMVECs were identified according to morphological by phase-contrast microscopy. Cell viability was assessed by Cell Counting Kit-8. For all experiments, cells were plated in six-well plates and grown to 80% confluence, and cells were serum deprived for 24 h before the addition of LPS.

2.6. Immunofluorescent staining

The cells were fixed in 4% paraformaldehyde (PFA) after rinsed in PBS, then blocked with 10% bovine serum in PBS for 1 h for combining the nonspecific binding. The cells were incubated with the primary antibodies overnight at 4 °C. The cells were rinsed in PBS, then incubated with secondary antibodies for 2 h in the dark. Finally, the cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei staining after being rinsed in phosphate buffered saline (PBS) adequately. Fluorescent images were taken with a fluorescent microscope (Nikon, Japan).

2.7. Western blotting

Proteins were obtained by the following procedures: the suspending cells were mixed with radio immunoprecipitation assay (RIPA) lysis buffer with 1 mM Phenylmethanesulfonyl fluoride (PMSF) on ice for 30 min, ultrasonicated 3 times for 5 s, and followed by centrifugation at 12,000 × *g* for 30 min. The protein concentrations were measured by BCA protein assay kit (Thermo Scientific) according to the manufacturer's instruction. Protein lysates were electrophoresed *via* 10% SDS-PAGE, and the separated proteins were transferred to a polyvinyl difluoride membrane (PVDF). After the membranes were blocked in 5% nonfat dry milk for 1 h at room temperature, they were incubated with

specific primary antibodies overnight at 4 °C, followed by treatment with the appropriate secondary antibodies for 1 h. The proteins expression were detected by enhanced chemiluminescence (ECL) and visualized by UVP Gel imaging system (Upland, CA).

2.8. Quantitative RT-PCR

Total RNA was extracted from the samples using Trizol reagent (Invitrogen), according to the manufacturer's instructions. The total RNA concentration was quantified by ultraviolet spectrophotometry. cDNA was synthesized using the total RNA by reverse transcription using RT-PCR kit (R&D Systems). All RT-PCR products were expressed a percentage change in gene expression relative to internal controls.

2.9. Cell viability assay (CCK-8)

PMVECs were seeded into 96-well plates (1×10^6 cells per well) and incubated overnight at 37 °C in media containing FBS. The cells were performed by the two-hit model of PMN-mediated TRALI as described above and DMSO (control) for 0.5, 1, 2, 4 and 6 h. The cell viability (%) was examined by CCK-8 assay (Beyotime, China) according to the manufacturer's instructions. The fluorescence of each well was measured using a spectrophotometer at an emission of 450 nm (Thermo, USA).

2.10. Transendothelial permeability assay in vitro

PMVECs (1×10^6) were cultured in the Transwell chambers (3-μm pore size, 12-mm diameter) and cultured at 37 °C in a humidified 5% CO_2 atmosphere, maintained until confluence. The cells were incubated with 2 μg/ml LPS for 6 h, then the HNA-3a$^+$ PMNs (1×10^6) were added incubated for 30 min; and the coculture was incubated with plasma from the donors with antibodies to HNA-3a$^+$ for 0.5, 1, 2, 4 and 6 h. The culture medium in the upper chamber of Transwell chambers was replaced by FITC-dextran (100 μl, 1 mg/ml), and samples were taken from the lower chamber. The amount of FITC-dextran was measured with a TECAN GeNios microplate reader (TECAN, Reading, United Kingdom) using an excitation wavelength of 485 nm and emission at 510 nm.

2.11. Statistical analysis

All data are expressed as the means ± SEM. The experimental data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis and graphs were done with Origin 8.0 software. Results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. The expression of Slit2 and Robo4 in TRALI

Firstly, we investigated the dynamic expression of Slit2 and Robo4 in the two-event *in vitro* model of TRALI. Immunofluorescent staining analysis revealed that the reduction of Robo4 gradually changed in time dependence TRALI (Fig. 1). Additionally, the data of RT-PCR and western blot also showed that the expression of Robo4 (Fig. 2A–D) and Slit2 (Fig. 2E) were significantly decreased over time. The viability of the cells in TRALI model was decreased in a time-dependent manner (Fig. 2F).

3.2. The observation of integrity and stability in TRALI

Then we examined the expression of VE-cadherin in PMN-mediated TRALI in different times. Immunofluorescent staining analysis revealed the expression of VE-cadherin was gradually decreased (Fig. 3A). By WB analysis, we observed that the expression of VE-cadherin in TRALI was decreased in a time-dependent manner (Fig. 3B and C). Transendothelial Permeability Assay showed that the PMVECs permeability was increased in a time-dependent manner. The PMVECs permeability was increased significantly at 6 h after PMN-mediated TRALI (Fig. 3D).

3.3. The effect of Slit2-N in PMN-mediated TRALI

On the basis of our previous data, the PMVECs permeability was increased significantly in PMN-mediated TRALI at 6 h. Subsequent, we investigated whether the integrity and stability of PMVECs in PMN-mediated TRALI were associated with the Slit2-N. The PMVECs in PMN-mediated TRALI were incubated with 0.5, 1, 4, 10 and 20 ng/ml Slit2-N for 24 h. Immunocytochemical analysis showed the expression of VE-cadherin was increased compared with the control group (Fig. 4A). WB analysis showed that the expression of VE-cadherin was increased in a time-dependent manner compared with the control group (Fig. 4B). Transendothelial Permeability Assay showed that the PMVECs permeability was decreased in a dose-dependent manner compared with the control group (Fig. 4C).

4. Discussion

In the current study, we investigated the pathogenesis of antibody-mediated TRALI. We observed that LPS-induced PMN-mediated TRALI can down-regulate Slit2 and Robo4 expression significantly in PMVECs, with down-regulate of cell adhesion protein VE-cadherin. The Slit2/Robo4 interaction is responsible for the maintenance of endothelium semipermeable barrier function effects. Exogenous Slit2-N can regulate Slit2/Robo4 to enhance endothelium semipermeable barrier function *via* up-regulate VE-cadherin expression. We conclude from these finds that, Slit2/Robo4 may play a pivotal role in the pathogenesis of PMN-mediated TRALI. To our knowledge, this is the first study to show the role of Slit2/Robo4 signaling pathway in TRALI.

Robo4 is a specificity vascular endothelial cell biomarker which is highly expressed in tumor blood vessels compared to normal blood vessels [14]. Slit2, as the ligand of Robo4, has the role of stabilizing the vascular endothelium by binding to Robo4. Gorbunova [13] reported that Slit2/Robo4 responses within PMVECs that inhibits andes virus induced permeability and adherens junctions disassembly. Bekes [7] and colleagues first time demonstrate that the role of Slit2/Robo4 signal pathway in antagonizing the action of HCG-induced VEGF on endothelial cell adhesion and luteal permeability. Many studies have revealed a role for Slit2/Robo4 in modulating vascular endothelial functions in different diseases, we first explored the effects of Slit2 and Robo4 in TRALI. Our results suggested that the Slit2 and Robo4 were both decrease in TRALI in a time-dependent manner.

Vascular integrity and stability are critical to capillary leakage which is redundantly regulated by factors, receptors and signaling responses that act in concert to maintain endothelium semipermeable barrier function. The VE-cadherin protein, one of the tight junction proteins, consisted of extracellular region, intracellular region, and the transmembrane region which plays a critical role in maintaining the endothelial barrier integrity and stability [32,33]. London et al. [24] found that Slit2-N can significantly reduce pulmonary vascular leak in LPS-treated Robo4+/+ mice and the effect of Slit2-N was lost in Robo4-null mice. *In vivo* and *in vitro* experiments demonstrated that Slit2-N promotes VE-cadherin expression by combining the Robo4, which decrease the cytokine-mediated endothelial permeability. Our study showed that VE-cadherin expression decreased significantly in a time-dependent manner in LPS-induced PMN-mediated TRALI. Exogenous Slit2-N significantly inhibited PMVECs hyperpermeability in a dose-dependent manner. We consider that Robo4 was necessary for the effect of Slit2-N and Slit2-N promotes VE-cadherin expression through Slit2/Robo4 signal pathway.

The PMVECs provide a critical semipermeable barrier between the

blood and tissue, the semipermeability of this barrier is determined by the cell surface adherens junction protein VE-cadherin [33,34]. Semipermeable barrier destruction was key event in the pathophysiology of TRALI. The destruction of VE-cadherin in PMVECs is always consequent with barrier hyper-permeability and lead to TRALI. Thus, the enhancement of the endothelial barrier function was critical in treatment for TRALI. Our study indicated that Slit2-N may be a new drug for the TRALI treatment, including other vascular hyperpermeability disease. But more prospective studies will be required to better understand the treatment effect.

5. Conclusions

In conclusion, our study showed that Slit2, Robo4 and VE-cadherin significantly decreased in the model of TRALI. Moreover, Robo4 was required for the effect of Slit2-N on VE-cadherin–mediated PMVECs semipermeable barrier function. These suggest that Slit2/Robo4 signaling plays an important role in modulating endothelial hyperpermeability in a two-event *in vitro* model of TRALI. In addition, Slit2-N appears to be a promising candidate for managing TRALI or other vascular diseases.

Conflict of interest

The authors declare no conflict of interest.

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CRediT authorship contribution statement

Jie Weng: Writing - review & editing. **Xiaoming Zhou:** Writing - review & editing. **Hui Xie:** Writing - review & editing. **Ye Gao:** Writing - review & editing. **Zhiyi Wang:** Writing - review & editing. **Yuqiang Gong:** Writing - review & editing.

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