IL-9 Promotes the Development of Deep Venous Thrombosis by Facilitating Platelet Function

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Abstract
The development of deep venous thrombosis (DVT) is a sterile inflammatory process related to cytokines, such as interleukin (IL)-6 or IL-17. IL-9 is a cytokine involved in many inflammatory diseases, including cystic fibrosis, ulcerative colitis, psoriasis and psoriatic arthritis. However, it remains unknown whether IL-9 is related to DVT. In this study, we characterized the role and mechanism of IL-9 in DVT. Analysis of the data of patients with and without DVT revealed that stasis, venous surgery as well as elevated IL-9 and sP-selectin levels were related to the development of DVT. We also showed for the first time that IL-9 receptor was expressed in mouse platelets, and it dramatically promoted the aggregation rate and expression of P-selectin (CD62P) in the presence of adenosine diphosphate, but otherwise exhibited no effect on platelets. This study also revealed that Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signalling pathway, not phosphoinositide 3-kinase/AKT pathway, was involved in the process. We also showed in a mouse model of stasis that the thrombus size (weight and length) and CD62P expression in the thrombus were higher and lower in the IL-9 group and IL-9 antibody group, respectively, than in the control group. All these findings indicated that IL-9 facilitated platelet function through the JAK2/STAT3 pathway, thus promoting the development of DVT.

Keywords
► deep vein thrombosis
► IL-9
► platelets
► P-selectin

Introduction
Deep venous thrombosis (DVT) is a major clinical problem with an increased incidence of 30/100,000 in the Chinese population.1 Pulmonary embolism is one of its complications, which often presents as sudden death.2 Our previous studies indicated that interleukin (IL)-17A could facilitate platelet activation and aggregation through the extracellular-signal-regulated kinase 2 pathway, thus promoting the development of arterial and venous thrombosis.3,4 IL-9 is also produced by Th17 cells, which secrete mainly IL-17A and IL-17F.5 When administered alone or with IL-6 and transforming growth factor-b1, IL-9 greatly enhances the production of IL-17 from Th17 cells in vitro.6 Therefore, we hypothesized that IL-9 could promote DVT development by facilitating IL-17A production.

Studies showed that elevated mean volume and count of platelets are related to DVT,7,8 and IL-9 promotes the differentiation of megakaryocytic cells to increase functional platelet count in circulation.9 Thus, we hypothesized that IL-9 could promote the development of DVT by elevating platelet count.

IL-9 promotes the expression of vascular endothelial cell adhesion molecule-1 (VCAM-1) in atherosclerosis,10 and many other studies indicate that the adhesion molecules VCAM-1 and P-selectin are related to DVT.11-16 Therefore, we also hypothesized that IL-9 could exhibit significant effect against DVT.

* The first three authors contributed equally to the study.

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Materials and Methods

Patients

A total of 43 DVT patients diagnosed with lower extremity venous colour Doppler ultrasonic showing the thrombus in at least one iliac vein, femoral vein, popliteal vein or tibial vein, and 35 patients without DVT were randomly recruited from the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The patients were not prescribed any anti-inflammatory, anti-platelet or anti-coagulant drug within 7 days prior to the experiment. Patients without DVT were used as the control group, they were admitted to the hospital for different reasons except arterial or venous thrombosis. The clinical data of patients were collected, and 5 mL blood sample was drawn from the antecubital vein and placed into a vacutainer tube (Beckon Dickinson, California, United States) containing 3.2% sodium citrate. The blood samples were centrifuged at 2,000 \( g \) for 5 minutes and the plasma was stored at \(-80^\circ C\) until analysis. Written informed consent was obtained from the patients and the research was performed in accordance with the guidelines of the Declaration of Helsinki. The study was approved by the Ethics Committee of Tongji Medical College at Huazhong University of Science and Technology.

Enzyme-Linked Immunosorbent Assay

Plasma levels of IL-9 and P-selectin were detected with IL-9 enzyme-linked immunosorbent assay (ELISA) kit (BioOcean, United States) and P-selectin ELISA kit (R&D Systems, United States), respectively, according to the manufacturers' instructions. The sensitivities of IL-9 and P-selectin detection were 0.06 pg/mL and 0.5 ng/mL, respectively. All samples were detected in triplicate.

Mice

Male BALB/c mice aged 6 to 8 weeks and weighing 20 to 25 g were purchased from the Experimental Animal Research Centre of Hubei, China. All mice were maintained in a pathogen-free environment. All experiments involving animals conformed to the guidelines of the Animal Care and Utilization Committee of Huazhong University of Science and Technology.

Preparation of Mouse Platelet, Erythrocyte and Leukocyte

Mouse blood was drawn from the retro-orbital plexus and placed into a vacutainer tube containing 3.2% sodium citrate and 1 mL HEPES–Tyrode buffer (145 mM NaCl, 10 mM HEPES, 5 mM D-glucose, 5 mM KCl, 0.5 mM Na2HPO₄, 1 mM MgSO₄, pH 6.5). Blood sample was centrifuged at 100 \( g \) and 20°C for 30 minutes to obtain the platelet-rich plasma (PRP), and the residue was further centrifuged at 2,000 \( g \) for 15 minutes to obtain the platelet-poor plasma (PPP). Erythrocytes were collected from the final residue, lysed with erythrocytes lysis buffer and centrifuged at 2,000 \( g \) for 5 minutes to obtain leukocytes. The PRP, erythrocytes and leukocytes were washed twice by centrifugation at 600 \( g \) for 10 minutes with phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) containing 100 nM prostaglandin E1. Finally, the washed platelets, erythrocytes and leukocytes were suspended in HEPES-Tyrode buffer. The platelet count was normalized to 10⁹/mL.

Platelet Aggregation Assay

Platelet aggregation was detected with a turbidimetric aggregation monitoring device (AggRAM, Helena Laboratories, Texas, United States). The mice platelets were used for platelet aggregation assay. The PPP was used as a standard for 100% aggregation. PRP was pre-warmed to 37°C in a resting state and then incubated with 0.05, 0.1, 0.15, 0.2 and 0.4 \( \mu g/mL \) IL-9 for 2 minutes respectively. Subsequently, 3 \( \mu M \) adenosine diphosphate (ADP) was added to the plasma to induce aggregation. To examine the Janus kinase 2 (JAK2) inhibitor AG490 (Sigma, United States), PRP was pre-incubated with 25 \( \mu M \) AG490 for 0.5 hour. Untreated PRP was used as a control (CTRL). Platelet aggregability was expressed as the maximum aggregation rate within 100 seconds.

Flow Cytometry

Mice washed with PRP were incubated with AG490 for 0.5 hour, treated with 0.2 \( \mu g/mL \) IL-9 (R&D Systems) for 2 minutes and incubated with ADP for 5 minutes. Untreated plasma sample was used as a control. Platelets were then incubated with purified anti-phycocerythrin-conjugated anti-CD62P antibody and fluorescein isothiocyanate-conjugated anti-CD61 antibody for 30 minutes, washed with HEPES–Tyrode buffer by centrifugation at 500 \( g \) for 5 minutes and re-suspended in 200 mL flow cytometry buffer. Finally, the platelet samples were analysed using a flow cytometer (BD Biosciences, California, United States).

Western Blotting

Mice platelets, erythrocytes and leukocytes were collected just as mentioned above, then the cells were lysed in radioimmunoprecipitation assay protein lysis buffer containing protease inhibitors and phosphatase inhibitor. Protein samples (30 μg) were electrophoresed using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and electrotransferred for 2.5 hours using semi-dry transfer (Bio-Rad, Hercules, California, United States). The membranes were blocked with Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-base, 100 mM NaCl and 0.01% Tween 20) containing 5% fat-free dry milk for 2 hours at room temperature, washed three times with TBST and incubated at 4°C for 24 hours with various primary antibodies, namely, IL-9 receptor (IL-9R) antibody (Santa Cruz Biotechnology, United States, 1:200), phosphor-JAK2 (CST, United States, 1:1,000), JAK2 (Abcam, United States, 1:1,000), phosphor-signal transducer and activator of transcription 3 (STAT3) (Abcam, 1:50,000), STAT3 (Abcam, 1:50,000), phosphor-AKT (CST, 1:1,000), AKT (CST, 1:1,000), IL-9R antibody (Santa, Europe, 1:200) and CD62P antibody (Novus, United States, 1:500). Afterwards, the membranes were washed in TBST three times and incubated with horseradish peroxidase-conjugated secondary antibodies (CST, 1:2,000) for 2 hours at room temperature. Finally, the protein membranes were washed and treated with enhanced cheluminescence reagent (Thermo Scientific, United States) for visualization.
The protein bands were semi-quantitatively analysed using densitometry.

**DVT Mouse Model**

A mouse stasis model of DVT was established using the following method. The male BALB/c mice were anaesthetized with 4% chloral hydrate at a dose of 12 mL/kg. Following a 2-cm-long median abdominal incision, the inferior vena cava (IVC) was exposed, separated from the abdominal aorta and ligated by a 4-0 Prolene suture below the renal veins. Thus, a complete stasis model of DVT was successfully established. IL-9 (500 ng/mouse), anti-IL-9 antibodies (100 μg/mouse) (R&D Systems) and PBS solution were subsequently injected intraperitoneally. At 24 hours post-injection, the thrombus was isolated from the IVC, weighed and measured for length using a vernier caliper.

**Immunohistochemical Staining of the Thrombus**

Immunohistochemistry was performed on paraffin-embedded thrombus sections. Following deparaffinization, thrombus sections were rehydrated with distilled water and graded ethanol. Endogenous peroxidases were blocked by 3% H2O2 and antigen was retrieved with 10 mM citrate buffer (pH 6.0) in a microwave oven. After being blocked with normal species-specific serum, the sections were incubated with anti-CD62P (Novus, 1:50) for 24 hours at 4°C. Finally, the sections were counterstained with haematoxylin, dehydrated and cover-slipped. The immunoreactivity was assessed on a light microscope (Olympus, Japan).

**Statistical Analysis**

Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using the two-tailed Student’s t-test or one-way analysis of variance for comparison between two groups or more than two groups, respectively, using SPSS 11.0 (IBM, United States). The statistical significance was defined as p < 0.05.

**Results**

**IL-9/sP-Selectin Plasma Levels and Clinical Characteristics of Patients**

To explore whether IL-9 and P-selectin were associated with DVT, we detected the plasma IL-9 and P-selectin levels of patients. The plasma levels of IL-9 (p = 0.032) (Fig. 1A) and sP-selectin (p < 0.0001) (Fig. 1B) in DVT patients (DVT group) were prominently higher than those in patients without DVT (control group). Clinical characteristics of the patients are listed in Table 1, in which stasis (p = 0.024) and venous surgery (p = 0.033) were found to be associated with the development of DVT (p < 0.05). Therefore, stasis, venous surgery as well as high levels of serum IL-9 and P-selectin were related with the development of DVT.

**IL-9R Expression on Platelets**

Western blotting was performed to detect the IL-9R expressed on platelets. IL-9R expression was detected on platelets and leukocytes (the positive group), but not on erythrocytes (the negative group) (Fig. 2A).

**Table 1** The clinical characteristics of patients

<table>
<thead>
<tr>
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<th>DVT (n = 43)</th>
<th>Control (n = 35)</th>
<th>p-Value</th>
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<tr>
<td>Sex, male (n)</td>
<td>22</td>
<td>16</td>
<td>0.632</td>
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<tr>
<td>Age, mean (y)</td>
<td>56.3 ± 2.1</td>
<td>59.1 ± 2.1</td>
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<td>Hypertension (n)</td>
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<td>8</td>
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<td>Diabetes (n)</td>
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<td>3</td>
<td>0.658</td>
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<tr>
<td>Smoking (n)</td>
<td>18</td>
<td>18</td>
<td>0.399</td>
</tr>
<tr>
<td>Stasis (n)</td>
<td>19</td>
<td>7</td>
<td>0.024</td>
</tr>
<tr>
<td>Venous surgery (n)</td>
<td>10</td>
<td>2</td>
<td>0.033</td>
</tr>
<tr>
<td>P-selectin (ng/mL)</td>
<td>39.31 ± 3.79</td>
<td>17.65 ± 1.56</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL-9 (pg/mL)</td>
<td>9.76 ± 1.03</td>
<td>6.95 ± 0.65</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Abbreviations: DVT, deep venous thrombosis; IL, interleukin; SEM, standard error of the mean.

Note: Data are presented as mean ± SEM or numbers.
IL-9 Enhancing Platelets Aggregation and Activation Induced by ADP

Platelet aggregation assay was performed to investigate the effect of IL-9 on platelet aggregation. We found that the effect of IL-9 on platelet aggregation induced by ADP was concentration-dependent, and it reached maximum at 0.15 μg/mL (Fig. 2B). We confirmed that IL-9 significantly enhanced platelet aggregation in the presence of ADP (n = 3, p = 0.0448) (Fig. 2E, F), but otherwise exerted no effect on platelet aggregation (n = 4, p = 0.8813) (Fig. 2C, D).

**Fig. 2** Interleukin-9 receptor (IL-9R) expression and aggregation rate of mouse platelets. (A) IL-9R was expressed on mouse platelets. IL-9R, IL-9 receptor; P, platelets; W, white blood cells (positive control); R, red blood cells (negative control). (B) Platelet aggregation induced by adenosine diphosphate (ADP) (3 μM) and combined with various concentrations of IL-9 at 0.05, 0.1, 0.15, 0.2 and 0.4 μg/mL, respectively, n = 3. *p < 0.05 versus ADP group. (C) Platelet aggregation in mice treated with IL-9 alone or CTRL (untreated). (D) The statistical analysis results of the ratio of platelet aggregation in mice treated with IL-9 alone or CTRL (untreated), n = 4. (E) Platelet aggregation in mice treated with ADP or ADP + IL-9. (F) The results of statistical analysis on the ratio of platelet aggregation in mice treated with ADP or ADP + IL-9, n = 3. *p < 0.05 versus ADP group.
IL-9 Promoted CD62P Expression in Mouse Platelets

To investigate the direct platelet-activating effect of IL-9, we detected the expression of the platelet membrane protein CD62P, a marker of platelet activation. CD62P levels were higher in platelets treated with IL-9 and ADP than those treated with ADP alone ($n = 3$, $p = 0.0161$). In contrast, no significant difference in CD62P levels was detected between CTRL and IL-9 group ($n = 3$, $p = 0.8886$) (Fig. 3A, B).

IL-9 Enhanced Platelet Function through JAK2-STAT3 Pathway

To explore the signalling pathways associated with the effects of IL-9 in platelets aggregation and activation, we used Western blotting to detect the phosphorylation levels of JAK2, STAT3 and AKT. The phosphorylation levels of JAK2 ($n = 3$, $p = 0.0335$) and STAT3 ($n = 3$, $p = 0.0264$) in the group treated with ADP and IL-9 were higher than those in the ADP group.
respectively. Data showed that the length of the thrombus were increased by IL-9 (n = 6, p = 0.0133) and decreased by IL-9 antibody (n = 6, p = 0.0426) (Fig. 6B).

To detect the expression of CD62P in the thrombus, immunohistochemical staining and Western blotting were performed. CD62P were expressed in all the thrombus, with the IL-9 group showing a larger positive area (n = 6, p = 0.0016) than that of the anti-IL-9 antibody group (n = 6, p = 0.0066) (Fig. 7A, B). CD62P expression was also measured by Western blot analysis. The data showed that CD62P expression was higher in the thrombus of the IL-9 group (n = 3, p = 0.0032) than in the IL-9 antibody group (n = 3, p = 0.0005) (Fig. 7C, D). These findings indicated that IL-9 facilitated the P-selectin expression in the thrombus.

**Discussion**

This study showed that IL-9 played a crucial role in the development of DVT. The clinical data showed that stasis and the serum levels of IL-9 and P-selectin were associated with the development of DVT. IL-9R was expressed in the mouse platelets, whereas IL-9 treatment markedly increased the aggregation rate and P-selectin expression of platelets in mice through the JAK2/STAT3 pathway. Furthermore, the study on DVT mouse model revealed that IL-9 increased the thrombus size and P-selectin level in the thrombus.

Stasis and venous surgery were found to be associated with DVT in this study. According to the Virchow triad, blood hypercoagulability, blood flow restriction and pro-thrombotic changes in the vessel wall play important roles in thrombosis. In this study, we revealed for the first time that the plasma IL-9 levels in DVT patients were notably increased than in patients without DVT. We thus concluded that IL-9 was associated with the development of DVT. In addition, we found that sP-selectin was also involved in DVT, which is in agreement with multiple published studies. P-selectin is a type of cell adhesion molecules belonging to the selectin family, which are mainly stored in the platelets and widely known to play a crucial role in DVT. Therefore, we suspected that there may be a relation between IL-9 and platelets in the development of DVT.

To reveal the direct effect of IL-9 on platelets, we examined the expression of IL-9R in mouse platelets. Our study is the first to prove that IL-9R was indeed expressed in platelets. Based on this result and a literature study, we concluded that IL-9 could significantly enhance the ADP-induced platelet aggregation, which is dependent on platelet activation and marked by the expression of adhesion molecule P-selectin (CD62P). Furthermore, the flow cytometry analysis in this study showed that membrane CD62P expression was increased in platelets treated with IL-9 and ADP than in those treated with ADP or IL-9 alone. P-selectin/P-selectin glycoprotein ligand 1 interaction plays a central role in thrombus formation. It promotes platelet–leukocyte aggregation and facilitates the formation of platelet thrombus. Taken together, the present results showed that IL-9 facilitated platelet aggregation and activation, thus promoting the development of DVT.
Fig. 5 The effect of AG490 on platelet function. (A) Representative pictures of the effect of AG490 on Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) and phosphoinositide 3-kinase (PI3K)/AKT phosphorylation. (B) The results of statistical analysis on the intensity ratio of JAK2/STAT3 in the AG490-treated group, $n = 3$. (C) Representation of the effect of AG490 on platelet aggregation. (D) The results of statistical analysis on the ratio of platelet aggregation in mice treated with adenosine diphosphate (ADP), ADP + IL-9 and AG490 + IL-9 + ADP, $n = 3$. * $p < 0.05$ versus ADP and IL-9 + ADP group.
The binding of IL-9 and IL-9R mainly activate the JAK/STAT pathway.\textsuperscript{18} Moreover, several studies showed that signalling pathways such as JAK2/STAT3 and phosphoinositide 3-kinase/AKT played important roles in platelet activation.\textsuperscript{19–21} In this study, the phosphorylation levels of JAK2 and STAT3 in the group treated with ADP and IL-9 were higher than those in the ADP group and IL-9 group, whereas the phosphorylation level of AKT was not significantly increased. Pre-incubation of platelets with AG490 also induced the aggregation and activation of platelet, suggesting that AG490 inhibited the promoting effect of IL-9 on the platelet aggregation and activation. We hence concluded that IL-9 facilitated platelet aggregation and activation through the JAK2/STAT3 pathway.

Platelets play a crucial role in the formation of DVT, especially in the initial stage.\textsuperscript{17,18} Brill et al indicated that von Willebrand factor–platelet interaction played a pathogenetic role in flow disturbance-induced venous thrombosis.\textsuperscript{17} To verify whether IL-9 promoted the development of DVT by facilitating platelet function, we established a mouse model of DVT. Based on the weight and length of the thrombus in the mouse model, we can say that IL-9 did promote DVT development, and the immunohistochemistry analysis of the thrombus indicated that IL-9 treatment resulted in a higher percentage of areas stained positive for P-selectin, indicative for an increased number of platelets in the thrombus.

There were some limitations to our study. Since we used exogenous IL-9 or anti-IL-9 neutralizing monoclonal antibody, the findings of this study should be verified by using IL-9 knockout or transgenic IL-9 mice. The mechanism underlying the synergetic effect of IL-9 and ADP on platelet aggregation and activation should also be further explored.

Despite these limitations, our study is still the first to prove the relationship between IL-9 and DVT. Furthermore, we showed that IL-9 facilitated platelet aggregation and activation through JAK2/STAT3 pathway, thus promoting the development of DVT. This study provided a new target for the treatment and prevention of DVT. The blocking of IL-9 in DVT patients may be a promising therapeutic method against the development of DVT.
Fig. 7  P-selectin expression in thrombus. (A) Representative immunohistochemical staining pictures of P-selectin expression in the different groups. (B) The statistical analysis results of the percentage of P-selectin positive area in thrombus, n = 6. The values were expressed as means ± standard error of the mean (SEM). *p < 0.05 versus CTRL. (C) Representative pictures of P-selectin expression in the different groups analysed by Western blotting. (D) The statistical analysis results of the Western blotting of P-selectin expression in the different groups, n = 3. *p < 0.05 versus CTRL (mice injected with phosphate-buffered saline [PBS]).
What is known about this topic?
- Our previous studies indicated that IL-17A could facilitate platelet activation and aggregation through the ERK2 pathway, thus promoting the development of arterial and venous thrombosis. IL-9 is also produced by Th17 cells, which secrete mainly IL-17A and IL-17F.
- IL-9 promotes the expression of vascular endothelial cell adhesion molecule-1 (VCAM-1) expression in atherosclerosis, and many other studies indicate that the adhesion molecule VCAM-1 and P-selectin are related to DVT.
- Studies showed that elevated mean volume and count of platelets are related to DVT, and IL-9 promotes the differentiation of megakaryocytic cells to increase functional platelet count in circulation.

What does this paper add?
- Our study is the first to prove the relationship between IL-9 and DVT.
- Our study is the first to prove that IL-9R was indeed expressed in platelets.
- We showed that IL-9 facilitated platelet aggregation and activation through JAK2/STAT3 pathway, thus promoting the development of DVT.

References