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M1-macrophage polarization is upregulated in deep vein thrombosis and contributes to the upregulation of adhesion molecules

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ABSTRACT

Deep vein thrombosis (DVT) is characterized by high acute fatality rate due to pulmonary embolism and by serious long-term complications. The risk of DVT development is increased in many medical conditions, such as trauma, cancer, and surgery. However, DVT can also occur as an idiopathic disease without clearly identifiable causes. To investigate the pathogenesis of idiopathic DVT, the involvement of circulating monocytes and macrophages was examined. Data showed that circulating monocytes and monocyte-derived macrophages from DVT patients presented significantly elevated M1-polarization, characterized by higher IL-6 and higher TNF-α than corresponding cells from controls. Macrophages from DVT patients were more potent at stimulating endothelial cell-mediated expression of adhesion molecules, including SELE, ICAM1, and VCAM1, than macrophages from controls. M1-polarization, but not M2-polarization, could profoundly upregulate the expression of adhesion molecules. This upregulation was dependent on direct cell-to-cell contact, as well as on contact-independent TNF-α expression. IL-10 expression, on the other hand, significantly reduced the upregulation of adhesion molecules. Together, this study demonstrated that circulating monocytes and macrophages could contribute to the pathogenesis of idiopathic DVT.

1. Introduction

Deep vein thrombosis (DVT) and pulmonary embolism (PE), a severe complication of DVT, together represent the third most prevalent pathology in the cardiovascular system [1]. The risk of DVT development is also increased in many medical conditions, such as trauma, cancer, and surgery, but in many patients, the DVT is considered idiopathic due to the lack of a clear explanation [2–4]. Stasis of the blood flow, likely due to lying position and immobility is considered the major cause of idiopathic DVT [3]. Currently, DVT is mainly treated using anticoagulants, such as thrombin inhibitors, Factor Xa inhibitors, and heparin [5]. These agents are effective at preventing further thrombosis but are unable to resolve existing thrombus. Complications, such as post-thrombotic syndrome and PE, can occur as the result of unresolved thrombus [6]. Extensive research is needed to elucidate the various mechanisms that participate in the initiation, progression, and resolution of DVT.

Inflammation is increasingly recognized as a pathway through which various conditions initiate and propagate DVT [7,8]. Damage to the endothelial wall and hypercoagulation are two of the preceding events in thrombus formation [5]. Inflammation can induce the expression of cell adhesion molecules (CAMs) on leukocytes and the expression of P- and E-selectins on endothelial cells, which function together to facilitate the infiltration of immune cells into the endothelial wall [9–11]. These immune cells can release chemokines and cytokines that further elevated the inflammation status in a positive-feedback loop. Additionally, proinflammatory cytokines, such as IL-6, IL-8, and TNF-α, may directly induce tissue factor and promote coagulation [12–15]. Overall, mounting evidence supports that DVT can develop due to pathological response from inflammatory molecules, immune cells, endothelial cells, and the coagulation system. Better understanding of the interplay between these factors is required for the development of therapeutic and preventative measures.

Macrophages are critical modulatory cells in the immune system.

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Activated macrophages display a spectrum of responses, with the classically activated M1 macrophages and the alternatively activated M2 macrophages at the polar ends [16,17]. The M1 macrophages express high level of MHC class II, which enables presentation to CD4 T cells, and high level of costimulatory molecules CD80/CD86. The expression of SOCS3 and NOS2 is also increased. Additionally, M1 macrophages secrete IL-1β, TNF-α, IL-12, IL-18, and IL-23. The M2 macrophages, on the other hand, preferentially express IL-4, IL-10, IL-13, and TGF-β. Both M1 and subsets of M2 macrophages can express IL-6. Via these molecules, macrophages can potently modulate the inflammation of both immune cells and non-immune cells, including the endothelial cells.

The underlying mechanisms, including the potential involvement of the immune system, remain unclear during the development of idiopathic DVT. In addition, since idiopathic DVT lacks identifiable risk factors, advanced warning signs and preventative measures are usually not in place prior to its development. We hypothesized that monocyte/macrophage responses could contribute to the development of idiopathic DVT. In this study, the expression of effector molecules by monocytes and macrophages from DVT patients, and their effects on endothelial cells, were investigated.

2. Method

2.1. Participants

The diagnosis of DVT was performed using ultrasound. Twenty-one patients with idiopathic unilateral lower extremity DVT but not PE were recruited at Qianfoshan Hospital. Twenty-one patients, including 11 females and 10 males between 41 and 65 (median 55) years of age were recruited. Twenty-one healthy controls, including 11 females and 10 males between 43 and 65 (median 55) years of age, were also recruited at the same hospital. Patients were considered idiopathic if the DVT occurred in the absence of hereditary thrombophilia, antiphospholipid syndrome, previous history of DVT, and other conditions that were known to associate with elevated DVT risk, such as cancer, inflammatory bowel diseases, paralysis, trauma and surgery within the past six months, and immobility due to prolonged flying within the past two weeks. Additional exclusion criteria were age outside of the 18 to 65 years range, severe hepatic or renal failure, pregnancy within 6 months of sample collection, and autoimmune diseases. All patients and controls provided written informed consent. The Ethics Committee of Qianfoshan Hospital approved this study.

2.2. Monocyte enumeration and isolation

Peripheral blood was collected from all controls and untreated patients immediately after the diagnosis was made by ultrasound. Peripheral blood mononuclear cells (PBMCs) were isolated via gradient centrifugation using Ficoll reagent (GE Healthcare). Untouched monocyte was isolated using EasySep Human Monocyte Enrichment kit (Stemcell) with purity greater than 96%, confirmed using flow cytometry staining. The number of live PBMCs and monocytes were counted using Trypan Blue (Thermo Fisher) staining, and the frequency of circulating monocytes in PBMCs was calculated as the number of live monocytes per 10^6 live PBMCs.

2.3. Derivation and stimulation of macrophages

Isolated monocytes were plated at 2×10^5 cells per mL in RPMI 1640 supplemented with 15% heat-inactivated FBS, 1% L-glutamine and 1% penicillin-streptomycin (Thermo Fisher). On day 6, monocyte-derived macrophages were either harvested or stimulated for 12 h with 0.1 μg/mL LPS (Sigma). For M1 and M2 polarization, the M1- or M2-Macrophage Generation Medium (PromoCell) was applied according to the manufacturer's instructions, and then stimulated with LPS as described above. Secretion of IL-6, TNF-α, and IL-10 in the supernatant was examined using Human IL-6, TNF-α, and IL-10 Quantikine ELISA kits (R&D Systems), respectively, according to the manufacturer's protocols.

2.4. Macrophage-HUVEC coincubation

Human umbilical vein endothelial cells (HUVECs; ATCC CRL-1730) were incubated in F-12K medium (ATCC) at 2×10^5 cells per mL. For coincubation with macrophages, HUVECs and monocyte-derived macrophages, M1-polarized macrophages, or M2-polarized macrophages were incubated at 1/1 ratio. In some experiments, macrophages and HUVECs were separated using a 1.0-μm pore-sized membrane in a Transwell plate (Corning). Neutralizing antibodies, including MAB2061 (αIL-6), AF-210-NA (αTNF-α), and MAB217 (αIL-10), were purchased from R&D Systems and applied at 5 μg/mL each in the experiments when indicated.

2.5. Gene expression

Monocytes, macrophages, and HUVECs were processed using RNeasy mini kit (Qiagen) for the collection of total RNA. cDNA was then synthesized from mRNA templates using Superscript III Reverse Transcriptase kit (Invitrogen). The following Taqman assays were purchased from Thermo Fisher and used to examine the gene expression in the cells, including Hs00174131_m1 (IL-6), Hs00174128_m1 (TNF), Hs00961622_m1 (IL10), Hs00174057_m1 (SELE), and Hs00164932_m1 (ICAM1), and Hs01003372_m1 (VCAM1). PCR reactions were processed using SYBR Green Master Mix (Thermo Fisher) in the 7500 Real-Time PCR System (Applied Biosystems). Results were analyzed via the standard $2_T^{-\Delta\Delta C}$ method.

2.6. Statistics

Data were presented as scatterplots overlaid on top of a box-and-whisker of min, 25-percentile, median, 75-percentile, and max values. D'Agostino-Pearson omnibus normality test was applied to determine data normality. Significance of the differences was calculated using the Mann-Whitney test or 2-way ANOVA, followed by Sidak's multiple comparison test. All tests were 2-sided. Significance was granted if p value is smaller than 0.05. The software Prism version 7.0a (GraphPad) was used for statistical analysis.

3. Results

3.1. Frequency and characteristics of circulating monocytes in DVT patients

Macrophages can be derived from circulating monocytes, which may directly migrate to thrombus-forming areas. In the first part, we investigated the frequency of circulating monocytes from control subjects and idiopathic DVT patients. Per mL of blood, control subjects contained a median of 0.365 (min 0.206 – max 0.793) million monocytes, and DVT subjects contained a median of 0.441 (min 0.226 – max 0.747) million monocytes (Fig. 1A). No significant difference between control subjects and DVT patients were found. The expression of M1 and M2-associated genes that coded for effector cytokines, including IL6, TNF, and IL10, was then examined ex vivo (Fig. 1B). The IL6 and TNF expression was significantly higher in DVT monocytes than in control monocytes. No significant difference in IL10 expression was found between DVT monocytes and control monocytes.

3.2. Macrophage activation in DVT patients

Functional macrophages can be derived from circulating monocytes. To examine the macrophage activation and polarization, we measured the expression of IL6, TNF, and IL10 in monocyte-derived macrophages,

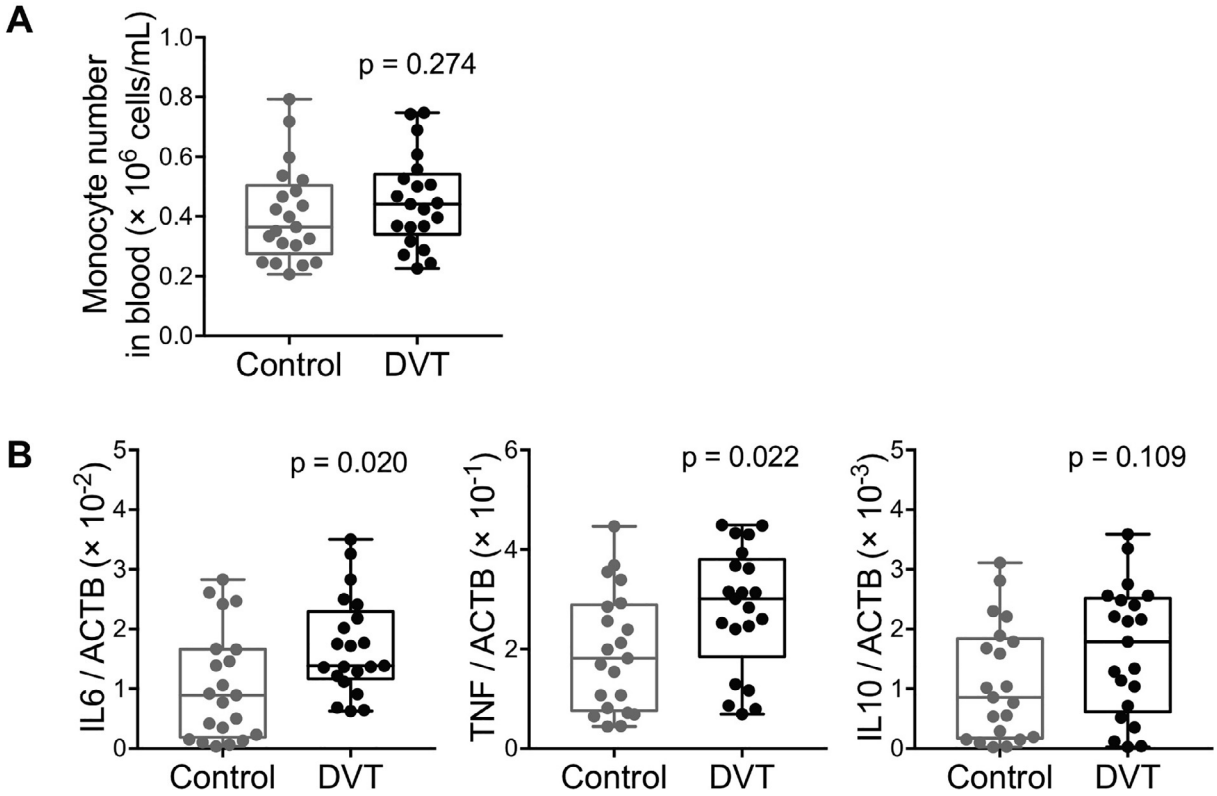

Fig. 1. Frequency and characteristics of circulating monocytes. (A) The frequency of monocytes in PBMCs from controls and DVT patients. (B) The expression levels of IL6, TNF, and IL10, examined in monocytes ex vivo via qPCR. For this and all figures below, the median, interquartile range, and min to max were presented as box and whiskers. Mann-Whitney test.

collected from DVT patients and healthy controls directly ex vivo (Fig. 2A). The IL6 and TNF expression was significantly higher in macrophages from DVT patients than in macrophages from controls. The IL10 expression, on the other hand, was comparable between DVT patients and controls. Subsequently, the macrophages were stimulated with LPS for 12 h, and the gene transcription and protein secretion was examined (Fig. 2B). Again, the expression of IL6 and TNF were significantly higher in macrophages from DVT patients than in macrophages from controls. We further measured cytokine concentration in the supernatant from stimulated macrophages of patients and controls (Fig. 2C). IL-6 secretion and TNF-α secretion were both significantly higher in macrophages from DVT patients than in macrophages from controls. IL-10 secretion, on the other hand, was not significantly different between the two groups.

3.3. Adhesion molecule expression by activated macrophages

Adhesion molecules can promote the initiation and propagation of venous thrombosis by the adhesion of leukocytes to the endothelial wall, causing vascular lesion, and increasing platelet accumulation [18]. Hence, we examined the effect of macrophages on the expression of adhesion molecules, by incubating macrophages from DVT patients and healthy controls with primary unbilical vein endothelial cells (HUVECs). In pure HUVECs, the baseline expression levels of SELE (coding for E-selectin), ICAM1, and VCAM1 were examined. The expression levels of these genes in the presence of macrophages were then expressed as fold over the levels in pure HUVEC. We found that the expression levels of SELE, ICAM1, and VCAM1 by HUVEC cells were significantly higher in the presence of macrophages, since the fold was greater than one in all controls and DVT patients (Fig. 3). Macrophages from DVT patients induced significantly stronger upregulation of these adhesion molecules than macrophages from control subjects.

3.4. M1/M2 polarization affected the expression level of adhesion molecules

Subsequently, we investigated the mechanisms used by macrophages to stimulate the expression of adhesion molecules. We first examined whether M1/M2 polarization could affect the expression of adhesion molecules, by incubating circulating monocytes in the presence of M1 or M2-polarization media. The M1-polarized macrophages presented significantly higher IL6 and TNF expression and significantly lower IL10 expression than the M2-polarized macrophages (Fig. 4A), demonstrating that the polarization media produced M1-like and M2-like macrophages with divergent effector gene expression. Subsequently, we incubated the HUVECs with M1 or M2-polarized macrophages, and found that the expression of SELE, ICAM1, and VCAM1 was significantly higher following incubation with M1-polarized macrophages than with M2-polarized macrophages (Fig. 4B). The above effects were found in both control subjects and DVT patients, and no significant differences between these two groups were observed.

3.5. The expression of adhesion molecules by HUVECs depended on direct contact with macrophages and cytokine expression

Subsequently, we examined the requirements of macrophage-mediated upregulation of adhesion molecules in HUVEC cells. By using transwell plates with a membrane that was permeable to solutes but not whole cells, we physically prevented the direct contact between macrophages and HUVECs while still allowing the interchange of secreted molecules. In this separated coculture system, the expression of adhesion molecules by HUVECs was still upregulated compared to that by pure unstimulated HUVECs, since the ratios over pure unstimulated HUVECs was greater than one (Fig. 5A). But compared to the expression levels of adhesion molecules in HUVECs following macrophage coculture in the same compartment (combined), the expression levels of

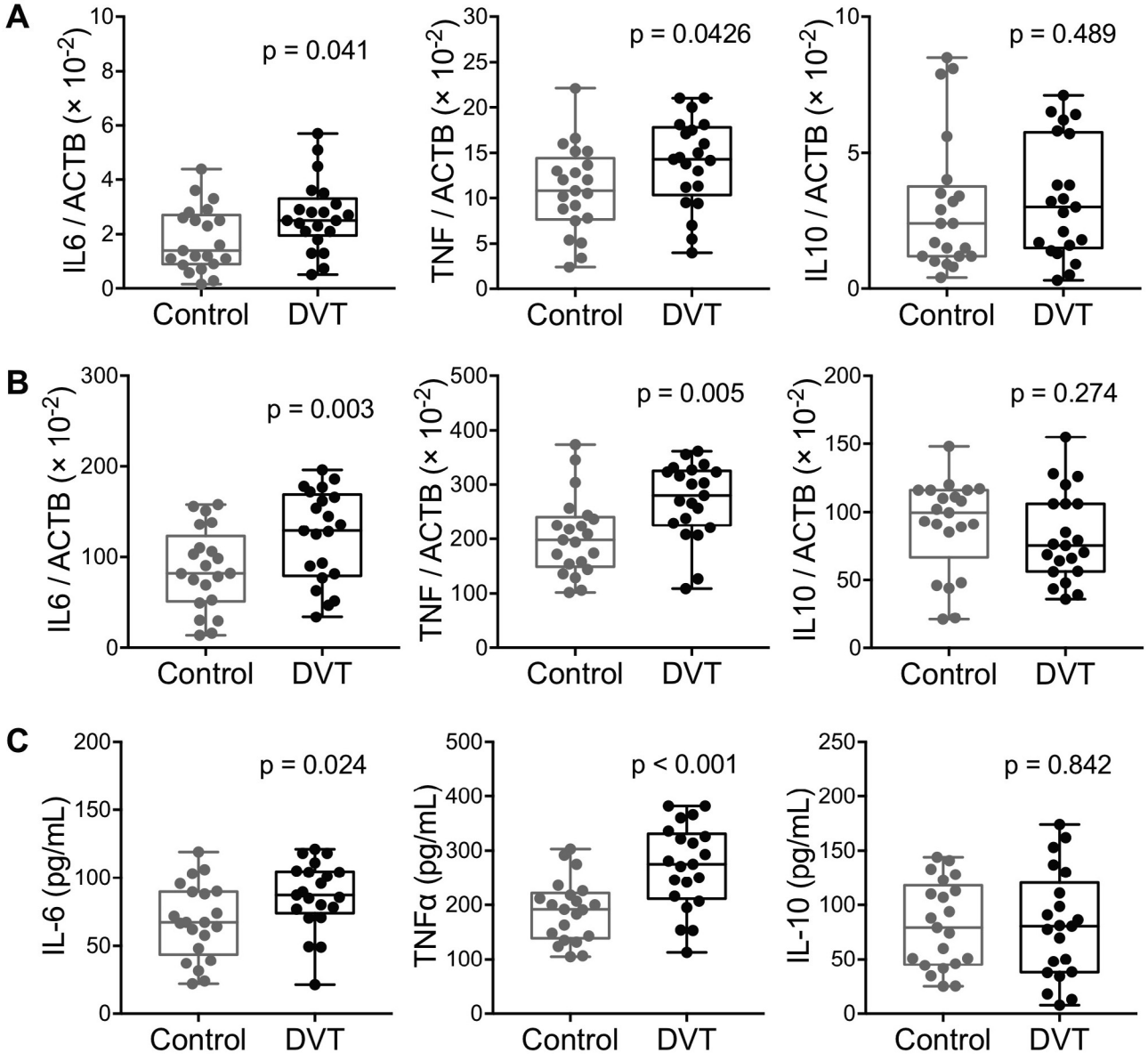

Fig. 2. Expression of M1 and M2-associated genes in monocyte-derived macrophages. Monocyte-derived macrophages were examined in 21 controls and 21 DVT patients. (A) The unstimulated expression levels of IL6, TNF, and IL10, examined via qPCR. (B) The LPS-stimulated expression levels of IL6, TNF, and IL10, examined via qPCR after 12-hour stimulation. (C) The secretion of IL-6, TNF-α, and IL-10 following 12-hour LPS stimulation. Mann-Whitney test.

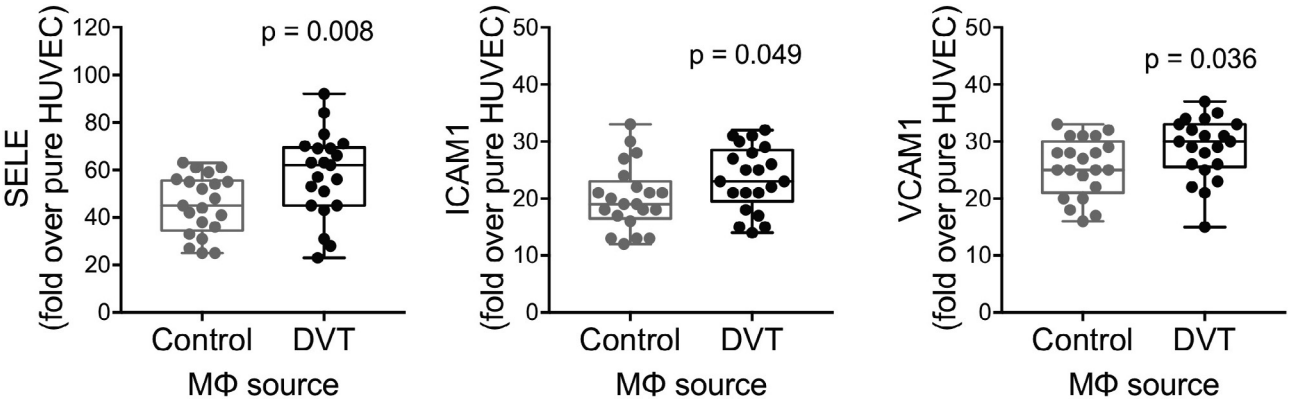

Fig. 3. Expression of adhesion molecules in HUVEC cells. HUVEC cells were incubated with LPS-stimulated macrophages (MΦ) for 12 h. The SELE, ICAM1, and VCAM1 expression levels following incubation with macrophages were then examined, and expressed as fold over the expression levels in pure HUVEC cells. Mann-Whitney test.

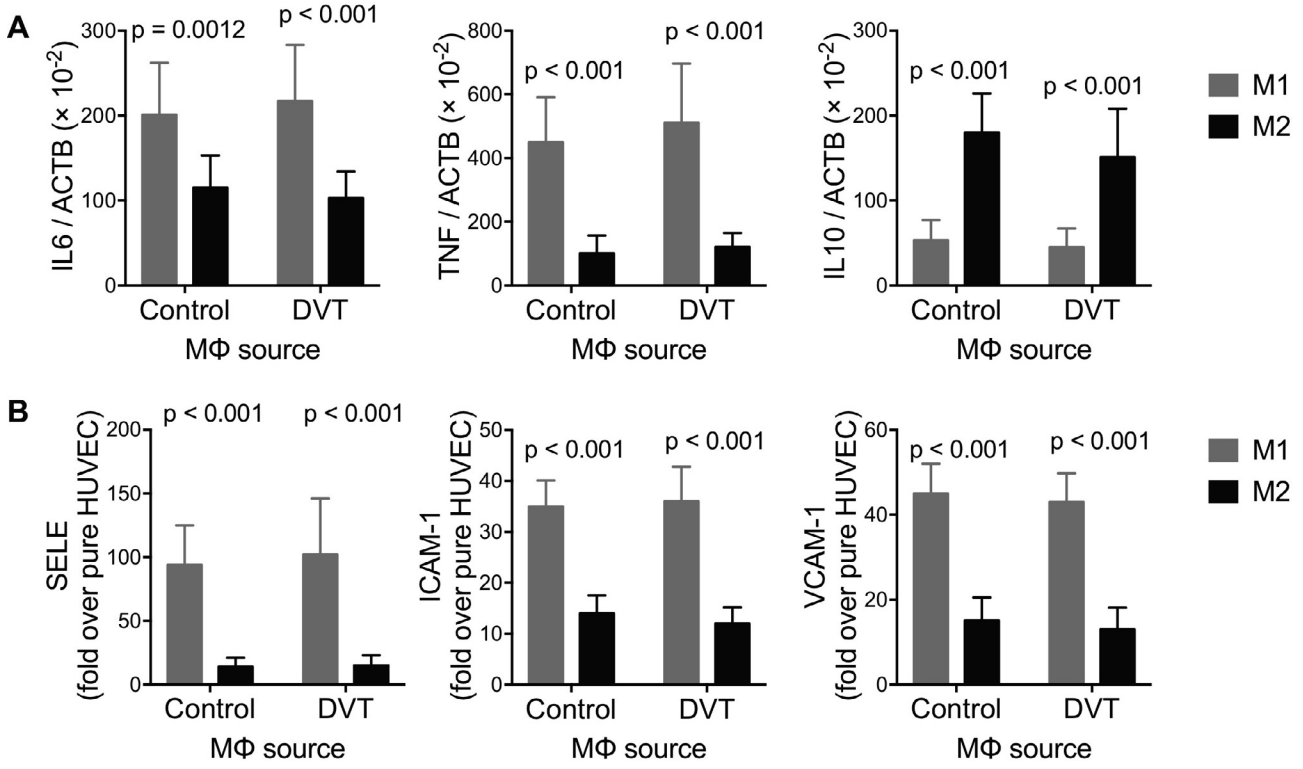

Fig. 4. M1/M2 polarization and adhesion molecule expression. (A) Monocytes were polarized into M1 and M2 macrophages, which were then stimulated with LPS for 12 h. The expression of IL6, TNF, and IL10 were examined via qPCR. (B) HUVEC cells were incubated with LPS-stimulated M1/M2-polarized macrophages for 12 h. The SELE, ICAM1, and VCAM1 expression levels were then examined following incubation with macrophages, and expressed as fold over the expression levels in pure HUVEC cells. 2-way ANOVA followed by Sidak's multiple comparison test.

adhesion molecules in HUVECs following separated coculture was significantly reduced, indicating a role of direct cell-to-cell contact in the upregulation of adhesion molecules. These effects were observed in both controls and DVT patients. In addition, the DVT macrophages in combined cultures induced significantly stronger upregulation of SELE, ICAM1, and VCAM1, than the control macrophages in combined cultures.

Subsequently, we neutralized the cytokines, including IL-6, TNF-α,

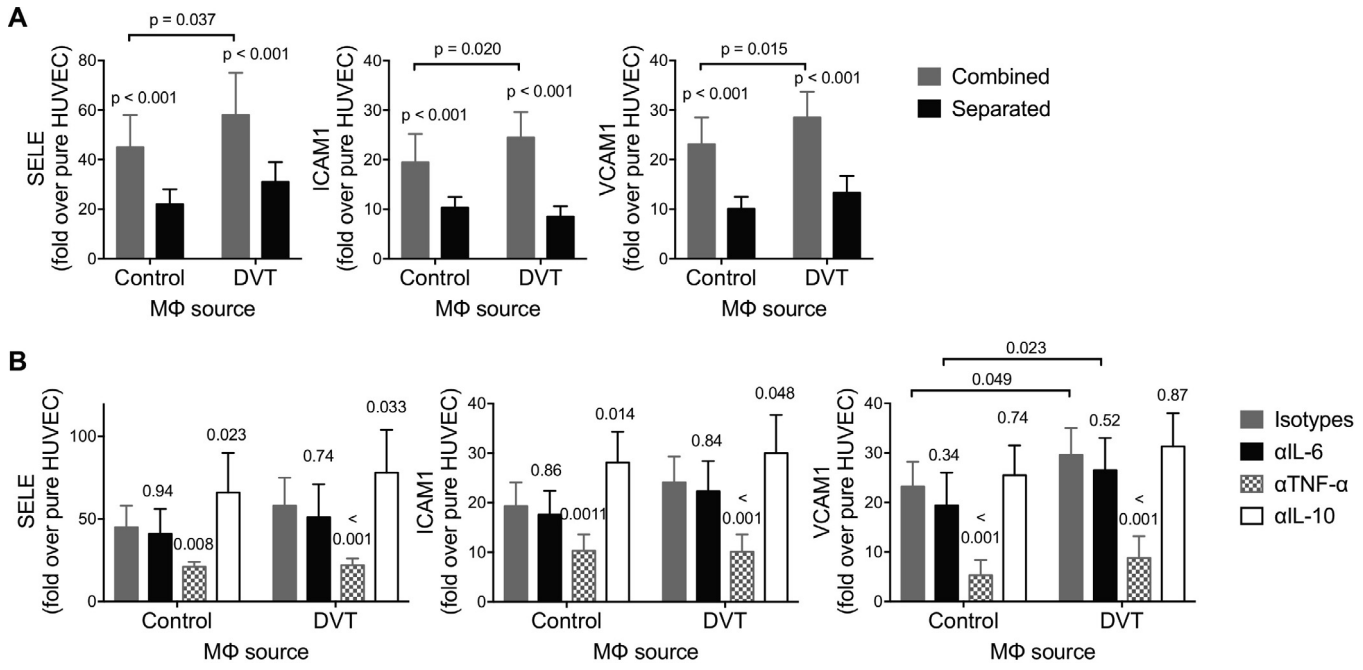

Fig. 5. Requirements for the upregulation of adhesion molecules. (A) Macrophages and HUVECs were incubated in the same compartment (combined) or in separate compartments (separated) of a transwell plate. The SELE, ICAM1, and VCAM1 expression levels were then examined after 12 h. (B) Neutralizing antibodies to human IL-6, TNF-α, or IL-10 were added to the macrophage/HUVEC coculture. The SELE, ICAM1, and VCAM1 expression levels were examined after 12 h. Numbers on top the each group presented the p values compared to the isotype group. 2-way ANOVA followed by Sidak's multiple comparison test.

and IL-10, with corresponding antibodies in the macrophage-HUVEC coculture. Compared to isotype controls, the inhibition of TNF-α significantly reduced the expression of SELE, ICAM1, and VCAM1, while the inhibition of IL-10 significantly increased the expression of SELE and ICAM1 (Fig. 5B). The inhibition of IL-6 did not produce a significant effect. These effects were observed in both controls and DVT patients. The VCAM1 upregulation in the presence of isotype control and in the presence of anti-IL-6 antibodies were significantly higher in DVT patients than in controls.

4. Discussion

The present study demonstrated that the expression of adhesion molecules by endothelial cells could be profoundly affected by the response from monocytes and macrophages. M1-polarization, but not M2-polarization, could profoundly upregulate the expression of adhesion molecules. This upregulation was dependent on direct cell-to-cell contact, as well as on contact-independent TNF-α expression. IL-10 expression, on the other hand, significantly reduced the upregulation of adhesion molecules. Interestingly, the circulating monocytes, as well as monocyte-derived macrophages, from DVT patients presented significantly higher IL-6 and TNF-α expression than the corresponding cells from control subjects. Despite the statistically significant findings, the ranges of values overlapped to a large degree between controls and DVT patients, which might indicate that the inflammation in DVT patients was a low-grade one, with slightly, but not markedly, elevated inflammation

It remains unclear whether the elevated IL-6 and TNF-α in monocytes and macrophages is a cause or effect of DVT. Previous studies demonstrated that obesity and smoking, two conditions that were associated with elevated IL-6 and TNF-α, were also associated with elevated DVT risk [19–21]. In addition, monocyte/macrophage activation and inflammation, including IL-6 and TNF-α production, were shown to upregulate adhesion molecule expression, a precursor to DVT formation [7], while the formation of DVT in itself does not seem to have a clear pathway to affect monocyte/macrophage gene expression systemically. Hence, it is the current opinion of the authors that the IL-6 and TNF-α inflammation is more likely a cause or contributing factor to DVT. To prove this hypothesis conclusively, cytokine knockout animal models are required.

To achieve macrophage stimulation, we used LPS, which is found on the Gram-negative bacteria's outer membrane, and activates macrophages via toll-like receptor (TLR)-4. Results from this study showed that LPS-mediated macrophage activation could significantly increase the expression of adhesion molecules by endothelial cells, which could explain the elevated risk of DVT and PE during conditions where the bacterial product could be found in the blood stream, such as in inflammatory bowel diseases, septic shock, trauma, and surgery. However, the patient group in our study presented idiopathic DVT without the above conditions in the past six months prior to sample collection or concurrently at the time of sample collection. Nevertheless, circulating monocytes from the idiopathic DVT patients displayed higher IL-6 and TNF-α transcription. The underlying causes of this phenomenon require further investigations.

We also demonstrated that the M2-polarized macrophages were more resistant to LPS-mediated activation. HUVECs incubated with LPS-stimulated M2 macrophages were significantly less effective at upregulating adhesion molecules. We also found that LPS-stimulated M2-polarized macrophages produced significantly lower TNF-α and higher IL-10 than LPS-stimulated M1-polarized macrophages. Therefore, it should be investigated whether polarization of monocytes/macrophages could be used as a potential treatment method for DVT. For example, the circulating monocytes could be polarized toward M2 macrophages, which are more resistant to TNF-α production mediated by LPS, and possibly, other microbial products and stimulants. In addition, it should be investigated whether M2 macrophage-mediated IL-10 could prevent or alleviate DVT, since DVT development was associated with low IL-10 expression [22,23]. It has been shown that two to four months after acute idiopathic DVT, patients still present significantly lower IL-10 than controls [22]. Also, in mice, IL-10 deficiency significantly increased the incidence of thrombosis [23].

The process of thrombus resolution remains unclear. Interestingly, this process may be critically dependent on macrophages and monocytes. Administration of peritoneal macrophages in rats, or injection of monocyte chemotactic protein (MCP)-1 in mice, resulted in significant reduction in the thrombus sizes [24]. Also in mice, myeloid-specific deletion of p53 significantly impaired the resolution of thrombi, together with a reduction in M2 macrophages [25]. Quinacrine could enhance thrombus resolution in a myeloid- p53-dependent manner and was associated with increased intrathrombus M2-like macrophages [25]. In future studies, whether M2-polarized macrophages could be used in treating DVT patients and directly reduce thrombi should be investigated. In the past, macrophages were polarized in vitro and reintroduced into experimental mice. Adoptive transfer of M2-polarized macrophages was shown to suppress diabetes development in NOD mice, and ameliorate neuropathic pain [26,27]. It remains to be seen whether macrophage adoptive transfer could ameliorate proinflammatory conditions in humans.

This study was focused on idiopathic DVT but not DVT patients with identifiable causes, who could also present macrophage polarization. We excluded those subjects for two reasons. First, in some patients with identifiable causes, such as those with recent surgery and immobility, their DVT may be avoided by increased assisted movements and increased education in advance, while those without clear causes are potentially at increased danger, and their pathogenesis is more of a mystery that awaits investigation. Second, to control for patients with identifiable causes, such as cancer, we could not use healthy subjects, but instead would need other cancer patients who did not develop DVT. Also, these non-idiopathic DVT might be a collection of separate diseases with distinctive etiology. This complicates the types of controls needed for this study, as each cause may need a separate control. In the future, whether these findings could expand to DVT patients due to clearly identifiable causes should be investigated.

Conflict of interest

None.

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