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The Release of Monocyte-Derived Tissue Factor-Positive Microparticles Contributes to a Hypercoagulable State in Idiopathic Membranous Nephropathy

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Aim: Idiopathic membranous nephropathy (IMN) is an immune-mediated inflammatory disease characterized by a high risk of thromboembolic complications. Microparticles (MPs), a type of extracellular vesicles, have procoagulant properties, especially when they display tissue factor (TF). This study aimed to investigate whether circulating TF-positive MPs contributed to the hypercoagulable state in patients with IMN.

Methods: Twenty adult IMN patients and fourteen healthy subjects were included in the study. The basic indexes of a routine biochemical examination and coagulative function were determined. The plasma levels of MPs were detected by flow cytometry, and TF activity of MPs was examined using an assay kit. The plasma levels of lipopolysaccharide (LPS) were measured by an enzyme-linked immunosorbent assay.

Results: Total circulating MPs were not increased in patients with IMN compared with healthy controls. Circulating $CD14^+/TF^+$MPs were significantly increased in IMN patients, but this achieved significance was not observed in $CD41^+/TF^+$MPs between the two groups. Interestingly, the circulating TF-positive MPs were increased significantly. Plasma MPs TF assays revealed high procoagulant activity, which was positively associated with the D-dimer level in IMN. In addition, circulating LPS in IMN patients were significantly higher than those in the controls. Furthermore, after two hours' incubation with healthy whole blood, LPS enhanced the release of circulating TF-positive MPs and the TF activity of MPs.

Conclusion: Increased circulating LPS may mediate the release of monocyte-derived TF-positive MPs, which further contributes to the hypercoagulable state in IMN patients. These findings provide an additional mechanism by which patients with IMN have a higher risk of thromboembolic complication.

Key words: Lipopolysaccharide, Tissue factor, Microparticles, Hypercoagulable state, Idiopathic membranous nephropathy

Introduction

Thromboembolic events are well recognized as a common complication in patients with idiopathic nephrotic syndrome (INS). Furthermore, venous thromboembolic events are reported to occur more frequently in idiopathic membranous nephropathy (IMN) than in other types of INS[1, 2]. In recent years, several studies have confirmed that patients with IMN have a higher risk of cardiovascular events (CVEs)[3, 4]. The underlying mechanisms are related to increased prothrombotic factors, decreased antithrombotic factors, and impaired thrombolytic activity. Furthermore, hypoalbuminemia, hyperlipidaemia, and immune complex activation of the clotting system participate in the hypercoagulable state of IMN[5].

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Traditionally, hypoalbuminemia has been one of the most important risk factors for the hypercoagulable state that results in thromboembolic events in IMN[2]. However, the mechanism accounting for the increased risk of thrombosis in IMN is still unknown. Moreover, some studies have indicated that even though there is no difference in serum albumin level, thromboembolic complications may be more likely to occur in patients with IMN[6], indicating that there might be other mechanisms involved.

Microparticles (MPs) are a heterogeneous population of small extracellular vesicles (0.1−1 μm in diameter) released from almost all cell types via outward membrane budding[7]. MPs mediate intercellular communication by delivering cargos from parent cells. Recently, circulating MPs have been proposed to promote coagulation by the exposure of phosphatidylserine (PS) and tissue factor (TF), which is the trigger of the clotting system. MPs TF activity was significantly increased in cirrhosis patients, although the number of circulating PS-positive MPs was not increased, which contributed to the procoagulant imbalance[8]. Circulating TF-positive MPs promote thrombosis at the site of injury[9, 10] and partially represent the hypercoagulability in diabetic vascular complications[11]. Therefore, we assumed that the circulating TF-positive MPs might be increased and involved in the hypercoagulable state in patients with IMN.

It is well known that IMN results from immune disorder-mediated glomerulonephritis. Some studies have shown the involvement of inflammatory mediators in the inflammatory process responsible for the progression of IMN, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-alfa (TNF-α)[12]. Lipopolysaccharide (LPS), one of the inflammatory mediators, augments the development of glomerulonephritis[13]. Meanwhile, LPS increased whole-blood monocyte TF surface expression and TF functional activity in circulating MPs[14]. Therefore, this study hypothesized that LPS may contribute to the hypercoagulable state in IMN patients by inducing the release of circulating TF-positive MPs.

We conducted a cross-sectional study to investigate the plasma levels of TF-positive MPs and their correlation with the hypercoagulable state in IMN. Furthermore, we examined the circulating LPS level and demonstrated its effect on the release of monocyte-derived TF-positive MPs *in vitro*. We also explored their contribution to thromboembolic complications in IMN patients.

Materials and Methods

Research Subjects

Twenty adult IMN patients (≥18 years old) with the diagnosis of IMN confirmed by renal biopsy were recruited for the study at the Institute of Nephrology, Zhongda Hospital, Southeast University, China. The control group included 14 healthy subjects who were not taking any medication and did not have any significant medical problems. The exclusion criteria were as following: severe acute or chronic infection; use of antibiotics or anticoagulant drugs 30 days prior to the study; an estimated glomerular filtration rate <60 ml/min/1.73 m^2; needed transfusion of platelets or plasma; secondary MN (malignancy, systemic lupus erythematosus, hepatitis B, hepatitis C, and human immunodeficiency virus); and a previous history of diabetes, coronary artery diseases, or autoimmune disease, alcohol abuse, and pregnancy. The ethical committee of Zhongda Hospital approved the study. All patients provided written informed consent. The following baseline data were collected: age, sex, hemoglobin, platelet count, serum albumin, high-density lipoprotein cholesterol (HDL-C), urea nitrogen, and serum creatinine.

Blood Sample Preparation

All the patients and healthy volunteers had fasted for at least 12 hours before blood collection. Peripheral venous blood was collected with or without 3.8% sodium citrate anticoagulant between 8 and 9 a.m. The blood samples were checked for routine biochemical indexes and coagulative function assay after centrifugation at 3,000 g for 10 minutes. The routine blood test was done using a Sysmex XE-2100 hematology analyzer (Sysmex). Blood biochemistry was detected by an automatic biochemical analyzer 7020 (Hitachi). D-dimer was measured using an automatic coagulation analyzer ACL TOP700 (Beckman).

Whole-Blood Experiments *In Vitro*

Whole blood was obtained from five healthy donors using sodium citrate or EDTA as anticoagulant. Each blood sample was divided into two parts, one for the control group and the other for LPS group. The samples were incubated with LPS (Sigma, 100 ng/ml) or PBS at 37℃ for two hours. Plasma was obtained by centrifugation as the above and subsequently used for the acquisition of MPs.

Collection and Quantification of Circulating MPs

Circulating MPs were isolated using differential centrifugation, as previously described[15]. Briefly, platelets were removed by two subsequent centrifugations at 2,500 g for 15 minutes at room temperature. Then

Table 1. Basic characteristics of IMN patients and control subjects

	Control ($n=14$)	IMN ($n=20$)	P value
Age (years)	44 ± 11	52 ± 14	0.082
Sex male, n (%)	11 (78.5)	16 (80)	0.662
Albumin (g/L)	46.7 ± 3.4	24.3 ± 4.2*	< 0.001
Hemoglobin (g/L)	148 ± 15.3	138 ± 5.4	0.158
Platelet (10^9/L)	212 ± 65.7	218 ± 44.4	0.737
BUN (mM)	4.8 ± 1.2	5.0 ± 1.5	0.207
Creatine (μM)	73.3 ± 14.3	79.2 ± 11.9	0.304
HDL-C (mM)	1.45 ± 0.32	1.66 ± 0.39	0.104

IMN, idopathic membranous nephropathy; BUN, blood urea nitrogen, HDL-C, high density lipoprotein cholesterol

the supernatant was centrifuged at 18,000 g for 60 minutes to pellet the MPs. The pellets were resuspended by annexin V binding buffer and then incubated with FITC-anti-annexin V (BD Pharmingen) and APC-anti-human TF (BioLegend) for 30 minutes at 4℃ in the dark. Then 0.8 μm and 3 μm beads were used for gating and counting control, respectively. Circulating TF-positive MPs were detected as annexin V^+/TF^+ particles by a FACS Calibur cytometer (BD Biosciences)[16]. To investigate the cellular origin of circulating TF-positive MPs, samples were incubated with PE-anti-Human CD41 or PE-anti-Human CD14 (BioLegend). Analysis was performed using FlowJo (Tree Star Inc.) software.

MPs TF Activity Assay

Plasma was collected using EDTA as the anticoagulant. TF activity of circulating MPs was analyzed as previously described using a tissue factor human chromogenic activity assay kit (Abcam)[17]. The assay measures the ability of lipoprotein TF/factor VIIa (F VIIa) to activate factor X (FX) to factor Xa. Briefly, the desired volume of assay mix was freshly prepared, including assay diluent 50 μL, FVII 10 μL, and FX 10 μL, and 70 μl of the assay mix was added to each well. Then 10 μL TF standard or sample was added to each well and mixed gently. The mixed reaction system was then incubated at 37℃ for 30 minutes in a humid incubator. After that, FXa 20 μL was added to each well and incubated at 37℃ for 30 minutes. Finally, the absorbance at 405 nm was assayed on a microplate reader.

Plasma Levels of LPS

Plasma levels of LPS were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Elabscience) according to the manufacturer's instructions. Briefly, 50 μL of the standard, the blank, or a sample was added to each well and incubated for 45 minutes at 37℃. After incubation, the samples were read at 450 nm using a microplate reader. The values are expressed as ng/ml. The detection limit for LPS was established at 0.94 ng/mL. The intra- and inter-assay coefficients of variation were within 10%.

Statistical Analysis

Categorical variables are expressed as percentages, and continuous variables are reported as the mean ± standard deviation (SD). Comparisons between the controls and the IMN patients were analyzed using Student's t-test or the Mann-Whitney U test for continuous variables and the chi-square test for categorical variables. Spearman's correlation analysis was used to perform the correlation calculation. $P<0.05$ was considered statistically significant. All the analyses were performed with SPSS 19.0 and GraphPad Prism 7.0.

Results

Circulating TF-Positive MPs were Increased in Patients with IMN

The basic characteristics of all the subjects are described in **Table 1**. Basic data for the controls and IMN patients were collected in terms of age, sex, hemoglobin levels, platelet counts, blood urea nitrogen, and HDL-C levels. The serum albumin levels in IMN patients were significantly lower than those in the controls, which was consistent with the diagnosis of nephrotic syndrome. As shown in **Fig. 1A** and **1B**, total circulating MPs indicated as PS-positive were examined using flow cytometry. There was no statistical difference between IMN patients and the controls ($P=0.078$). However, we found that there were a portion of TF-positive MPs in total MPs. Plasma levels of TF-positive MPs were much higher in IMN patients than that in the controls ($(17.1 \pm 5.7) \times 10^6$/mL versus $(10.9 \pm 5.3) \times 10^6$/mL, $P=0.008$, **Fig. 1C**). To investigate the cellular origin of circulating TF-positive MPs, antibodies against CD14 (monocytes) or CD41 (platelets) were also used. Circulating $CD14^+/TF^+$MPs were sig-

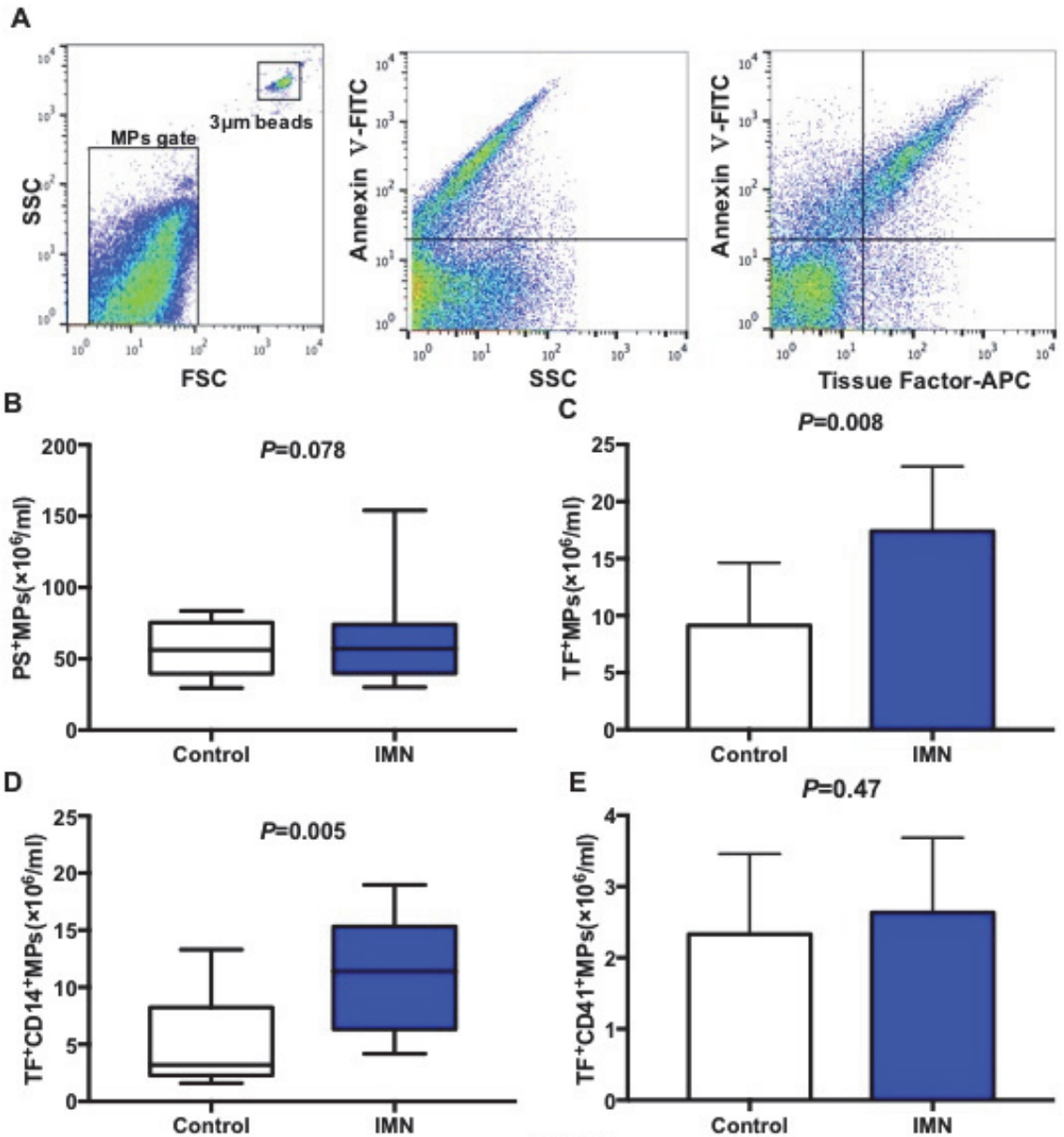

Fig. 1. Circulating TF-positive MPs were increased in patients with IMN.

The plasma level of MPs in the controls ($n=14$) and the IMN patients ($n=20$) were measured using flow cytometry. (A) 0.8 μm beads were used for the MPs gate setting, while 3 μm beads were used for counting controls. Total MPs were indicated by PS-positive particles that were in the MPs gate. Circulating TF-positive MPs were indicated as both TF and PS-positive particles. (B) Quantification of total MPs in the two groups. (C) Quantification of total MPs in the two groups. Cell origins of TF-positive MPs were also detected by CD14 (D) and CD41 (E). (B and D) The data are shown as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). (C and E) The data are shown as the mean ± SD. *P*, significance level.

nificantly increased in IMN patients compared with the controls ($P=0.005$, Fig. 1D). However, this achieved significance was not observed in CD41$^+$/TF$^+$MPs between these two groups ($P=0.47$, Fig. 1E). These results suggest that the circulating TF-positive MPs were increased in patients with IMN, which were primarily derived from monocytes but not platelets.

Circulating MPs TF Activity was Correlated with the Hypercoagulable State in Patients with IMN

The data shown in Fig. 2A indicated that D-dimer level was significantly elevated in IMN patients compared with controls ($P=0.001$), suggesting a hypercoagulable state in patients with IMN. Correlation analysis revealed that the number of TF-positive MPs was

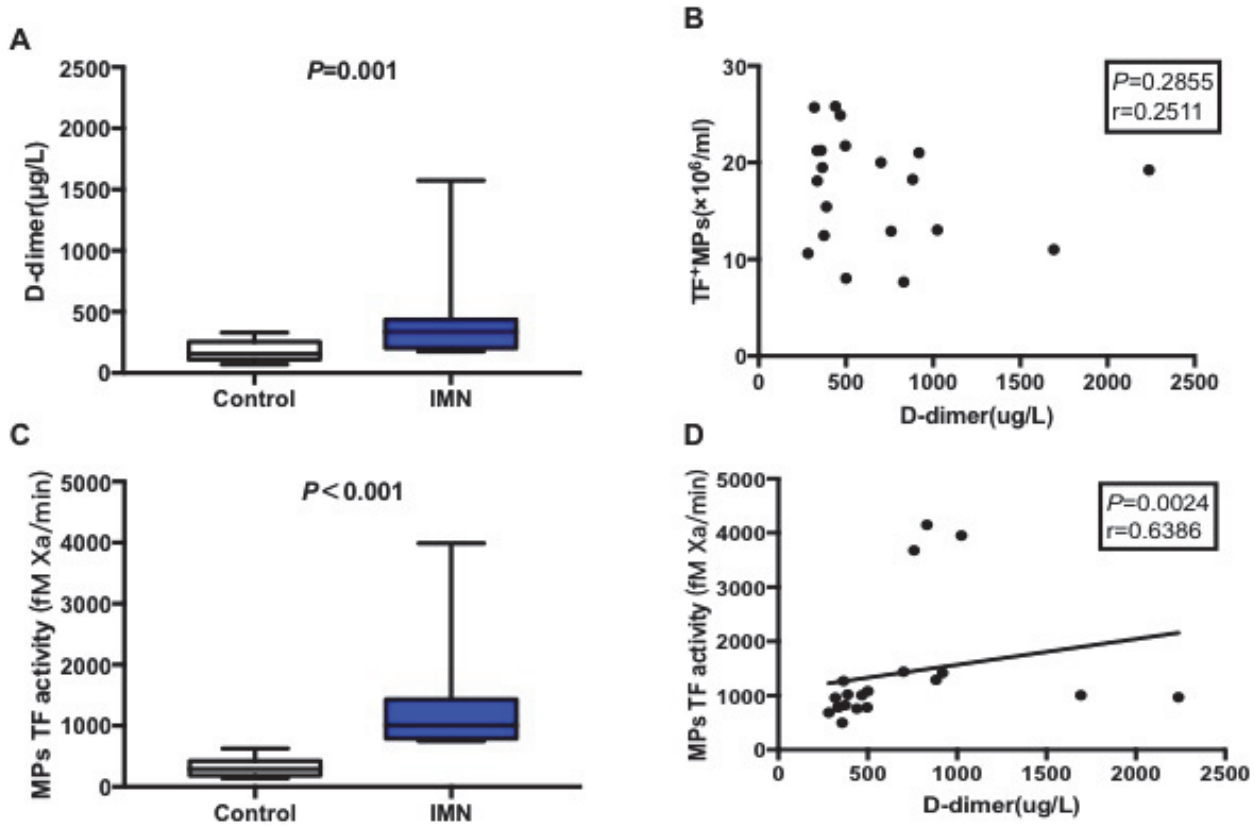

Fig. 2. Circulating MPs TF activity was correlated with the hypercoagulable state in patients with IMN.

(A) The plasma D-dimer level in the controls ($n=14$) and the IMN ($n=20$) patients was detected using an automated coagulation analyzer. (B) The correlation analysis between the plasma D-dimer and TF-positive MPs in IMN patients ($n=20$). (C) The Boxplot shows MPs TF activity in each group. The data are shown as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). (D) The correlation analysis between D-dimer and MPs TF activity in IMN patients. *r*, correlation coefficient; *P*, significance level.

not correlated with the plasma D-dimer level in patients with IMN (**Fig. 2B**, $r=0.2511$, $P=0.2855$). Subsequently, TF activity of MPs was also detected. Plasma MPs TF activity was very low in healthy controls, whereas it was significantly increased in IMN patients (**Fig. 2C**, $P<0.001$). Interestingly, Spearman's correlation analysis showed that the higher MPs TF activity was positively correlated with the plasma D-dimer level in IMN patients (**Fig. 2D**, $r=0.6386$, $P=0.0024$). This suggests that the hypercoagulable state in patients with IMN is partially due to the increased circulating MPs TF activity.

Increased Circulating LPS Level was Positively Associated with TF-Positive MPs in IMN Patients

To explore the cause for increased levels of monocyte-derived TF-positive MPs in IMN, circulating LPS level was examined, which is one of the most prominent pro-inflammatory components. Compared with the healthy control, the plasma level of LPS was increased in patients with IMN (**Fig. 3A**, $P=0.039$). Furthermore, the increased circulating LPS level was positively correlated with the number of TF-positive MPs (**Fig. 3B**, $r=0.6932$, $P=0.0007$) and MPs TF activity (**Fig. 3C**, $r=0.7153$, $P=0.0004$) in IMN patients. These data indicate that increased circulating LPS level might be the reason for increased procoagulant TF-positive MPs in IMN.

LPS may Mediate the Release of TF-Positive MPs

To confirm the effect of LPS on the release of circulating TF-positive MPs, we collected MPs from human whole blood, which was incubated with LPS *in vitro*. After two hours' incubation, the number of monocyte-derived TF-positive MPs was increased significantly (**Fig. 4A**, $P<0.001$). MPs TF activity was also elevated in LPS group compared with the control group (**Fig. 4B**, $P<0.001$). These results suggest that LPS may mediate the release of TF-positive MPs in IMN.

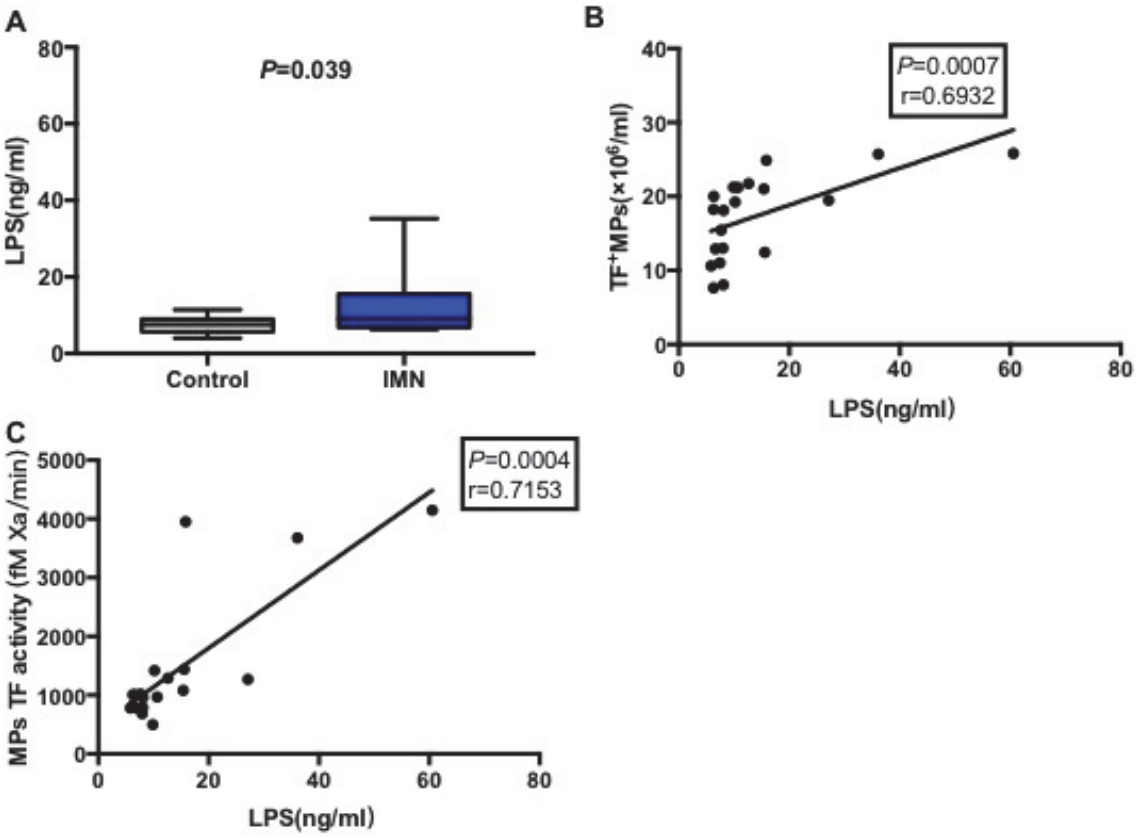

Fig. 3. Increased circulating LPS was positively associated with TF-positive MPs in IMN patients.

(A) Plasma LPS level in the controls ($n=14$) and the IMN patients ($n=20$) was measured using an ELISA assay kit. The data are shown as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). (B) The correlation analysis between the plasma levels of LPS and monocyte-derived TF-positive MPs in IMN patients ($n=20$). (C) The correlation analysis between LPS and MPs TF activity in IMN patients. *r*, correlation coefficient; *P*, significance level.

Discussion

In the present study, we provided the first evidence that increased circulating TF-positive MPs contribute to the hypercoagulable state in patients with IMN. More importantly, we demonstrated that elevated circulating TF-positive MPs and their increased TF activity may be a consequence of elevated plasma LPS.

Previous studies have suggested that the hypercoagulable state in INS[18], which may result in the development of thromboembolic events, primarily depends on hypoalbuminemia and hyperlipidaemia, especially in IMN patients. In this study, plasma level of D-dimer was significantly increased in IMN, which indicates hypercoagulable state and secondary hyperfibrinolysis. TF, trigger of the coagulation cascade, plays a crucial role in the development of thrombosis[19]. Some studies have demonstrated that shedding of TF-containing MPs rather than spliced TF is the main source of TF activity[20] MPs, as a novel medium for intercellular communication, possess procoagulant effect[21].

In the current study, we did not find a statistical difference in the number of circulating PS-positive MPs between the patients with IMN and healthy controls. Interestingly, TF-positive MPs were increased in IMN. Some studies reported that cancer patients with higher levels of circulating TF-positive MPs had a sevenfold increased risk of thrombosis[22]. Further analysis also indicated that circulating TF-positive MPs primarily derived from monocytes. A recent study showed that increased circulating MPs in human endotoxemia model expressed both TF and CD14[23]. Shet and colleagues also reported that monocyte-derived TF-positive MPs were elevated in sickle cell crisis[24]. Previous studies and our results indicate that monocytes are likely to be the major source of circulating TF-positive MPs in health and diseases.

However, there was no correlation between the number of circulating TF-positive MPs and the D-dimer

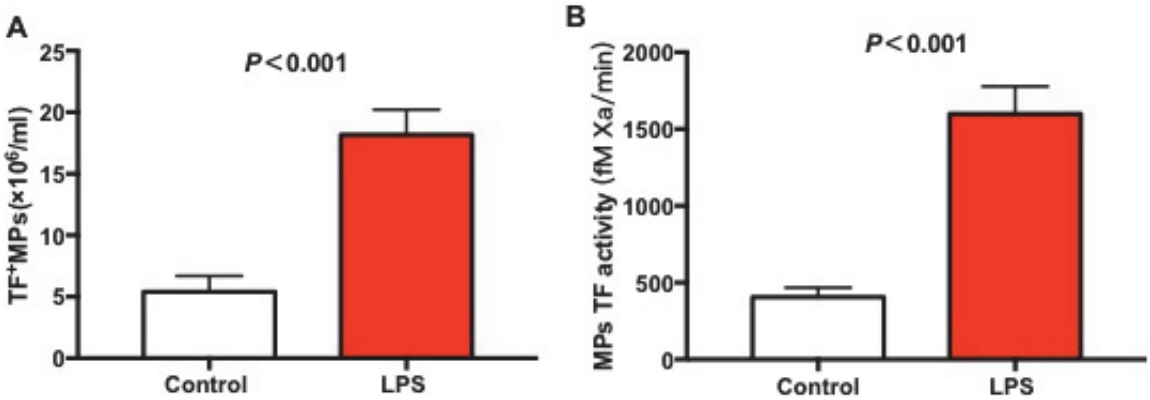

Fig. 4. LPS mediated the release of TF-positive MPs.

Whole blood obtained from healthy donors was incubated with LPS (100 ng/ml) or PBS for two hours at 37℃. (A) Circulating TF-positive MPs were detected using flow cytometry. (B) Plasma MPs TF activity was examined using assay kit. Data are shown as the mean ± SD. $n = 5$. *P*, significance level.

level in IMN. As we know, the procoagulant activity of TF is to form a complex with FVII and FX, which is then activated to F Xa[10]. Functional assays of MPs TF activity are more specific and sensitive than the antigenic assays. Then we further examined the plasma MPs TF activity, and the results showed that plasma MPs TF activity was increased in IMN. Interestingly, the increase of MPs TF activity was much higher than the increase of TF-positive MPs numbers in IMN patients. There might be a difference between absolute count and functional evaluation for MPs as the limitation of single detection methods[25]. Bharthuar *et al.*[26] reported that pancreatic cancer patients had higher levels of MPs TF activity than healthy controls, whereas the TF antigen assay failed to detect a difference. Thus, TF antigen level and functional assay in MPs should both be used to characterize them.

Increased MPs TF activity was positively correlated with the D-dimer level in IMN. Elevated circulating MPs TF activity, but not PS^+MPs, was associated with thrombosis and worsened survival in patients with pancreaticobiliary cancer[27]. MPs TF activity also contributed to the hypercoagulable state in acute liver injury[28]. Huisse *et al.* found that MPs TF activity was increased in patients with acute myocardial infarction[29]. These findings suggest that increased plasma MPs TF activity in patients with IMN contributes to the hypercoagulable state, which might be predictive of thrombosis.

Systemic inflammation induces the release of MPs from parent cells. Circulating LPS has been considered to be associated with chronic inflammation, dyslipidaemia, and obesity[30]. LPS is an important microbial trigger that stimulates the innate immunity of the host. The IMN is characterized as an immune-mediated inflammatory disease. Under healthy conditions, only small quantities of LPS pass through the intestinal barrier during nutrient ingestion[31]. In this study, we found for the first time that the plasma LPS was significantly increased in IMN patients compared with the controls. Previous studies suggested that enhanced intestinal permeability may account for the increase of circulating LPS[32].

Both immunoinflammatory reaction and hypoalbuminemia might be the reason for the increased intestinal permeability. LPS is commonly used as an inducer for experimental glomerulonephritis models[33], which might be correlated with the LPS receptor toll-like receptor (TLR) 4 and its coreceptor CD14 expressed in podocytes[34]. Our findings suggested that there might be some potential correlation between LPS and the pathogenesis of IMN. Previous studies found that the serum LPS in type 1 diabetic patients was increased and positively correlated with the renal injury[30]. This makes us further identify whether elevated LPS contributed to the release of monocyte-derived TF-positive MPs in IMN.

Correlation analysis showed that LPS was positively associated with the number of TF-positive MPs and MPs TF activity in patients with IMN. *In vitro* studies further confirmed that LPS mediated the release of TF-positive MPs and their procoagulant activity. Landsem *et al.* demonstrated that LPS administration activated the coagulation system by upregulation of TF expression and activity in circulating MPs[14]. Previous studies also reported that LPS was crucial for inducing monocyte- and MPs-associated TF activity[35]. In accordance with our hypothesis, the increased plasma LPS enhanced the release of monocyte-derived TF-positive MPs in IMN.

This study also has some limitations. Besides IMN, other types of INS were not studied. The small sample size in this study remains a limitation. Larger, prospective studies are needed to learn more about the role of LPS and monocyte-derived TF-positive MPs in IMN.

In conclusion, increased circulating LPS may mediate the release of monocyte-derived TF-positive MPs which further contribute to the hypercoagulable state in IMN patients. These findings provide an additional mechanism by which patients with IMN have a higher risk of thromboembolic complication.

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