Phosphatidylserine-exposing cells contribute to the hypercoagulable state in patients with multiple myeloma

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Abstract. Multiple myeloma (MM) is characterized by an increased incidence of thromboembolic events, particularly when treated with immunomodulatory drugs (IMiDs) in combination with dexamethasone. The optimal prophylactic strategy to prevent the hypercoagulable state of patients with MM is still debated. The aim of the current study was to investigate the definitive role of phosphatidylserine (PS) in supporting procoagulant activity (PCA) in patients with MM. Patients with MM (n=20) and healthy subjects (n=15) were recruited for the present study. PS analyses were performed by flow cytometry and confocal microscopy. The PCA was evaluated by clotting time, purified coagulation complex assays and fibrin production assays. The percentage of PS+ blood cells was significantly higher in patients with MM than in healthy subjects. Additionally, the patient serum induced more PS exposure on endothelial cells (ECs) *in vitro* than serum from healthy subjects. Isolated blood cells from patients with MM and ECs cultured with patient serum *in vitro* demonstrated significantly shortened coagulation time, greatly intrinsic/extrinsic factor Xa generation and increased thrombin formation. In addition, the levels of PS+ erythrocytes, platelets, leukocytes, and ECs incubated with IMiDs and dexamethasone were higher than with IMiDs alone. The findings support the hypothesis that increased PS exposure on blood cells and ECs participates in the hypercoagulable state in patients with MM. Thus, blocking PS may be a novel therapeutic target for the prevention of thrombosis in these patients.

Introduction

Venous thromboembolism (VTE) is a common complication in patients with multiple myeloma (MM), who can have up to a 28-fold increase in risk of VTE (1,2). The occurrence of thrombosis seriously impacts quality of life and increases mortality of patients with MM (3). Notably, the incidence of VTE in patients with MM treated with immunomodulatory drug (IMiD) monotherapy is 2-6%, while dexamethasone (Dex)-containing regimens dramatically increase the risk of VTE up to 17-26% in individual clinical trials (4,5). Previous studies have reported that patients with MM have several risk factors for VTE, including increased levels of factor VIII and von Willebrand factor, hypofibrinolysis and acquired activated protein C resistance (6-9). However, these molecular mechanisms are not sufficient to reflect the changes in plasma procoagulant components and there is limited research studying the cellular mechanism of VTE in patients with MM. In addition, the evidence to guide optimal selection of thromboprophylaxis when managing patients with MM is still lacking. Thus, it is important to investigate the precise molecular and cellular mechanism of the hypercoagulable state in these patients.

Phosphatidylserine (PS) is exposed by the action of scramblase on the cell's surface during biological processes such as apoptosis and cell activation (10). Once exposed on the outer membrane, PS acts as a catalytic surface for factor Xa and thrombin formation in the coagulation cascade (11). Our previous studies have demonstrated that PS expression on the surface of circulating cells is associated with a risk of developing venous thrombotic complications in various disorders, including nephrotic syndrome and

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acute promyelocytic leukemia (12,13). Previous studies have demonstrated that peripheral blood cells and endothelial cells (ECs) can be injured or activated by high levels of monoclonal paraprotein and inflammatory cytokines circulating in the blood of patients with MM (14-18). However, relatively little is known about the extent to which PS is exposed on these cells, or whether PS$^+$ cells contribute to a hypercoagulable state in patients with MM. Although other studies reported that IMiDs with Dex increased PS exposure on monocyte and EC cell lines (19,20), the inhibition assays were not performed to clarify whether PS was responsible for cell-associated procoagulant activity (PCA). Furthermore, it is unclear whether IMiD-based regimens will further aggravate the amount of PS exposure on blood cells or ECs from patients with MM treated with patient serum. Thus, lactadherin, a superior PS probe (21), was used to detect the exposure of PS on cells and define whether there is an association between PS levels, cell-associated PCA and development of the hypercoagulable state in patients with MM.

The main objective of the current study was to evaluate the PCA of PS$^+$ blood cells and ECs and to assess a possible association with the development of venous thrombosis. Furthermore, the effect of treatment with IMiDs with or without Dex on PS exposure in blood cells and ECs was evaluated, and their relevance to PCA of these cells. Additionally, inhibition experiments were performed to evaluate the anticoagulant property of lactadherin. The results suggested that PS exposure on blood cells and ECs has a pivotal role in the hypercoagulability in patients with MM.

Materials and methods

Study subjects. The study included 20 newly diagnosed patients with MM (range 31-78, 45% males) according to the standards of the International Myeloma Working Group who were admitted to the First Affiliated Hospital of Harbin Medical University between October 2016 and February 2017, and 15 healthy volunteers (range 28-75, 46.67% males) (22). The exclusion criteria were a recent (<6 months) thrombotic event, current anticoagulant therapy, and associated disease, including antiphospholipid syndrome, chronic renal disease, heart disease, malignant or systemic disease, diabetes, acute infection, immobilization, surgery, hereditary thrombophilia and hyperviscosity, among other conditions. Harbin Medical University Ethical Committee (Harbin, China) approved the study, and patients provided written, informed consent.

Reagents. Thalidomide (Tha), lenalidomide (Len) and Dex were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Human umbilical vein cells (HUVECs), ECs medium, and poly-L-lysine were from ScienCell Research Laboratories, Inc. (San Diego, CA, USA). RPMI-1640 medium, fetal bovine serum (FBS) and bovine serum albumin (BSA) were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Alexa Fluor 488 or Alexa Fluor 647-conjugated lactadherin were prepared in our laboratory. Human factors (F) Va, VIIa, VIII, IXa, X, Xa, prothrombin, thrombin, fluorescein EGR-chloromethylketone and biotinylated EGR-chloromethylketone were all from Haematologic Technologies, Inc. (Burlington, VT, USA). Mouse anti-fibrin II chain (cat. no. NYBT2G1) was from

Accurate Chemical & Scientific Corporation (Westbury, NY, USA). Isotype control antibody (cat. no. X0931) was from Dako (Agilent Technologies, Inc., Santa Clara, CA, USA). Fluorescein-maleimide, fluorescein isothiocyanatephalloidin, DAPI, Alexa 647-labeled isotype-matched control antibody (cat. no. MA5-18168) were from Molecular Probes (Invitrogen; Thermo Fisher Scientific, Inc.). Propidium iodide (PI) was obtained from Shanghai Dobio Biotech Co., Ltd. (Shanghai, China). Chromogenic substrates S-2765 and S-2238 were from Diapharma Group, Inc. (West Chester, OH, USA). Percoll was from GE Healthcare Life Sciences (Uppsala, Sweden). Tyrode's buffer containing 1 mM HEPES was prepared in our laboratory and filtered through a 0.22-mm syringe filter from EMD Millipore (Billerica, MA, USA).

Protein purification and labeling. Lactadherin was purified from bovine milk and labeled with Alexa Fluor 647 or Alexa Fluor 488 as described previously (12). The ratio of fluorescein to lactadherin was 1.2/1 or 1.1/1.

Preparation of blood cells. Blood was drawn prior to therapy with a 21-gauge needle and was collected into an anticoagulant tube containing 3.2% citrate (5 ml; BD Biosciences, San Jose, CA, USA). Blood cell isolation was performed ≤30 min post-blood collection. Following centrifuging at 200 x g for 15 min at 20˚C, platelet-rich plasma (PRP) was aspirated carefully from the upper layer and erythrocytes were collected from the bottom layer of blood samples. Mixed peripheral blood leukocytes were isolated from the blood of study subjects using gradient centrifugation with 30% percoll and 68% percoll, according to the manufacturer's protocol. Erythrocytes, PRP, and leukocytes were analyzed immediately by flow cytometry and confocal microscopy following isolation. To prepare platelet-free plasma (PFP), PRP was centrifuged for 20 min at 1,500 x g at room temperature. For microparticle-depleted plasma (MDP) preparation, 600 μl of PFP was centrifuged for 30 min at 20,000 x g at 20˚C. The supernatant (400 μl) was collected, snap-frozen in liquid nitrogen, and then stored at -80˚C prior to use.

Preparation of immunomodulatory drugs and Dex. Tha and Len were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and Dex was resuspended in 1X PBS. The drugs were stored at -20˚C prior to use. Tha or Len and Dex were diluted in culture medium to reach the final concentrations of 1.0 and 10 μM, respectively. The final concentration of DMSO was 0.01% in all experiments as preliminary experiments had demonstrated that DMSO concentrations of 0.01% had no significant effect on PS exposure on cells (data not shown).

HUVEC culture and reconstitution experiments. HUVECs were maintained in EC medium in poly-L-lysine-coated cell culture flasks at 37˚C and 5% CO_2 in a humid environment. HUVECs were incubated in growth media containing 20% pooled serum obtained from patients with MM or healthy subjects at room temperature for 24 h. Serum-cultured ECs were then treated with DMSO (0.01%), Tal (1.0 μM), Len (1.0 μM), Tal (1.0 μM)/ Dex (10 μM), or Len (1.0 μM)/Dex (10 μM) at room temperature for 24 h. Cells in the logarithmic growth phase were used

for all experiments. All cell culture results presented are based on at least three individual experiments.

Blood cells of incubation and reconstitution experiments. Erythrocytes, platelets and leukocytes from patients with MM were cultured separately with DMSO (0.01%), Tal (1.0 μM), Len (1.0 μM), Tal (1.0 μM)/Dex (10 μM), or Len (1.0 μM)/Dex (10 μM) in a 5% CO_2 atmosphere at 37°C. Erythrocytes (10^6/ml) were incubated *in vitro* for 24 h at a hematocrit of 0.4% in Ringer's solution (23). Platelets (10^6/ml) were suspended in Tyrode's buffer for 1 h (24). Leukocytes (10^6/ml) were incubated for 24 h in RPMI-1640 medium containing 10% FBS (25). Erythrocytes, platelets and leukocytes were washed twice before detection.

Flow cytometric analysis of PS exposure on blood cells and ECs. To quantify PS exposure on blood cells and ECs, 5 μl of each cell suspension (0.5-1x10^6/ml) in Tyrode's buffer was incubated with 5 μl Alexa Fluor 488-conjugated lactadherin for 15 min at room temperature in the dark. A total of 10,000 events per sample were acquired by flow cytometry and analyzed with BD FACSDiva software 6.0 (BD Biosciences).

Clotting time and inhibition assays. PCA of various cell types was evaluated using a one-stage recalcification time assay in a KC4A-coagulometer (Amelung; Labcon GmbH, Heppenheim, Germany). Cell suspensions [100 μl of erythrocyte (1x10^8), platelet (1x10^7), leukocyte (1x10^6), or EC (1x10^6)] were incubated with 100 μl MDP from healthy volunteers at 37°C. After 3 min, 100 μl of warmed 25 mM $CaCl_2$ was added to start the reaction, and the time to subsequent fibrin strand formation was recorded. All clotting assays were performed in triplicate. For inhibition assays, cells were preincubated with lactadherin (final concentration 128 nM) prior to the assay.

Factor Xa and prothrombinase formation and inhibition assay. For the intrinsic Xa formation, a total of 10^4 platelets/leukocytes/ECs or 10^5 erythrocytes were incubated with 1 nM factor IXa, 5 nM factor VIII, 0.2 nM thrombin, 130 nM factor X, and 5 mM $CaCl_2$ in factor Xa buffer (TBS with 0.2% BSA) for 5 min at 20°C. The reaction was stopped by the addition of EDTA (7 mM final concentration). Immediately following the addition of 10 μl S-2765 (0.8 mM) chromogenic substrate, factor Xa generation was determined using a kinetic absorbance reading at 405 nm on a SpectraMax M5 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The assay to measure formation of extrinsic factor Xa was analogous to that for intrinsic factor Xa except that cells were mixed with 1 nM factor VIIa, 130 nM factor X and 5 mM $CaCl_2$. For the prothrombinase assay, the samples were incubated with 1 nM factor Va, 2 nM factor Xa, 1 μM prothrombin and 5 mM $CaCl_2$ in prothrombinase buffer (TBS with 0.05% BSA) for 5 min at 25°C. Thrombin production was evaluated after the addition of EDTA using the chromogenic substrate S-2238 (0.8 mM). For the inhibition assays of intrinsic/extrinsic factor Xa and prothrombinase formation, cells suspensions were pre-incubated with lactadherin (final concentration 128 nM) for 10 min at 25°C in Tyrode's buffer. The mixture was then incubated with the specified clotting factors according to the above protocols. The quantity of thrombin or factor Xa formation was assessed as previously described (12).

Fibrin generation assay. Fibrin formation was quantified by turbidity as described (26). For assays in normal human MDP, blood cells and cultured ECs were added to recalcified (10 mM, final) normal human MDP (88% MDP, final) in the absence or presence of lactadherin (final concentration 128 nM). Fibrin production was measured by turbidity at 405 nm in a SpectraMax 340PC plate reader (Molecular Devices).

Confocal microscopy. To locate PS, erythrocytes, platelets or leukocytes were incubated with Alexa Fluor 488-lactadherin (final concentration 4 nM) or PI, and ECs were incubated with the final concentrations of 4 nM Alexa Fluor 488-lactadherin and Alexa Fluor 647-CD31 [purified CD31, (clone L133.1), BD Biosciences)] for 10 min at room temperature in the dark. Then, cells were washed to remove unbound dye and imaged immediately. Observation of PS exposure on cells by confocal microscopy was performed as previously described (12). For fibrin generation experiments *in vitro*, ECs were cultured on 1% gelatin-coated coverslips in media containing 20% normal or patient serum for 24 h. Following rinsing with Tyrode's buffer, the ECs were then overlaid with prewarmed MDP (15%) in the presence of 3 mM calcium. Fibrin networks were imaged using laser confocal microscopy in the presence of Alexa Fluor 647-conjugated anti-fibrin (final concentration, 1 μg/ml; prepared in our laboratory) at 37°C for 15 min. Background signal was calculated using a similarly labeled isotype matched control antibody (final 1 μg/ml) at 37°C for 15 min. To observe the location of coagulation factor binding sites, the ECs were co-stained with factor Va-fluorescein-maleimide (final concentration, 2 nM) and factor Xa-EGRck-biotin (final concentration, 2 nM, complexed to Alexa Fluor 647-streptavidin) at 37°C for 10 min. Samples were excited with 488 or 568 nm emission lines of a krypton-argon laser and narrow bandpass filters were used for restricting emission wavelength overlap. Images were obtained using the LSM 510 system (Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis. Results are presented as mean ± standard deviation of at least triplicate measurements. Statistical analysis was performed with Student's t-test or one-way analysis of variance followed by Fisher's least significant difference post hoc tests for multiple comparisons as appropriate. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Patient characteristics. The characteristics of the study participants (20 patients with MM and 15 healthy subjects) are detailed in Table I. Patients with MM had a high serum concentration of M-component. Compared with healthy subjects, patients with MM had significantly higher levels of β2-microglobulin, fibrinogen, D-dimer, von Willebrand factor and factor VIII and shortened prothrombin time. However, patients with MM had lower levels of erythrocytes and hemoglobin than healthy subjects.

PS exposure of blood cells from healthy subjects and patients with MM patients, and in cultured ECs. As lactadherin binds

Table I. Clinical characteristics of healthy subjects and patients with multiple myeloma.

Characteristic	Healthy subjects	MM
Total n	15	20
Age (years)	57±7	60±11
Male, n (%)	7 (46.67%)	9 (45%)
International Staging System stage I/II/III, n (%)	NA	4/7/9 (20%/35%/45%)
M-protein class, n (%)		
IgG	NA	14 (70%)
IgA	NA	6 (30%)
Serum M protein (g/l)	NA	25±12
β2-microglobulin (mg/l)	1.65±0.55	6.88±5.08[a]
Erythrocytes (x10^{12}/l)	4.10±0.44	3.31±1.01[a]
Platelet (x10^9/l)	228±25	188±82
Leukocytes (x10^9/l)	5.79±0.63	5.31±1.38
Albumin (g/l)	42.4±3.6	37.6±7.4
Hemoglobin (g/l)	124±8	101±29[a]
Prothrombin time (sec)	13.48±0.76	12.52±1.04[a]
Activated partial thromboplastin time (sec)	33.9±2.0	31.6±4.5
Fibrinogen (g/l)	2.61±0.32	3.07±0.96
D-dimer (mg/l)	0.20±0.07	1.81±1.26[a]
Von Willebrand factor (U/ml)	1.06±0.26	2.23±0.71[a]
Factor VIII (U/ml)	0.87±0.18	2.10±0.62[a]

[a]$P<0.05$ vs. healthy subjects. NA, not applicable. Data are presented as numbers (percentages) or mean ± standard deviation.

to PS with high affinity (21), fluorescence-labeled was used lactadherin to detect the level of PS exposure on various blood cells using a flow cytometer. Patients with MM had a significantly higher percentage of PS$^+$ blood cells than healthy subjects (RBC, 1.12±0.58 vs. 0.54±0.29%; PLT, 10.24±3.37 vs. 2.35±1.10%; WBC, 19.26±2.55 vs. 5.46±1.41%; $P<0.05$ for all cell types; Fig. 1A). The percentage of PS$^+$ ECs after treatment with patient serum was 4.30-fold higher than after treatment with healthy plasma ($P<0.05$; Fig. 1B). The absolute number of PS$^+$ blood cells was also significantly higher in patients with MM compared with healthy subjects ($P<0.05$, for all; Table II). To observe PS binding on the outer membrane of cells, erythrocytes, platelets, or leukocytes were incubated with Alexa Fluor 488-lactadherin and PI, and imaged using confocal microscopy. Alexa Fluor 488-lactadherin staining was not observed on the membranes of platelets, erythrocytes and leukocytes from healthy subjects (Fig. 1C), whereas light green fluorescence was observed on platelets, leukocytes and erythrocytes from patients with MM (Fig. 1D). These results further confirmed that there is increased PS exposure on blood cells in patients with MM compared with healthy subjects.

ECs incubated for 24 h with serum from patients with MM or healthy control were stained with CD31-Alexa Fluor 647 and PS exposure was detected using lactadherin-Alexa Fluor 488. Confocal laser-scanning microscopy demonstrated that there was limited lactadherin staining on ECs cultured with normal serum (Fig. 1C), while large amounts of lactadherin, exhibited

Table II. Absolute number of PS-positive blood cells in study subjects.

PS$^+$ blood cells	Healthy subjects	Multiple myeloma
Erythrocyte (x10^{10}/l)	2.20±1.30	3.71±5.93[a]
Platelet (x10^9/l)	5.33±2.96	19.24±9.69[a]
Leukocytes (x10^8/l)	3.16±0.93	10.23±3.34[a]

[a]$P<0.05$ vs. healthy subjects. Data are presented as the mean ± standard deviation. PS, phosphatidylserine.

by green fluorescence, was observed on ECs treated with patient serum (Fig. 1D). Notably, the percentage of PS$^+$ ECs was higher than that of erythrocytes, platelets and leukocytes in patients with MM.

Coagulation time and inhibition assay. To examine whether the increase in PS$^+$ cells contributes to enhanced PCA, recalcification-time assays were performed using a KC4A-coagulometer. Compared with samples from healthy subjects, patient suspensions of erythrocytes, platelets and leukocytes (cell number controlled) exhibited significantly shorter coagulation time ($P<0.05$; Fig. 2A). ECs cultured in serum from patients with MM induced a shorter coagulation time when cultured in
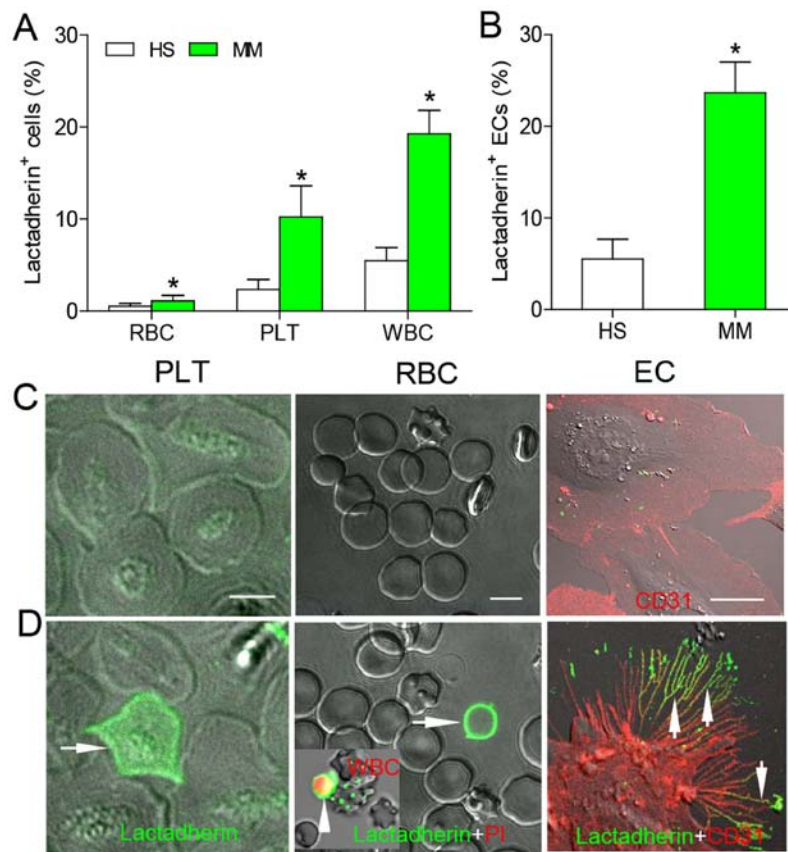
Figure 1. PS exposure on blood cells and HUVECs was analyzed by flow cytometry and confocal microscopy. Blood cells of (A) HS and patients with MM and (B) HUVECs treated with HS or MM serum were incubated with Alexa 488-conjugated lactadherin and assessed by flow cytometry. Data are presented as the mean ± standard deviation, *P<0.05 vs. HS. Alexa Fluor 488-lactadherin staining was performed on blood samples from (C) HS and (D) patients with MM; almost no staining was observed on cells from HS; WBCs were identified using PI staining (red fluorescence, inset). ECs with (C) HS or (D) patient serum were stained with CD31-Alexa Fluor 647 and PS exposure was detected using lactadherin-Alexa Fluor 488 at 24-h incubation; almost no lactadherin staining was observed on normal HUVECs. Treatment of HUVECs with patient serum led to retraction of cell margins, extension of filopods, and lactadherin (green) binding on filopods (arrows); scale bar, 5 μm. PS, phosphatidylserine; HUVEC, human umbilical vein endothelial cells; HS, healthy subjects; MM, multiple myeloma; PLT, platelets; RBCs, red blood cells (erythrocytes); EC, endothelial cell; WBCs, white blood cells (leukocytes); PI, propidium iodide.
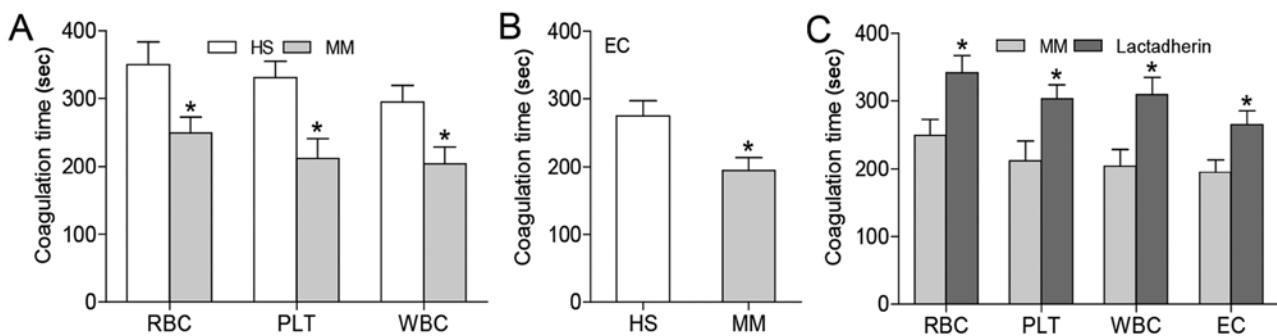

Figure 2. Coagulation time and inhibition assay. (A) Coagulation times of RBCs, PLTs, or WBCs from healthy subjects and patients with MM were evaluated. Blood cells of MM patients had more procoagulant activity than those from healthy subjects. (B) ECs cultured with patient serum for 24 h showed more PCA than those cultured with healthy serum. (C) Procoagulant activity of blood cells from MM patients and cultured ECs in patient serum for 24 h was detected in the absence or presence of lactadherin (128 nM); lactadherin entirely inhibited procoagulant activity induced by blood cells and ECs. Data are representative of triplicate independent experiments, and are displayed as the mean ± standard deviation. *P<0.05 vs. HS. HS, healthy subjects; MM, multiple myeloma; RBCs, red blood cells (erythrocytes); PLT, platelets; WBCs, white blood cells (leukocytes); EC, human umbilical vein endothelial cells.

serum from healthy subjects (P<0.05; Fig. 2B). To confirm whether this increased PCA was attributed to membrane PS exposure, inhibition assays were performed where cells were incubated with 128 nM lactadherin prior to coagulation testing. Lactadherin prolonged the coagulation time of PS⁺ cells to similar values as healthy subjects (P<0.05; Fig. 2C).

Role of PS in formation of procoagulant enzyme complexes. In order to further evaluate the capacity of blood cells and ECs to support the formation of procoagulant enzyme complexes, intrinsic/extrinsic factor Xa and thrombin formation assays were performed using purified coagulation factors. The production of all three procoagulant enzyme complexes was
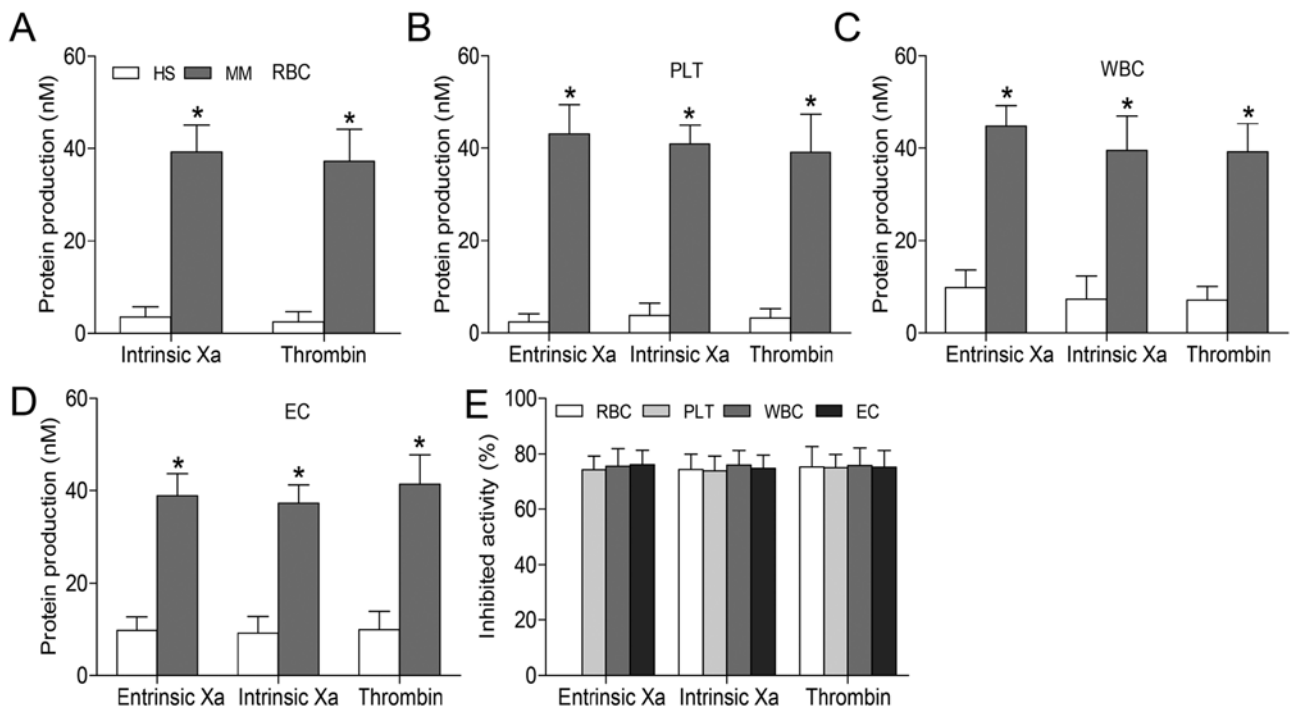
Figure 3. Formation and inhibition assays of procoagulant enzyme complexes. Xa and thrombin production in the presence of (A) $2x10^5$ RBCs, (B) $2x10^4$ PLTs or (C) $2x10^4$ WBCs from healthy subjects and patients with MM. Experiments were also performed using (D) $2x10^4$ ECs cultured with normal or patient serum for 24 h. Intrinsic Xa formation was measured in the presence of intrinsic Xa, FVIII and thrombin. Extrinsic Xa production was assessed in the presence of FVIIa. Thrombin generation was investigated in the presence of Xa and FVa. (E) Capacity of lactadherin (128 nM) to block procoagulant enzyme complexes on cells from patients with MM was evaluated; in each cell type, lactadherin decreased activity of the procoagulant enzyme complexes by ~75%. Results displayed as the mean ± standard deviation, *$P<0.05$ vs. HS. FVIII, factor VIII; FVa, factor Va; HS, healthy subjects; MM, multiple myeloma; RBCs, red blood cells (erythrocytes); Xa, factor Xa; PLT, platelets; WBCs, white blood cells (leukocytes); EC, human umbilical vein endothelial cells.
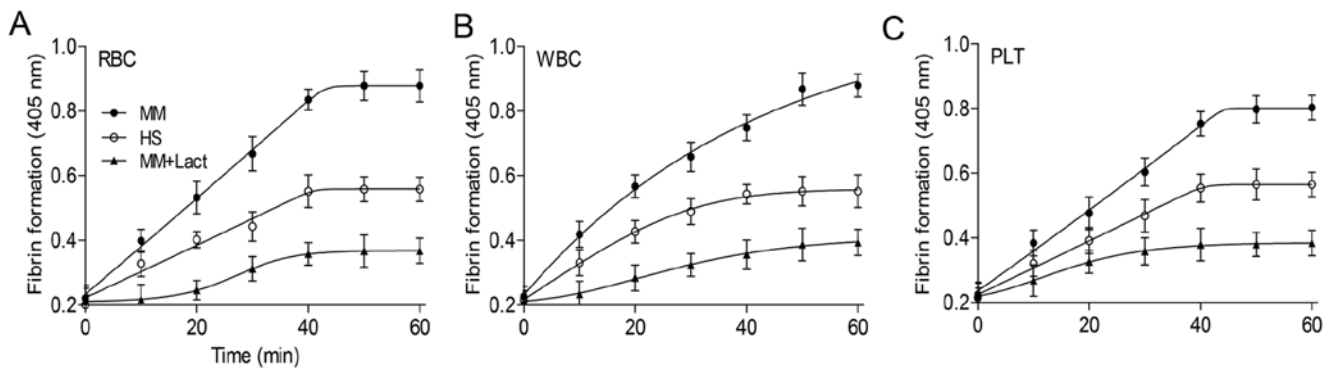

Figure 4. Fibrin formation and inhibition assays. Fibrin production supported by blood cells from healthy subjects and patients with MM was measured by turbidity. (A) RBCs, (B) WBCs and (C) PLTs from patients with MM supported more fibrin generation than those from HS. Preincubation with lactadherin (128 nM) reduced fibrin generation on blood cells from patients with MM to lower than HS. Data are presented as the mean ± standard deviation. RBCs, red blood cells (erythrocytes); HS, healthy subjects; MM, multiple myeloma; Lact, lactadherin; WBCs, white blood cells (leukocytes); PLT, platelets.

increased in MM groups compared with healthy subjects ($P<0.05$; Fig. 3A-D). These findings suggest that PS$^+$ blood cells contribute to coagulation, and that the relative number of these blood cells may increase thrombosis risk. In inhibition assays for all cell types, lactadherin (at 128 nM) blocked production of the three procoagulant enzyme complexes by ~75% (Fig. 3E). PS blockade almost entirely inhibited the formation of these complexes, suggesting that PS independently increases PCA in patients with MM.

Role of PS in fibrin formation on blood cells. Fibrin constitutes the primary structural protein of blood clots and intravascular

thrombi and its formation involves the concerted action of coagulation factors and blood cells. Therefore, fibrin formation was evaluated in normal MDP. More fibrin was deposited on erythrocytes, platelets and leukocytes from patients with MM than those from healthy subjects ($P<0.05$; Fig. 4). The inhibition assays demonstrated that lactadherin reduced fibrin formation of all cells to a level lower than healthy subjects.

Factor Xa/factor Va binding and fibrin deposition around the PS on ECs. Confocal microscopy provided more evidence that PS on ECs has an important role in fibrin clot formation. Limited factor Va (red) or factor Xa (green) binding was
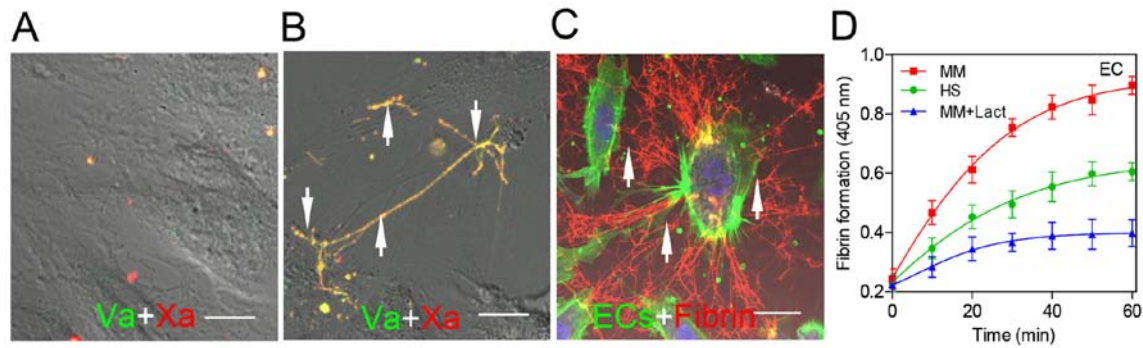
Figure 5. FVa/FXa binding and fibrin deposition on ECs treated with patient serum. FVa/FXa binding on cultured ECs treated with (A) normal serum and (B) serum from patients with MM, detected using confocal microscopy; more FVa and FXa colocalization (yellow) is observed on filopods (arrows) of near the retracted edges of ECs treated with patient serum than normal serum. (C) Cultured ECs were incubated with plasma in the presence of calcium and Alexa 647-conjugated anti-fibrin at 37˚C for 15 min; ECs and nuclei were visualized by actin (green) staining with fluorescein isothiocyanate-phalloidin and DAPI (blue) and marked fibrin strands were observed along the filopodia of cultured ECs (arrow); scale bar, 2 μm. (D) Fibrin production on ECs cultured with patient serum was detected in the presence of recalcified MDP with or without 128 nM lactadherin. Data are presented as the mean ± standard deviation. ECs, human umbilical vein endothelial cells; Va, factor Va; Xa, factor Xa; MM, multiple myeloma; HS, healthy subjects; Lact, lactadherin.
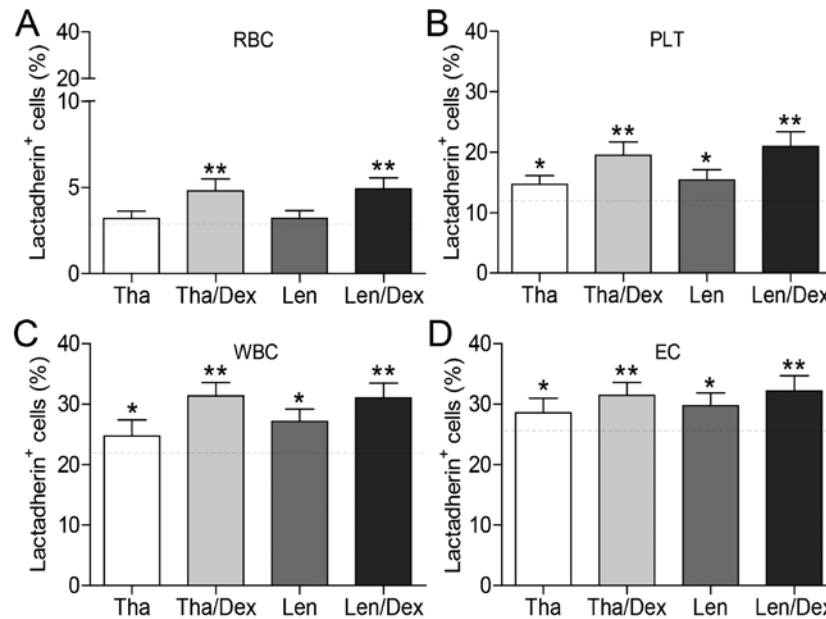

Figure 6. Effect of immunomodulatory drugs-based treatments on PS exposure of blood cells and ECs. (A) RBCs (24 h), (B) PLTs (1 h), (C) WBCs (24 h) from patients with MM were incubated with dimethyl sulfoxide (0.01%), Tha (1.0 μM), Len (1.0 μM), Tal (1.0 μM)/Dex (10 μM), or Len (1.0 μM)/Dex (10 μM) at 37˚C. (D) ECs were incubated in five groups containing 20% pooled serum obtained from patients with MM at room temperature for 24 h. The gray dashed line represents PS exposure with no drug treatment as control. PS exposure was measured as the % of cells that were positive for Alexa Fluor 488-lactadherin using flow cytometry. Data are presented as the mean ± standard deviation, *P<0.05 vs. control, **P<0.05 vs. Tha or Len. PS, phosphatidylserine; MM, multiple myeloma; RBC, red blood cells (erythrocytes); Tha, thalidomide; Len, lenalidomide; Dex, dexamethasone; PLT, platelets; WBC, WBCs, white blood cells (leukocytes); EC, human umbilical vein endothelial cells.

observed on ECs treated with serum from healthy subjects (Fig. 5A, arrow). When ECs were cultured with the serum from patients with MM, factor Va and factor Xa were observed to be bound to the PS-enriched areas of the cell membrane (Fig. 5B, arrow). ECs were incubated with plasma in the presence of calcium and Alexa 647-conjugated anti-fibrin. Considerable fibrin fibrils (red) were spread along the filopodia of ECs visualized using actin and DAPI staining (Fig. 5C, arrow). The patient serum resulted in a 1.48-fold higher fibrin generation in ECs than the serum from healthy subjects (P<0.05; Fig. 5D).

Effects of IMiDs with or without Dex to PS exposure on blood cells and ECs. The effects of the IMiDs (Tha and Len, with or without Dex treatment) on PS exposure of blood cells *in vitro* were determined. Erythrocytes (24 h), platelets (1 h), and leukocytes (24 h) from patient with MM were cultured with Tha (1.0 μM), Len (1.0 μM), Tha (1.0 μM)/Dex (10 μM), or Len (1.0 μM)/Dex (10 μM) at 37˚C. Flow cytometry analysis demonstrated that, compared with control or either IMiD alone, Tha or Len plus Dex increased the level of PS exposure on erythrocytes (P<0.05; Fig. 6A). IMiD treatment alone did not significantly increase PS exposure on erythrocytes compared with controls. However, Tha and Len significantly increased PS exposure on platelets and leukocytes compared with controls (P<0.05; Fig. 6B and C). Treatment of platelets and leukocytes with an IMiD in combination with Dex resulted in higher PS

exposure compared with IMiD treatment alone. The effects of IMiDs and Dex, either alone or in combination, on PS exposure on ECs were determined by flow cytometry. Incubation of ECs with an IMiD alone resulted in increased PS exposure compared with controls (P<0.05; Fig. 6D). Furthermore, an increased percentage of PS⁺ ECs was induced by the combination of an IMiD and Dex, as compared with Tha or Len alone. The results indicate that IMiDs and Dex used in combination can further increase PS exposure on blood cells from MM patients and ECs *in vitro*.

Discussion

The findings of the current study demonstrated that PS⁺ blood cells may potentially have a role in the induction of a hypercoagulable state in patients with MM, and that patient serum components can induce PS exposure in cultured ECs. Compared with healthy subjects, patients with MM had markedly higher levels of PS⁺ blood cells, and serum from patients with MM increased PS⁺ cultured ECs compared with healthy serum. In addition, the percentage of PS⁺ ECs was higher than that of erythrocytes, platelets and leukocytes in patients with MM. Exposed PS contributes to tenase and prothrombinase complex formation, leading to a shorter coagulation time and greater intrinsic/extrinsic factor Xa, thrombin and fibrin generation. In addition, combined treatment with IMiDs plus Dex induced greater PS exposure on platelets, leukocytes and erythrocytes than treatment with IMiDs alone. Finally, lactadherin inhibited production of all three procoagulant enzyme complexes by ≥75%, further confirming the procoagulant role of PS in patients with MM.

Although platelet dysfunction often occurs in MM (27), it remains unclear whether platelets contribute to thrombosis in patients with MM. To the best of our knowledge, this is the first study evaluating the role of PS⁺ platelets in the prothrombotic state in patients with MM. The percentage of PS⁺ platelets was significantly higher in patients with MM than in healthy subjects, which indicates abnormal activation or apoptosis of platelets. In a previous study, the serum level of platelet factor 4 was significantly elevated in patients with MM (15), supporting this finding. Notably, PS⁺ platelets had the capacity to increase tenase and prothrombinase complex formation, leading to greater factor Xa, thrombin and fibrin generation. Activated platelets release thrombin leading to platelet aggregation and three-dimensional clot formation (28). Thus, we hypothesize that PS⁺ platelets may recruit more circulating platelets by producing thrombin. On this basis, platelet PS exposure is an important mediator of the hypercoagulable state in patients with MM.

Clinical studies have reported that activated leukocytes are associated with venous thrombosis (29). However, the pathogenic pathways linking leukocyte abnormalities to thrombosis in patients with MM remain unclear. The results of the current study indicated that injured or activated leukocytes increased PCA through PS exposure. This process increases the serum concentration of three procoagulant enzyme complexes by PS⁺ leukocyte-derived PCA and potentially forms the nidus upon which the thrombus develops. Typically, erythrocytes are considered as passive participants in coagulation, merely providing bulk material for the obstructive clot. The observa-

tions in the current study provide evidence that erythrocytes may be actively involved in the hypercoagulable state of patients with MM. PS exposure on erythrocytes provides a catalytic surface to support the assembly of blood coagulation factors, thus promoting the coagulation cascade activation and thrombin generation (10). Previous studies reported that PS⁺ erythrocytes are also more adhesive to endothelial cells and prone to form erythrocyte aggregates in chronic uremia and obesity, supporting the conclusions of the present study (30,31).

To investigate the role of vascular endothelium dysfunction in MM-associated thrombosis, cultured ECs were incubated with patient serum, which resulted in a significant increase in PS exposure compared with incubation in serum from healthy subjects. A previous study has indicated patients with MM exhibit pathologically enhanced von Willebrand factor, a marker of ECs activation, which is consistent with the findings of the present study (7). In addition to promoting the formation of thrombin, the results of the current study demonstrate that patient serum-treated ECs highly expressed PS, which supported binding of factor Va and factor Xa, and fibrin deposition. Following activation, imbalance or alteration of 'differential expression of procoagulants and anticoagulants in the endothelium' may modulate endothelial thromboresistance from an anticoagulant state into a procoagulant one (32,33). Thus, it seems reasonable to hypothesize that PS has a pivotal role in the localized procoagulant phenotype of ECs by bringing clotting factors together, resulting in fibrin formation.

It was previously reported that patients with MM have higher levels of endogenous thrombin potential in a global assay of thrombin generation (34). The data of the current study demonstrated that PS⁺ blood cells and ECs from patients with MM supported the formation of intrinsic/extrinsic factor Xase and prothrombinase, essential components of the coagulation cascade that lead to increased thrombin generation. In addition, PS⁺ cells from patients with MM have a shorter coagulation time and higher production of fibrin than samples from healthy subjects. Blockade of PS with lactadherin prolonged coagulation time and decreased fibrin formation to control levels and inhibited ~75% of the procoagulant enzyme production. By contrast, in a previous study, anti-tissue factor antibody had a negligible effect on PCA of cells from patients with MM; this may be explained by the fact that plasma exposed tissue factor is generally quiescent, with little or no detectable PCA, unless it resides in a membrane containing PS (35,36). The results indicate that increased PS contributes to the hypercoagulable state in patients with MM.

The exact mechanism of the increased risk of VTE in patients with MM treated with IMiDs-based regimens is not yet fully understood. The results of the current study have demonstrated that treatment with IMiDs plus Dex induces higher levels of PS⁺ blood cells and ECs than controls or IMiDs alone, *in vitro*. IMiDs with Dex has been previously reported to increase P-selectin expression on platelets and induce EC injury, supporting the findings of the present study (37,38). In addition, IMiDs stimulate T-cell production and activation of natural killer cells (39). Thus, we hypothesize that treatment with Dex may aggravate the abnormal activation or apoptosis of blood cells in patients with MM using IMiDs-based regimens. Considering the high cost of treatment (40), the number

of patients that receive IMiDs-based regimens is low in China. The *in vivo* effect of IMiDs-based regimens on PCA of blood cells and ECs from patients with MM will be investigated in future studies.

In summary, PS$^+$ blood cells and PS-associated PCA were increased in the circulation of patients with MM and patient serum induced PS exposure on cultured ECs. In addition, IMiDs-based treatment increased PS exposure on blood cells and ECs *in vitro*. Increasing levels of PS$^+$ cells were associated with a hypercoagulable state in patients with MM. Furthermore, PS inhibition assays using lactadherin suggest that blockage of PS may constitute a novel therapy for preventing thrombosis. Future clinical trials are required to investigate this hypothesis.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

Study concept and design (LG, JS); data acquisition, clinical data and sample collection, statistical analysis (LG, DT, MY, CW, PZ, JJ, BL, YL, RL); data interpretation and manuscript drafting (LG, JS); critical revision of the manuscript (LG, VN, DT, MY, YZ, TL, JS); YZ, TL, ZD, YT, JZ, YB and JK provided valuable advice for this study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Harbin Medical University Ethical Committee (Harbin, China) approved the study, and patients provided written, informed consent.

Consent for publication

Not applicable.

Competing interests

JS has a patent for the use of lactadherin as a probe for phosphatidylserine. Other authors declare that they have no competing interests.

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