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Type
Research paper

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AMI, IL-33, TF, EMPs

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Activated endothelial cells and EMPs they released simultaneously express TF, which is a risk factor for cardiovascular disease.
IL-33 / ST2 Signaling Promotes TF Expression by Regulating NF-κB Activation in Coronary Artery Endothelial Microparticles

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Conclusion: Activated endothelial cells and EMPs they released simultaneously express TF, which is a risk factor for cardiovascular disease.

**Keywords:** IL-33, TF, EMPs, AMI

### 1. Introduction

Atherosclerosis is a chronic inflammatory disease of atherosclerotic plaque\(^1\) that remains the leading cause of death worldwide. It is the leading contributor to Coronary Vascular Disease (CVD), and its treatment is an essential step towards appropriate management and prevention of CVD\(^2\). The Burden of disease study in China showed a 20.6% increase in ischemic heart disease mortality from 1990 to 2017\(^3\). According to the Statistics of the American Heart Association, approximately 2.5 million people were hospitalized for Acute Myocardial Infarction (AMI) each year, among which 18% of women and 23% of men in a population with an average age of over 40 years died within one year of being diagnosed with AMI\(^4\).

Microparticles (MPs) are cell membrane phosphatidylserine ranging from 0.1 to 1.0µm, which contain information like as mRNA, microRNAs (miRNAs), receptor and specific proteins of parent cell\(^5\,6\). MPs from endothelial cells, erythrocytes and platelets play an important role in the process of atherosclerosis\(^7\,8\). MPs shed from activated or apoptotic cells containing complex procoagulant and proinflammatory properties\(^9\,10\). The first step in the development of atherosclerotic lesions is endothelial dysfunction, which is a key factor in the development of coronary atherosclerosis disease\(^11\). Stimulating endothelial cells (ECs) to release endothelial microparticles (EMPs)
can not only be used as an early alternative to endothelial dysfunction, but also as a biological mediator to regulate inflammation and coagulation after early ECs injury.

Tissue Factor (TF), an integral cell-surface glycoprotein and the major in vivo initiator of coagulation, plays a central role in hemostasis and thrombosis\textsuperscript{12,13}. Plaque rupture reveals TF to flowing blood, resulting in coronary thrombosis and occlusion with consequent AMI. Thrombosis is linked to inflammation in many clinical conditions\textsuperscript{14}.

Interleukin-33 (IL-33) is released in the extracellular space following cell injury\textsuperscript{15}. IL-33 and ST2 are found locally in human atherosclerotic plaques\textsuperscript{16}. Related studies have shown that circulating IL-33 levels are associated with thrombotic complications after rupture of coronary and carotid atherosclerotic plaques\textsuperscript{17}, and are associated with ST segment elevation myocardial infarction (STEMI) mortality\textsuperscript{18,19}. These studies find that IL-33, locally expressed in atherosclerotic plaques, activates ECs by up-regulating the inflammatory system, through which promotes leukocyte adhesion to ECs and thereby regulates ECs proteolysis and promotes angiogenesis, which ultimately accelerates the development of atherosclerotic plaques\textsuperscript{16,20}.

Inflammation and coagulation are interdependent, which jointly determine the formation of atherogenic plaque lesions and the clinical progress of arterial thrombosis complications such as AMI, unstable angina and stroke\textsuperscript{21,22}. Therefore, we investigated the effect of IL-33 on TF release of EMPs, which may be a new link between inflammation and coagulation.

2. Materials and methods

2.1 Study population

The study population were the patients admitted to the Department of Cardiology of People’s Hospital of Xinjiang Uygur Autonomous Region from June 2018 to January 2020. According to the inclusion and exclusion criteria, a total of 27 patients with AMI and 30 patients with stable coronary artery disease (SCAD) were included in this study. The trial was conducted in
accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of People’s Hospital of Xinjiang Uygur Autonomous Region (No.2017041), and all patients provided a signed informed consent form.

Inclusion criteria were as follows:

(1) AMI: Measurement of elevated cardiac biomarkers (troponin preferred) that exceed the 99th percentile of the reference upper limit and fulfil at least one of the following conditions: 1) Symptoms of myocardial ischemia; 2) New ischaemic ECG changes; 3) Pathological Q wave appeared in electrocardiogram; and 4) Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischaemic aetiology; 5) Identification of a coronary thrombus by angiography or autopsy.

(2) SCAD: a clinical syndrome of transient ischemic and hypoxia caused by increased myocardial load on the basis of fixed and severe coronary artery stenosis. Patients undergoing coronary angiography for the diagnosis of atherosclerotic heart disease and stent implantation were included in the study (refer to guidelines for the Diagnosis and Treatment of Stable Coronary Artery Disease, Chinese Journal of Cardiovascular Diseases, 2018).

The exclusion criteria were as follows: 1) Serious liver or kidney dysfunction; 2) Cancer or other debilitating disease; 3) Diseases of the haematopoietic system; 4) Uncontrolled infection; 5) Infarction in another location of the body, such as cerebral infarction or pulmonary embolism; and 6) Coronary artery spasm.

2.2 Sample Collection

Circulating blood: Venous blood was collected within 24 hours after admission for general biochemical test.

Coronary blood: During PCI, the study subjects entered the coronary artery via the radial artery during the operation and the guide wire reached the lesion site. The balloon entered the lesion plaque to dilate the balloon, which was
suitable for the lesion vessels. After the balloon was rapidly discharged, 10ml of coronary blood was extracted, and the balloon was evacuated from the guide wire. The specimens were centrifuged at 3500×g 15 min at 4℃, and the supernatant was stored in the EP tube with EDTA of three grades. One sample was to obtain MPs and stored at -80℃ for qualitative and quantitative determination of MPs, and the remaining two samples were used for IL-33 and TF protein content detection.

2.3 Quantitation of CD31+ EMPs by flow cytometry

The samples were dissolved at room temperature. 500ul samples were taken from the EP tube, which were centrifuged at 2700×g for 15min and then 20,000×g for 20 min at 4℃. After centrifugation, the supernatant was gently removed, and 100 ul PBS was added. The extracted MPs were added with endothelial cell-specific monoclonal antibody(CD31) (1:50 dilution) at room temperature and incubated at 4℃ for 30min. Immediately after adding 200 ul PBS, the BD FACS AccuriC6 flow cytometer was used for qualitative and quantitative MPs detection.

The number of cells in the portal was 10000wh each time, which was read at a flow rate of 35μl /min for 30 seconds. The number of CD31+EMPs was counted and the fluorescence percentage of ECs labelled with specific monoclonal antibodies were analysed to further characterize CD31+EMPs. The final EMPs were expressed as percentages.

2.4 TF and IL-33 protein assays

TF protein levels in cell lysates were determined by using a specific ELISA (Human TF, cusabio CSB-E07913h) . The IL-33 protein levels were determined with a specific ELISA (Human IL-33, cusabio CSB-E13000h).

2.5 Cell culture

Human Coronary Artery Endothelial Cells (HCAECs) were purchased from GuangZhou Jennio Biotech Co. Ltd and cultured in M199 medium (Hyclone, SH30025) containing 10% fetal bovine serum (FBS), 100U / mL penicillin and 100ug / mL streptomycin. Cells were grown in 5% CO2, 95% air humidified
incubator at 37°C.

2.6 Treatment of cells

HCAECs were treated with 100ng/ml of recombinant human (rh) IL-33 (peprotech, 200-33) and blank control for 3, 6, 9, 24h respectively. In the experiment, the cell supernatant was collected and centrifuged at 500g for 20min to remove residual cells and pellets, and then the supernatant was transferred into a centrifuge tube and centrifuged at 2000g for 20min. The cell supernatant was transferred to a new centrifuge tube and centrifuged at 20000g after 50 min, where the supernatant was removed, the precipitate resuspended by adding PBS, and centrifugation repeated once to precipitate into MPs.

For blocking the transmembrane receptor ST2, 5ug/mL soluble extracellular domain ST2 coupled to the Fc fragment of human IgG1 (sST2) (G-Bioscience, BAN1479) and 5g/ml IgG (Beyotime, A7028) were added to the pre-incubated cultured cells and shaken evenly. In addition, the NF-Kb inhibitor dimethyl fumarate (DMF) (Selleck S6192), 100ng/ml rh IL-33 and DMF+IL-33 upon being shaken evenly were cultured in an incubator. Upon the collection of the cell superfine, the MPs were obtained by centrifugation with the same method as above. TF protein level in cell lysates was determined using the specific ELISA.

2.7 TF activity assays

Add 100ul PBS and resuscitate MP. TF Antibody (Ab) (Absolute antibody, ab00516-10.6) and 1ug/ml IgG (Beyotime, A7028) were added to the two groups, incubated at room temperature for 2h. Then RIPA protein was lysed for 30min for subsequent activity detection. After incubation in a 37°C incubator for 30min, 20 leds FVIIa reaction substrate were added. The OD value of absorbance was detected at 0min at OD 405nm wave length by Thermo fisher (Multiskan 51119000). OD value was detected at intervals of every 30min until 2h.
2.8 TF and TFPI protein assays

HCAECs were treated with 100ng/ml of rh IL-33 and blank control for 3, 6, 9, 24h respectively. Samples of rh IL-33 treating were removed from the -80°C refrigerator and placed at room temperature. Detection of TF and Tissue Factor Pathway Inhibitor (TFPI) protein used the Human TF (cusabio, CSB-E07913h) and Human TFPI sandwich ELISA Kit(R&D Systems, DTFP10), respectively.

Standards and samples were pipetted into the wells, and TF, TFPI present were bounded by the immobilized antibody. After removing any unbound substances, the wells were added with the biotin-conjugated antibody specific avidin conjugated Horseradish Peroxidase (HRP). Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of TF and TFPI bounded in the initial step. Absorbance read at 450nm was compared to those values obtained with recombinant TF and TFPI standard.

2.9 Statistical analysis: Continuous variables were expressed as mean ± standard deviation, and the data were compared in terms of Student’s t-test or Rank sum test in SPSS 21.0 statistical package for Windows. Chi-square test was adopted to analyse the association between categorical variables. Pearson’s correlation coefficient was calculated to determine significant correlations. Value of \( p \leq 0.05 \) was considered significant.

3. Results

3.1 The baseline characteristics of the patients included in this study were shown in Table 1 according to AMI and SCAD. The striking difference between the two groups was observed in the low-density lipoprotein cholesterol (LDL), Gensini score and the usage of prior statin. No significant differences were noted, such as age, sex, body BMI, hypertension, diabetes, smoking, TG, TC, HDL, CRP, Cr, Hb1c, LVEF, LVEDD, LVESD and the usage of prior antiplatelet.

3.2 AMI patients showed significantly higher levels of CD31+EMPs, TF protein
and IL-33 protein than SCAD patients (11.10[8.27, 13.20] versus 3.92[2.80,7.02], P<0.001 for CD31+EMPs. 303.80±42.04 versus 197.12±38.05, P<0.001 for TF protein. 138.29±47.64 versus 96.93±28.87, P<0.001 for IL-33 protein [Table 2 and Figure1].

3.3 To test whether the level of IL-33 is associated with the level of CD31+ EMPs or TF in coronary blood, we assessed the levels of IL-33, CD31+ EMPs and TF in 27 patients with AMI. The levels of IL-33 protein and CD31+ EMPs showed significant positive correlation (r = 0.794, p < 0.01) [Figure 2a]. The level of IL-33 protein did not correlate with TF protein (r=0.064, p=0.752) [Figure 2b].

3.4 To further investigate the efficiency of CD31+EMPs, TF protein and IL-33 protein as potential biomarkers of AMI, we performed ROC curve analysis on the patients with AMI. According to the outcome of ROC curve analysis, we found that the areas under the curve (AUC) of CD31+EMPs, TF protein and IL-33 protein were 0.888, 0.962, and 0.778 [Figure 3].

3.5 IL-33 increased TF activity of HCAEC derived MPs

HCAECs were treated with 100ng/ml of rh IL-33 and blank control for 3, 6, 9, 24h respectively. MPs were isolated from cell culture supernatants. TF Ab and IgG were added to the MPs of the two groups in each time point to test TF expression, which reflected the developmental stages that the TF was expressed. The results showed that the TF activity increased gradually with time of intervention [Figure 4]. TF activity was significantly higher compared to controls (p < 0.05), but the time point of 24h was not statistically significant.

3.6 IL-33 upregulated TF protein and downregulated TFPI protein in HCAEC derived MPs

HCAECs were treated with the presence or absence of rh IL-33. MPs were isolated from cell culture supernatants. TF and TFPI protein were tested by ELISA, and the expression of which was presented by the histogram. As Figure 5 showed, the viability of TF was significantly increased upon stimulation with rh IL-33 and the TFPI protein levels decreased conversely (p <
3.7 IL-33-induced TF expression ST2 and NF-κB

100ng/ml DMF or 5ug/ml sST2 was added to HCAECs which were treated with rh IL-33 and blank control. The TF protein level of adding DMF and sST2 were significantly lower compared to controls (p < 0.05) [Figure 6].

4. Discussion

Our results suggested that the level of EMPs, TF protein and IL-33 protein in AMI were higher in patients with SCAD. We examined the diagnostic value of EMPs, TF protein and IL-33 protein in discriminating patients with AMI from patients with SCAD. We could also show that IL-33 positively correlated with the level of CD31+ EMPs in patients with AMI. No such correlation was found for the level of IL-33 and TF. Furthermore, this evidence demonstrated that the higher levels of TF protein, EMPs and IL-33 protein could more likely to be potential biomarkers to distinguish patients with AMI from patients with SCAD.

In this study, we demonstrated that the pro-inflammatory cytokine IL–33 induced TF expression and TF activity in MPs of HCAECs as well as the release of procoagulant EMPs. We found that IL-33 upregulated TF protein level and downregulated TFPI protein level in HCAECs MPs. Total cellular TF protein was increased in HCAECs after 3, 6, 9 and 24 hours (h) of treatment with IL-33 as compared to the control. TFPI protein levels slightly but significantly declined in MPs of HUVECs after 3, 6, 9 and 24 h of treatment with rh IL-33.

The other studies show previously that IL-33 exerts its effects via binding to its cell surface receptor ST2. In order to investigate whether the TF of EC-derived MPs induction by IL-33 was also ST2-mediated, we incubated EMPs with a specific anti-ST2 antibody in the presence or absence of rh IL-33, with the stimulatory effect of IL-33 on TF protein level inhibited. This indicated that the increased TF production was a specific effect of IL-33 on HCAECs,
which could be blocked by sST2. In addition, the NF-κB inhibitor DMF was added to the HCAECs, which showed that IL-33/ST2 signaling promoted TF expression by regulating NF-κB activation.

High concentrations of EMPs may cause vascular damage and aggravate endothelial dysfunction. ECs and EMPs are the predominant sources of circulating blood-borne TF and contribute to the formation of a prothrombotic environment in patients with cardiovascular disease through the propagation of coagulation upon plaque rupture.25, 26.

TF expressed on the surface of MPs are the main activator of blood coagulation pathway. More and more studies have found that TF plays an important role in the process of thrombosis on the basis of atherosclerosis. It is found that the expression of TF in coronary plaques of patients with acute coronary syndrome (ACS) is higher than that of patients with stable angina.27 TF not only promotes the generation of thrombin and the formation of fibrin, but also causes instability of atherosclerotic plaques without depending on the coagulation mechanism. The process includes TF, which leads to vascular smooth muscle migration, vascular hyperplasia, activation of protease receptor and inflammatory response.28,29.

A prospective study suggests that the level of TF and MPs could serve as biomarkers for thrombosis risk.30 Activated ECs and their released MPs simultaneously express TF, which is a risk factor for cardiovascular disease.3

Studies have confirmed that IL-33 binds to the ST2 receptor and activates the NF-κB pathway, which causes the expression of TF on the surface of coronary artery endothelial cells and umbilical vein endothelial cells and their source MPs. The study have found that the expression level of TF mRNA is positively correlated with the expression of IL-33 mRNA in carotid atherosclerotic plaques, which proves that IL-33 acts on the TF activity at endothelial cell surface, enhances the coagulation function of endothelial cell and mediates the formation of thrombus in atherosclerotic plaques.32

In summary, this study shows that the levels of EMPs, TF protein and IL–33
increased in the AMI patients and IL–33 was positively correlated with the circulating levels of CD31+EMPs in the AMI patients. In addition, it presents evidence of an ST2/NF-κB mediated up-regulation of TF protein expression and activity in HCAECs MPs after treatment with IL-33. Furthermore, it suggests that IL-33 treatment increased the release of procoagulant HCAECs-derived MPs. The above findings provide a possible pathophysiologic explanation for a clinical association between IL-33 and atherosclerosis thrombotic events in patients with cardiovascular disease.

5. **Conclusion**

Our study shows that the levels of EMPs, TF protein and IL–33 increased and IL–33 was positively correlated with the circulating levels of CD31+EMPs in the AMI patients. In addition, we present evidence of an ST2/NF-κB mediated up-regulation of TF protein expression and activity in HCAECs MPs after treatment with IL-33. Furthermore, it suggests that IL-33 treatment increased the release of procoagulant HCAECs-derived MPs.
<table>
<thead>
<tr>
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<th>AMI(n=27)</th>
<th>SCAD(n=30)</th>
<th>Z/t/χ² Value</th>
<th>P Value</th>
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<tr>
<td>Age</td>
<td>58±12</td>
<td>62±8</td>
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<td>0.166</td>
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<tr>
<td>Male/female</td>
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<td>20/10</td>
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<td>BMI (Kg/m²)</td>
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<td>7,23.33</td>
<td>1.275</td>
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<td>Smoking(n,%)</td>
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<td>14,46.67</td>
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<td>CRP(mg/l)</td>
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<td>2.85[2.50,3.95]</td>
<td>-1.565</td>
<td>0.118</td>
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<td>Cr(umol/l)</td>
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<td>58.90[54.03,73.58]</td>
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<td>TG(mmol/L)</td>
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<td>1.45±0.99</td>
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<td>TC(mmol/L)</td>
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<td>HDL(mmol/L)</td>
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<td>LVEF%</td>
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<td>55[49,59]</td>
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<td>LVEDD(mm)</td>
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<td>48[45,51]</td>
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<td>Prior medication</td>
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<td>17,56.67</td>
<td>7.005</td>
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AMI: acute myocardial infarction, SCAD: stable coronary artery disease, CRP: C-reactive protein, Cr: creatinine, TG: triglycerides, TC: total cholesterol, LDL: Low density lipoprotein-cholesterol, HDL: High density lipoprotein-cholesterol, LVEF: left ventricular ejection fraction, HbA1C: Hemoglobin A1C, LVEDD: left ventricular end-diastolic diameter, LVESD: Left ventricular end-systolic diameter
Table 2 The levels of CD31+ EMPs, TF and IL-33 protein in patients with AMI and SCAD

<table>
<thead>
<tr>
<th></th>
<th>AMI</th>
<th>SCAD</th>
<th>Z/t Value</th>
<th>P Value</th>
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<td>CD31+EMP%</td>
<td>11.10[8.27,13.20]</td>
<td>3.92[2.80,7.02]</td>
<td>-5.019</td>
<td>&lt;0.01</td>
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<td>TF (pg/ml)</td>
<td>303.80±42.04</td>
<td>197.12±38.05</td>
<td>-10.057</td>
<td>&lt;0.01</td>
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<tr>
<td>IL-33 (pg/ml)</td>
<td>138.29±47.64</td>
<td>96.93±28.87</td>
<td>-3.911</td>
<td>&lt;0.01</td>
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</table>

![Graphs showing CD31+ EMPs, TF, and IL-33 levels in AMI and SCAD](image-url)
Figure 1. The levels of CD31+EMPs, TF protein and IL-33 protein in AMI and SCAD groups. a) The flow cytometry result in AMI patients. b) The flow cytometry result in SCAD patients. c) The level of CD31+EMPs in coronary blood of AMI and SCAD groups. d) The level of TF protein in coronary blood of AMI and SCAD groups. e) The level of IL-33 protein in coronary blood of AMI and SCAD groups. **means P<0.01.

Figure 2. IL-33 is positively correlated with CD31+EMP in patients with AMI. CD31+EMPs, IL-33 protein and TF protein are determined in coronary blood of patients with AMI. a) The level of IL-33 is correlated with CD31+EMPs. b) In contrast, IL-33 did not correlate with TF protein. Pearson’s correlation coefficient is calculated to determine significant correlations. p<0.05 is considered significant.
Figure 3. ROC curve analyses of CD31+EMPs, TF protein and IL-33 protein in AMI patients. We investigate the efficiency of CD+31 EMPs, TF protein and IL-33 protein as potential biomarkers of AMI through ROC curve. According to the areas under the curve (AUC) of EMPs, TF protein and IL-33 protein are 0.888, 0.962, and 0.778.

Figure 4. The TF activity by treated TF Antibody (Ab) and control. HCAECs are treated with 100ng/ml of rh IL-33 and blank control to obtain MPs, and then added with TF Ab and IgG. The number of time-points examined for each TF reflects the developmental stages that the TF is expressed for 3, 6, 9, 24 hours. The TF activity increase gradually with time of intervention for 3, 6, 9 hours, but the time point of 24h is not statistically significant.
Figure 5. HCAECs incubated for 3h, 6h, 9h, 24h in the absence (Co) or presence of rh IL-33 (100 ng/ ml). a) rh IL-33 induces TF expression and the release of TF-positive MPs in HCAECs. b) rh IL-33 induces TFPI expression and the release of TFPI-negative MPs in HCAECs.

Figure 6. HCAECs incubated for 3h, 6h, 9h, 24h in the absence (Co)/ presence of DMF (100 ng/ ml), 5ug/ml sST2 or rh IL-33 (100 ng/ ml). a) Isolated MPs are incubated in the absence (Co) or presence of sST2 (5ug/ml). b) Isolated MPs are incubated in the absence (Co) or presence of DMF (100 ng/ ml) and rh IL-33 (100 ng/ ml).

Conflicts:
The authors do not have any possible conflicts of interest.

Acknowledgements:
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Author Contribution:
Yujuan Yuan and Nijiati Muyesai conceived and designed the present study, performed relevant experiments, carried out statistical analysis, prepared original manuscript, and revised the submission. Nijiati Muyesai participated in the acquisition of fund support for the present study. Yujuan Yuan, Hui Cheng and Jing Tao, participated in experimental operations and data analysis. All the authors have read and approved the final manuscript.

**Abbreviations:**
- IL-33 Interleukin-33
- TF tissue factor
- TFPI tissue factor pathway inhibitor
- EMPs endothelial microparticles
- AMI acute myocardial infarction
- SCAD stable coronary artery disease
- HCAECs Human coronary artery endothelial cells
- DMF dimethyl fumarate
- CVD coronary vascular disease
- MPs microparticles
- MiRNAs microRNAs
- ECs endothelial cells
- STEMI ST segment elevation myocardial infarction
- PCI percutaneous coronary intervention
- Ab antibody
- HRP horseradish peroxidase
- LDL low-density lipoprotein cholesterol
- CRP C-reactive protein
- Cr creatinine
- TG triglycerides
- TC total cholesterol
- HDL high density lipoprotein-cholesterol
- LVEF left ventricular ejection fraction.
References


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