Supplemental Material to He, Si, Jiang et al. “Phosphotidylserine exposure and neutrophil extracellular traps enhance procoagulant activity in patients with inflammatory bowel disease” (Thromb Haemost 2016; 115.4)

Suppl. Methods

Study population
IBD patients were diagnosed based on the presence of clinical symptoms as well as endoscopic, radiological, and histopathological criteria according to European Crohn’s and Colitis Organization (ECCO) guidelines (1, 2). Twelve healthy volunteers were enrolled as controls. There were no significant differences in sex and age between patients’ groups and healthy controls. Exclusion criteria included: hypertension; diabetes mellitus; malignant disease; pregnancy; blood transfusion in the last six months; treatment with anti-platelet, anticoagulation drugs, statins, or other medications that might affect coagulation and infection within the past three months. All participants on steroids or immunosuppressant at the beginning of the study or during the three months prior to it were excluded. Individuals with the previous diagnosis of asthma, chronic obstructive airway disease, or rheumatoid arthritis were also excluded because we could not assess their disease activity on the basis of corticosteroid prescriptions. Finally, 51 consecutive patients with IBD (28 with UC, and 23 with CD) and 12 control individuals were included in the study.

Assessment of disease activity
In patients with IBD and in controls, the assessment included the presence of other diseases, cigarette smoking habits, current use of medications, and the measurement of the body mass index (BMI). In the UC and CD groups, disease activity, location, duration, as well as complications and past surgical procedures were determined.

Disease activity was assessed by using the Mayo Score (MS) for UC patients and the Crohn’s Disease Activity Index (CADI) for CD patients (3, 4). The MS is a composite score based on the daily number of stools, visible blood in stool, appearance of the colonic mucosa at endoscopy, and the physician’s global assessment. Each variable scores from 0 to 3 points, so that the total index score ranges from 0 to 12. The CDAI is a calculated index, combining patient’s vital parameters (i.e. temperature, body weight, and white blood cell count), clinical findings (abscess/fistula, abdominal mass, body weight/temperature) and medical history (extraintestinal symptoms), to reflect general disease activity.

The site of inflammatory lesions in 28 UC and 23 CD were determined. Fourteen percent of UC patients had proctitis, 14% proctosigmoiditis, 25% left sided colitis, 18% extensive colitis, and 29% pancolitis. CD was ileal in 35% of the patients, ileocolonic in 48%, and colonic in 17%. Complications were defined as the presence of abscess, stenoses resulting in postobstructive symptoms, fistulas, and IBD-associated extraintestinal diseases. Seven out of 17 active UC are PR3-ANCA-positive patients and 3 out of 14 active CD are PR3-ANCA-positive patients.
Based on the Mayo score of patients with UC and CDAI score of patients with CD, the groups were subsequently divided into 2 subgroups: inactive UC (MS <6) and active UC (MS ≥6), and inactive CD (CDAI <150) and active CD (CDAI ≥150).

Reagents
Human umbilical vein endothelial cells (HUVECs), EC medium, and Poly-L-Lysine were from Scien Cell (San Diego, CA, USA). Megamix beads (mix of 0.5, 0.9 and 3.0 µm beads) were from Biocytex (Marseille, France). Trucount Tube (Cat. No. 340334), purified CD31 (clone L133.1), CD41a (clone HIP8), CD14 (clone M5E2), CD3 (clone 1F4), CD19 (clone HIB19), CD66b (clone G10F5), CD235a (clone GA-R2), and mouse IgG1/IgG2a (clone X40/X39) were from Becton Dickinson Biosciences (San Jose, CA, USA), and all these monoclonal antibodies were labeled with Alexa Fluor 647 or Alexa Fluor 488 in our laboratory. Alexa Fluor 488 or Alexa Fluor 647-conjugated lactadherin and fluorescein-labeled fibrinogen were prepared in our laboratory. Rabbit anti-neutrophil elastase (NE) mAb (ab131260) was from Abcam (Cambridge, MA, USA). Fluorescein isothiocyanate phalloidin, 4',6-diamidino-2-phenylindole (DAPI), Alexa 647–labeled isotype-matched control antibody were from Molecular Probes (Invitrogen, Eugene, OR, USA). Percoll were from GE Healthcare (Uppsala, Sweden). Human factors Va, VIIa, IXa, X, prothrombin, thrombin, fluorescein EGR-Chloromethylketone, and biotinylated EGR-Chloromethylketone were all from Haematologic Technologies (Burlington, VT, USA). Recombinant human factor VIII was from American Diagnostica Inc. Tyrode’s buffer containing 1 mM Hepes was from our laboratory and was filtered through a 0.22-µm syringe filter from Millipore (UK). Chromogenic substrates S-2765 and S-2238 were from Instrumentation Laboratory Company (MA, USA). Tumor necrosis factor-α (TNF-α) was from Sigma-Aldrich (St Louis, MO, USA). Unlabeled lactadherin (128 nM) was used in the inhibition assays as we previously described (5), and DNase I (15000 U/ml, sigma) was diluted in phosphate-buffered saline (PBS) containing 0.1% BSA to a concentration of 100 U/ml for inhibition experiment as described (6).

Blood collection
By using a 21-gauge needle, peripheral blood samples for peripheral blood cells (platelets, erythrocytes, neutrophils, lymphocytes and monocytes) and MPs isolation, MP-depleted plasma (MDP) and serum preparation were collected from antecubital venous blood. After discarding the first 3 ml, blood was drawn into a tube containing 3.2% sodium citrate. Patients had fasted for at least 12 hours.

MPs isolation, MP-depleted plasma and serum preparation
MPs isolation was conducted as we previously described. In order to isolate the MPs, 250 µl of PFP was thawed on ice for 60 min and then centrifuged at 20,000 g for 45 min at 20 ºC (7, 8). Subsequently, 225 µl of supernatant (MP-depleted plasma i.e. MDP) was aspirated and the remaining 25 µl MPs pellet was washed once by centrifugation and resuspended in 75 µl of Tyrode’s buffer (MP enriched suspension). The MDP collection included the plasma from 12 healthy donors, serving as control samples for IBD patient plasma analysis. For serum preparation, blood was collected in vacutainer tubes and separated by centrifuging clotted blood at 1100 g for 10 minutes at room temperature to obtain serum.
Isolation of human peripheral blood cells (PBCs)
Platelet-rich plasma (PRP) and erythrocytes were prepared within 30 minutes (min) of blood collection by centrifugation for 13 min, 200 g at room temperature, and were analyzed immediately after isolation. Platelet-free plasma (PFP) was prepared as described (7, 9). Briefly, samples were centrifuged for 20 min at 1,500 g, and plasma was harvested and re-centrifuged for 2 min at 13,000 g to remove all residual platelets. PFP were snap-frozen in liquid nitrogen, and then stored at -80 °C until use. Neutrophils were separated with a Percoll density gradient as previously described (10). In brief, the whole blood was isolated by spinning on a Histopaque 1119 cushion and centrifuged for 20 min at 800 g. The neutrophils-rich layer was collected, washed, and layered on a discontinuous Percoll gradient (85-65% in PBS) for centrifugation. The neutrophils were aspirated from the interphase between 85% and 80% layers. After washing with Tyrode’s buffer, the cells were counted and kept in Tyrode’s buffer before experiments. PMN purity and viability for all experiment was >98%, as determined by Giemsa staining and trypan blue exclusion separately. The peripheral blood mononuclear cells and peripheral blood lymphocytes were separated by density gradient centrifugation over Ficoll Hypaque as described according to standard procedures (11, 12).

Endothelial cell culture and reconstitution experiments
Human umbilical vein endothelial cells (HUVECs) from ScienCell were cultured in endothelial cell medium (ScienCell, San Diego, CA, USA) at 37 °C in a 5% CO₂ humidified atmosphere. In those experiments performed to assess the effect of IBD serum, ECs were incubated in growth media containing 20% of pool serum obtained from IBD patients and healthy donors at room temperature for 24 h, respectively. At designated time points (every four hours) PS exposure was detected by flow cytometer.

Protein purification and labeling
Bovine lactadherin was purified as previously described (13, 14), and was labeled with Alexa Fluor 488 or Alexa Fluor 647 according to package instructions. The ratio of fluorescein to lactadherin was 1.2/1 or 1.1/1.

Flow cytometric analysis of PS exposure on MPs and their phenotype
Five microliter of MPs-enriched suspension was resuspended in 35 μl Tyrode’s buffer and incubated for 15 min at 4 °C in the dark with Alexa Fluoro 647-conjugated lactadherin (5 μl); Alexa Fluoro 647-conjugated CD41a (5 μl)/Alexa Fluoro 488-conjugated CD31 (5 μl); Alexa Fluoro 647-conjugated CD3 (5 μl)/Alexa Fluoro 488-conjugated CD14 (5 μl); Alexa Fluoro 488-conjugated CD19 (5 μl); Alexa Fluoro 647-conjugated CD66b (5 μl)/Alexa Fluoro 488-conjugated CD235a (5 μl); Alexa Fluoro 488 or Alexa Fluoro 647-conjugated IgG2a/IgG1 isotype controls (5 μl) together with beads (1 μm) in a Trucount Tube. Then, the suspension was diluted in 150 μl of Tyrode’s buffer and analysed immediately. Washed MPs was performed on flow cytometer (FACS Aria, Becton Dickinson), using Megamix polystyrene beads (0.5, 0.9, and 3 μm) to gate the MP region (15). MPs were initially gated as those particles that were less than 1 μm in size in a plot of side scatter (logarithmic scale; x-axis) and forward scatter (logarithmic scale; y-axis). To distinguish MPs from events due to exosome or noise, MPs were identified by lactadherin positivity as described previously (5). Briefly, these particles were then displayed on a plot of Alexa Fluoro 488-fluorescence (logarithmic scale; x-axis) and side scatter (logarithmic scale; y-axis) for further gating. Lactadherin-binding was used to
numerate PS\(^+\) circulating MPs, regardless of their cellular origin. Platelet-, endothelial-, monocyte-, T lymphocyte-, B lymphocyte-, neutrophil- and erythrocyte-derived MPs were defined as smaller than 1\(\mu\)m and lactadherin\(^+\) CD41a\(^+\), lactadherin\(^+\) CD31\(^+\)CD41a\(^+\), lactadherin\(^+\) CD14\(^+\), lactadherin\(^+\) CD3\(^+\) CD31\(^+\)CD41a\(^-\), lactadherin\(^+\) CD66b\(^+\) and lactadherin\(^+\) CD235a\(^+\), respectively. The number of MPs per microliter of plasma was calculated through Trucount tube (with a precise number of fluorescent beads 48678 to determine the number of MPs in a sample) by using the following formula: Number/\(\mu\)l= (number of events in region containing MPs x number of beads per test)/ (number of events in absolute count bead region x test volume).

**Flow cytometric analysis of PS exposure on MP-origin cells**
To quantify PS exposure on circulating cells, PBCs were resuspended in Tyrode’s buffer adjusted to 0.5-1\(\times\)10\(^6\)/ml. Platelets and erythrocytes were stained with 6 nM Alexa Fluor 488-labeled lactadherin. Neutrophils and monocytes were mixed with fluorescein-labeled CD66b and CD14, respectively, and with fluorescein-labeled lactadherin. To characterize T cells or B cells, isolated lymphocyte was mixed with fluorescein-labeled CD3 and fluorescein-labeled lactadherin, or fluorescein-labeled CD19 and fluorescein-labeled lactadherin. ECs were cultured in medium with normal serum or IBD serum for 24 h, respectively. At indicated time points (0, 4, 8, 12, 16, 20, 24 h), ECs were collected and incubated with Alexa Fluor 488-labeled lactadherin. After incubation for 15 minutes in the dark, cells were diluted and evaluated by flow cytometry. Ten thousand events per sample were acquired and analyzed with BD FACSDiva Software.

**Confocal microscopy**
Platelets/erythrocytes/leukocytes were stained with Alexa Fluor 488-labeled lactadherin or PI. MPs were stained with Alexa fluor 488-labeled lactadherin. PS exposure on cultured ECs was also determined by incubation of HUVECs with Alexa Fluor 488-labeled lactadherin and Alexa Fluor 647-labeled CD31. After washing with Tyrode’s buffer, samples were imaged by using LSM 510 system (Carl Zeiss Jena GmbH, Jena, Germany). To observe FXa and FVa binding, ECs were co-stained with factor Va-fluorescein-maleimide and factor Xa-EGRck-biotin (complexed to Alexa 647-streptavidin). All above sample were excited with 488 or 568 nm emission lines of a krypton-argon laser, and narrow band pass filters were used for restricting emission wavelength overlap. Images were obtained in LSM 510 SYSTEM (Carl Zeiss Jena GmbH, Jena, Germany).

**Wright-Giemsa staining**
Peripheral PMNs from IBD patients were collected by centrifugation as mentioned above, and then cell smears were prepared. After fixation in methanol-glacial acetic acid solution for 10 mins, cells were stained with Wright-Giemsa for 1 min and then washed with PBS. Cell samples were dried in air, and the cell morphology was examined under light microscope.

**NET structures generation, reproduction and quantification**
NETs generation, preparation and visualization were performed as follows. After isolation, neutrophils were seeded on poly-L-lysine-coated coverslips (1\(\times\)10\(^5\) cells/well) and incubated at 37 \(^\circ\)C, 5% CO\(_2\) for 15 min. IBD neutrophils were fixed right away with 4% paraformaldehyde and healthy neutrophils were incubated in
5% CO₂ at 37 °C with a total volume of 500 µl of RPMI in the presence of 10% serum from UC or CD patients and healthy donor for 1, 2 or 3 h, followed by fixation (16). Samples were then washed with phosphate buffered saline and blocked with 2% bovine serum albumin (BSA). Neutrophils were incubated with anti-NE (1:100) or isotype control for 30 min at room temperature (RT), followed by incubation with secondary fluorochrome-conjugated Ab for 30 min at RT. Nuclear DNAs were detected by incubating cells with DAPI (1:100) for 10 mins at room temperature. Visualization was performed with a fluorescence microscope (Leica). NETs were identified as structures positive for both neutrophil elastase and DAPI. To reproduce the NETs, PMNs isolated from controls were incubated in the presence of serum from control subjects or patients with IBD for 1, 2 or 3 h, and the following procedures are as described above. For quantification, NETs were counted from six different fields in triplicate wells for each condition and expressed as percentage of NET-forming PMNs per total number of cells in the field.

Coagulation time assays
Procoagulant activity (PCA) of MPs and MP-origin cells were evaluated by one-stage recalcification time assay in a KC4A-coagulometer (Amelung, Labcon, Heppenheim, Germany). One hundred microliter of MPs-containing suspensions (10 µl of MPs-enriched suspension was resuspended in 90 µl Tyrode’s buffer), erythrocytes (1×10⁸), platelets (1×10⁷), neutrophils (1×10⁶), monocytes (1×10⁶), lymphocytes (1×10⁶) isolated from each group or ECs (1×10⁸) cultured with serum from each group for 24 h was incubated with 100 µl of MDP from healthy volunteers at 37 °C, respectively. After incubation for 3 mins, 100 µl of preheated 25 mM CaCl₂ was added and the time to fibrin strand formation was recorded simultaneously. All clotting assays were performed in triplicate.

Fibrin formation
Fibrin formation was quantified by turbidity as described (17). Isolated MPs, circulating cells and cultured ECs (as used in coagulation time assays) were added to re-calcified (10 mM, final) MDP (88% MDP, final) in the circumstance of MDP isolated from healthy donors. Fibrin formation was measured by turbidity at 405 nm in a SpectraMax 340PC plate reader. Each test was performed in triplicate. For in vitro fibrin generation experiments, ECs cultured on 1% gelatin-coated coverslips chamber with 20% pooled IBD or normal serum for 24 h were rinsed with Tyrode’s buffer. Rinsed ECs were then overlaid with prewarmed MDP (15%) in the presence of 3 mM calcium. Fibrin networks were imaged by using laser confocal microscopy in Alexa Fluor 647-conjugated fibrinogen. Background signal was calculated by using a similarly labeled isotype matched control antibody.

Thrombin assays
For the prothrombinase assay, a total of 1×10⁵ erythrocytes, 1×10⁴ platelets, 1×10³ neutrophils/monocytes/lymphocytes, 10 µl MPs-enriched suspension isolated from each group and 1×10⁵ ECs cultured with serum from each group were incubated with 1 nM FVa, 0.05 nM FXa, 1 µM prothrombin and 5 mM Ca²⁺ in prothrombinase buffer for 5 mins at 25 °C, respectively. The reaction was stopped by EDTA at a final concentration of 7 mM. EDTA and S-2238 were added to each microplate, and thrombin production was
assessed as previously described (13). The quantity of thrombin formed was also determined immediately at 405 nm in kinetic mode on a Universal Microplate Spectrophotometer (PowerWave XS; Bio-Tek). Results were evaluated against the rate of substrate cleavage of a standard dilution of thrombin. Each test was performed in triplicate.

**Intrinsic and extrinsic FXa assays**
The formation of intrinsic FXa in the presence of cells was performed as follows. Isolated MPs, blood cells and cultured ECs (as used in the thrombin assays) was incubated with 1 nM FIXa, 130 nM FX, 5 nM FVIII, 0.2 nM thrombin and 5 mM Ca\(^{2+}\) in FXa buffer for 5 min at 25 °C, respectively. The reaction was stopped by EDTA at a final concentration of 7 mM. After the addition of 10 µl of S-2765 (0.8 mM) to each reaction, the quantity of FXa formed was determined immediately at 405 nm in kinetic mode on a Universal Microplate Spectrophotometer (PowerWave XS; Bio-Tek). Results were evaluated against the rate of substrate cleavage of a standard dilution of FXa. Measurement of extrinsic FXa formation was analogous to that for intrinsic FXa except that cells or MPs were incubation with 130 nM FX, 1nM FVIIa and 5 mM Ca\(^{2+}\). Each test was performed in triplicate.

**Inhibition assays**
For the inhibition assay of coagulation time and fibrin formation, MPs and blood cells isolated from active UC or ECs pretreated with active UC serum for 24 h suspensions were incubated with lactadherin or DNase I to inhibit the coagulation reaction and fibrin production in the circumstance that MDP was isolated from healthy donors or active UC, respectively. The quantity of coagulation time and fibrin formation was calculated as above mentioned. Each test was performed in triplicate. Inhibition assays of coagulation complexes by lactadherin were performed as follows. Each kind of MPs or their original cells (as used in coagulation and fibrin inhibition assays) was incubated with lactadherin or DNaseI for 10 min at 25 °C in Tyrode’s buffer, respectively. The quantity of thrombin or FXa formation was assessed as above after incubated with the specified clotting factors. Each test was performed in triplicate.

**Isolation or depletion of IgG**
Normal IgG was prepared from plasma of healthy donors. ANCA-IgG was prepared from plasma of patients with active PR3-ANCA-positive IBD. Plasma was filtered through a 0.22 µm syringe filter (Gelman Sciences, Ann Arbor, MI) and applied to a High-Trap-protein G column on an AKTA-FPLC system (GE Biosciences, South San Francisco, USA). IgG depletion from IBD patients was performed according to the methods described previously (18, 19).

**Stimulation of cultured ECs and neutrophils**
To establish the role of TNF-α and ANCA-IgG inducing PS exposure or NETs in cultured ECs or neutrophils separately, ECs were cultured in the control serum in the absence or presence of TNF-α (10 ng/ml) or ANCA-IgG (100 µg/ml) for 24 h. Neutrophils from healthy donors were treated with with 100 µg/ml normal IgG or ANCA-IgG in the absence or presence of TNF-α for 2 h at 37 °C.
### Suppl. Table 1. Characteristics of the subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls (n = 12)</th>
<th>Inactive UC (n = 11)</th>
<th>Inactive CD (n = 9)</th>
<th>Active UC (n = 17)</th>
<th>Active CD (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female, %)</td>
<td>6/12 (50%)</td>
<td>4/11 (36%)</td>
<td>4/9 (44.4%)</td>
<td>8/17 (47%)</td>
<td>6/14 (42.9%)</td>
</tr>
<tr>
<td>Ages (years)</td>
<td>44.2±14.4</td>
<td>43.3±11.3</td>
<td>45.8±17.1</td>
<td>44.4±14.7</td>
<td>45.5±13.6</td>
</tr>
<tr>
<td>Duration of IBD (months)</td>
<td>0 (0-0)</td>
<td>43 (34.5-50)</td>
<td>45.5 (29-55)</td>
<td>45 (32-57)</td>
<td>49 (36.5-56)</td>
</tr>
<tr>
<td>Neutrophils (10⁹/L)</td>
<td>3.6±0.9</td>
<td>3.8 ± 1.1</td>
<td>4.6 ± 1.2</td>
<td>6.5 ± 3.8**</td>
<td>5.1 ± 1.5**</td>
</tr>
<tr>
<td>Lymphocytes (10⁹/L)</td>
<td>1.9±0.4</td>
<td>2.0±0.7</td>
<td>2.1±0.6</td>
<td>1.5±0.5</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Monocytes (10⁹/L)</td>
<td>0.5±0.1</td>
<td>0.4±0.2</td>
<td>0.5±0.2</td>
<td>0.7±0.4**</td>
<td>0.7±0.3***</td>
</tr>
<tr>
<td>Platelets (10⁹/L)</td>
<td>232.9±38.8</td>
<td>277.2±47.9*</td>
<td>280.5±45.6*</td>
<td>346.7±104.3*</td>
<td>390.4±180.9*</td>
</tr>
<tr>
<td>Erythrocytes (10¹²/L)</td>
<td>4.5±0.4</td>
<td>4.0±0.6</td>
<td>4.3±0.4</td>
<td>4.3±0.8</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>149.3±13.2</td>
<td>140.3±12.2</td>
<td>138.7±13.3</td>
<td>125.0±27.9*</td>
<td>100.7±23.1**</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>8.0±2.4</td>
<td>20.0±4.6*</td>
<td>18.5±3.4*</td>
<td>48.9±21.9*##</td>
<td>36.2±13.1**</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>4.1±0.7</td>
<td>16.6±5.1*</td>
<td>14.5±2.3*</td>
<td>75.3±34.1##</td>
<td>123.2±41.6##</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>46.7±2.8</td>
<td>44.3±2.8</td>
<td>43.7±3.7</td>
<td>36.3±6.7***</td>
<td>31.4±6.8***</td>
</tr>
<tr>
<td>PT (s)</td>
<td>11.1±1.0</td>
<td>11.0±0.8</td>
<td>10.9±0.8</td>
<td>10.5±1.4</td>
<td>10.8±1.5</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>32.5±3.1</td>
<td>33.4±5.2</td>
<td>35.5±4.2</td>
<td>32.7±5.0</td>
<td>35.5±4.3</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.8±0.6</td>
<td>3.1±0.6</td>
<td>3.0±0.7</td>
<td>4.2±1.0***</td>
<td>4.1±1.1***</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>90.0±70.1</td>
<td>175.6±98.6*</td>
<td>190±99.7*</td>
<td>526.9±254.9*</td>
<td>512.6±196.4*</td>
</tr>
</tbody>
</table>

Data are expressed by mean ± standard deviation [SD], percentage or median (interquartile range [IQR]). *P < 0.05, **P < 0.01, ***P < 0.001 versus controls; P < 0.05, #P < 0.01 versus inactive UC or inactive CD.

ESR, erythrocyte sedimentation rate; CRP, C reactive protein.

### Suppl. Table 2. Associations of CRP with parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CRP</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PS⁺ MPs</td>
<td>0.639</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>Total PS⁺ blood cells</td>
<td>0.517</td>
<td>0.069</td>
<td></td>
</tr>
<tr>
<td>PS⁺ ECs</td>
<td>0.886</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>NET releasing PMNs</td>
<td>0.683</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>Coagulation time</td>
<td>-0.623</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>Final fibrin formation</td>
<td>0.597</td>
<td>0.016</td>
<td></td>
</tr>
</tbody>
</table>

CRP, C reactive protein.
Suppl. Figure 1. Flow cytometry analyses of PS exposure on MPs and subgroups in a sample from an active UC patient. A representative set of scattergrams in a sample from an active UC patient was shown to illustrate MPs and subpopulation definition. (A) Forward and side scatter were used to define the events, with a size smaller than 1 μm and were gated in the P1 window. (B) Events were then selected for their lactadherin binding, determined by positivity for lactadherin-Alexa Fluor 488 (P3). Lactadherin-positive (PS+) MPs were further examined for the expression of other antigens by co-labeling with Alexa Fluor 488- and Alexa Fluor 647-labeled antibodies as shown here for (C) PMPs (CD41a-Alexa Flouro 647+) and EMPs (CD31-Alexa Flouro 488+/CD41a-Alexa Flouro 647+). (D) T lymphocyte origin-MPs (CD3-Alexa Flouro 647+) and monocyte origin-MPs (CD14-Alexa Flouro 488+/CD41a-Alexa Flouro 647+). (E) MPs derived from B lymphocyte (CD19-Alexa Flouro 647+). (F) MPs derived from neutrophil (CD66b-Alexa Flouro 647+) and erythrocytes (CD235a-Alexa Flouro 488+).
Suppl. Figure 2. PS\(^+\) MPs and MP-origin cells between UC and CD. The circulating MPs and MP-origin cells were reanalyzed from 28 UC (including 17 active UC and 11 inactive UC) and 23 CD (including 14 active CD and 9 inactive CD). Boxes plots of (A) total PS\(^+\) MPs and their origin of platelets, (B) erythrocytes/monocytes, (C) T lymphocytes/B lymphocytes, (D) neutrophils/endothelial cell were assessed between UC and CD. Data are given as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). *P<0.05 versus UC. (E) Lactadherin-binding (PS\(^+\)) percent of blood cells were calculated between UC and CD groups. Data are expressed as mean ± SD (*P < 0.05 versus UC). (F) Lactadherin-binding (PS\(^+\)) percent of ECs were compared when incubated with sera from UC and CD groups. Each point represents mean ± SD for triplicate samples of independent experiments. All sera used for cell culture was MP depleted.
Suppl. Figure 3. Neutrophil extracellular traps (NETs) release after in vitro stimulation of polymorphonuclear cells (PMNs) with sera from patients with inflammatory bowel disease (IBD). NET formation by control PMNs was treated with sera from IBD patients with high disease activity (17 active UC sera and 14 CD sera). One out of 17 representative aUC independent experiments are shown. PMNs were incubated for (A) 1, 2 or 3 h with active IBD sera. Immunostaining of extracellular DNA traps were characterized by DAPI (blue) and granule-marker elastase (green). Original magnification 100×. Scale bar represents 20 μm. (B) Comparison of percentage of NET releasing control neutrophils treated with sera for 1, 2 or 3 h from aUC patients (n=17) or aCD (n=14) respectively. (C) Percentage of NET releasing PMNs in plasma samples obtained from, and of NET releasing control PMNs pretreated with sera from 28 UC and 23 CD patients, separately. Data are given as median (horizontal bar), 25th and 75th percentile (boxes), and 10th and 90th percentile (error bar). *P<0.001 versus controls.
Supplementary Figure 4. Integrated procoagulant activity of MPs and MP-origin cells between UC and CD groups. (A) Coagulation time and (B) fibrin formation of MPs and their originating cells of platelets, erythrocytes, neutrophils, monocytes, lymphocytes, and cultured ECs in 28 UC and 23 CD were evaluated with MDP from healthy donors. Data are expressed as mean ± SD. All sera and plasma used for cell culture or stimulations was MP depleted.
Suppl. Figure 5. Integrated procoagulant activity of MPs and MP-origin cells in patients with aUC in different MDP. Inhibited coagulation time (A) and fibrin production (B) of MPs and MP-origin cells of platelets, erythrocytes, neutrophils, monocytes, lymphocytes and cultured ECs in aUC (n = 17) was detected in the absence or presence of lactadherin or DNase I by addition of MDP from healthy donors. Procoagulant activity of PMN from aUC was not inhibited by DNase I in the circumstance of MDP from healthy donors. (C) Coagulation time and (D) fibrin formation of MPs and their originating cells from aUC (n = 17) were evaluated in the circumstances of healthy MDP or aUC MDP. PMN from active UC patient had more procoagulant activity in the circumstances of MDP from aUC than in that from healthy donors. *P<0.001 versus aUC or ctrl MDP group.
Suppl. Figure 6. Thrombin and FXa production formation and inhibition assays of MPs and their originating cells between UC and CD groups. (A) Thrombin, (B) intrinsic FXa and (C) extrinsic FXa production of MPs, platelets, erythrocytes, neutrophils, monocytes, lymphocytes, and cultured ECs between 28 UC and 23 CD are shown.
Suppl. Figure 7. Thrombin and FXa production formation and inhibition assays of cultured ECs and PMNs with stimulations. (A) Thrombin, (B) intrinsic FXa and (C) extrinsic FXa production of ECs incubated with control sera plus TNF-α and PR3-ANCA-positive IgG in the absence or presence of lactadherin or DNase I. (D) Thrombin, (E) intrinsic FXa and (F) extrinsic FXa production of PMNs incubated with control sera plus TNF-a and PR3-ANCA-positive IgG in the absence or presence of lactadherin or DNase I. Data are displayed as mean ± SD (*P < 0.01 versus the group without lactadherin or DNase I).
Suppl. References


