Supplementary material

Increase in circulating ACE-positive endothelial microparticles during acute lung injury

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Detailed Methods

Analyses for angiotensin-converting enzyme (ACE) expression on human primary endothelial cells by flow cytometry.

Human pulmonary microvascular endothelial cells (HPMECs), human dermal blood endothelial cells (HDBECs) and human pulmonary artery endothelial cells (HPAECs) were purchased from PromoCells (Heidelberg, Germany). Endothelial Cell Growth Medium MV 2 (PromoCells, containing 2% fetal calf serum (FCS)) was used as the culture media for HPMECs and HDBECs. Endothelial Cell Growth Medium 2 (PromoCells, containing 2% fetal calf serum (FCS)) was used as the culture media for HPAECs. We used primary endothelial cells at passage 4 for examination of the levels of ACE cell-surface expression before and after stimulation. For stimulation, we plated $1.0 \times 10^5$ cells onto each well of a 6-well cell culture plate (area of each well: 9.6 cm$^2$; Corning, Tewksbury, MA, USA). We used 2 ml culture media for each well. We cultured the cells at 37 °C in 5% CO$_2$. We changed the media once 24 h after plating the cells. At 72 h after plating, the endothelial cells were 80 % confluent. Then, we removed the culture supernatant and washed the cells with media twice. We then replaced the media containing either 100 ng/ml of LPS (extracted from Escherichia coli 055:B5; Sigma-Aldrich, Saint Louis, MO, USA) or 25 ng/ml of recombinant human TNF-α (Sigma-Aldrich) for stimulation followed by incubation for 24 h. Vehicle control samples were cultured with 2ml media with 20 μl PBS. After incubation, the culture supernatant was harvested for analyses of the EMPs. The cells were then washed with PBS twice and incubated with 0.5 ml 0.05 % Trypsin-EDTA (Thermo-Fischer Scientific, Waltham, MA, USA) for 5 minutes at 37 °C to harvest the single cell suspension. The
cells were washed with PBS twice. Cell viability was examined by Trypan Blue Solution (0.4%, Thermo Fisher Scientific). Each cell sample was resuspended with 100 μl of staining buffer for flow cytometry (PBS with 2% FCS) and incubated with 10 μl of human APC-conjugated anti-human ACE (CD143) IgG1 antibody (clone REA522, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4 °C in the dark for detecting the cell surface expression of ACE. We used human APC-conjugated isotype control antibody (REA Control (S) antibody, clone REA293, Miltenyi Biotec) for the staining control. After incubation, the cell samples were washed with PBS and resuspended with 800 μl of staining buffer. We added 8 μl of 7-AAD Viability Staining Solution (Biolegend, San Diego, CA, USA) and incubated the cells for 10 minutes in the dark before analysis to distinguish and exclude dead cells during flow cytometry. The cell surface expression of ACE on the live cells was measured by flow cytometry (BD LSRFortessa). We utilized each result of the staining with isotype control antibody to determine the boundary fluorescent value for distinguishing whether the cells were positive for ACE or not. The boundary fluorescent value was set when the fluorescence of more than 99% of the total cells in the staining with the isotype control antibody was less than the boundary value. We examined both the percentage of ACE-positive cells in the HPMEC or HDBEC populations and the geometric mean of the ACE expression levels normalised by the corresponding isotype control Ab staining on HPMECs or HDBECs.

**Enumeration and characterization of endothelial microparticles (EMPs).**

The counting and characterization of EMPs in the cell culture supernatant, mouse serum and human serum were performed by flow cytometry (BD LSRFortessa; BD
Biosciences, San Jose, CA, USA). Megamix-Plus SSC fluorescent polystyrene beads (0.16, 0.20, 0.24, and 0.5 µm beads; Biocytex, Marseille, France) were used to calibrate the BD LSRFortessa flow cytometer as described in a previous report [1]. Briefly, we set the regions around each of the singlet bead populations on FL1/FITC vs. SSC-H plot (online supplementary figure S2a). The gate for MPs on the FSC-H vs. SSC-H plot is defined based on the peaks and the distribution of 0.16 (purple), 0.20 (blue), 0.24 (light green) and 0.5 µm (red) bead singlets according to the manufacturer’s instructions (online supplementary figure S2b). The lower limit of the SSC-H level for the MP gate = the SSC-H median of 0.16 µm beads + (0.3 x (SSC-H median of 0.20 µm beads – SSC-H median of 0.16 µm beads)). The upper limit of the SSC-H level for the MP gate takes as the highest boundary the end (99 percentile) of the 0.5 µm SSC-H peak. The gate for MPs covers a major part of the theoretical MP size range (0.1 to 1.0 µm). In cell culture-conditioned medium samples, EMPs were defined as CD31-positive MPs. In the serum samples, which also contained platelet-derived MPs, EMPs were defined as CD31-positive and CD41- (platelet marker) negative MPs. More detailed staining strategies of the flow cytometry analysis for EMPs are described in a later paragraph for each type of sample. To quantify the absolute MP number, counting spheres with a known concentration (1000 spheres/µl; Flow-Count Fluorospheres; Beckman Coulter, Brea, CA, USA) were added to each sample as an internal standard. Samples were processed on the flow cytometer for 2 min. We examined the same supernatant or serum sample three times and calculated the average counts of the sample.

**Analyses for EMPs and ACE⁺ EMPs in culture supernatants of human endothelial cells.**
Because the counts of EMPs in the culture supernatants of human endothelial cells were too low to examine by flow cytometry, a concentration step was needed to obtain an MP-rich fluid. To prepare an MP-rich fluid from the cell culture supernatants, we first centrifuged 2 ml of culture supernatants at 400 g for 15 min to remove cell pellets, followed by centrifugation of the supernatant at 21,000 g for 45 min to precipitate the MPs. MPs were then resuspended with 100 μl of 0.2 μm filtered PBS. The samples of the MP-rich fluid were incubated with human APC-conjugated anti-human ACE antibody and mouse FITC-conjugated anti-human CD31 antibody (clone WM59, BD Biosciences) for 30 min in the dark at room temperature. We used human APC-conjugated isotype control antibody and mouse FITC-conjugated isotype control antibody (clone MOPC-21, BD bioscience) for staining the control (online supplementary figure S2c-g). For further characterization of the EMPs, mouse APC-conjugated anti-human CD9 Antibody (Clone HI9a, Biolegend), mouse Brilliant Violet 421 (BV421) anti-human CD45 Antibody (Clone HI30, Biolegend) and mouse BV 421-conjugated isotype control antibody (clone MOPC-21, Biolegend) were also used for staining. We utilized staining with an isotype control antibody to determine the boundary fluorescent value for distinguishing whether microparticles were positive for marker proteins. The boundary fluorescent value was set when the fluorescence of more than 99% of the microparticle events in the staining with isotype control antibody was less than the boundary value. To enumerate the number of microparticles, positive events with isotype controls were subtracted from the positive events identified with the corresponding antibodies. The samples were diluted to 300 μl with 0.9% saline solution, and 100 μl of the diluted samples were combined with an equal amount of Flow-Count Fluorospheres solution (Beckman Coulter). The samples were analysed by flow
cytometry to count the numbers of EMPs and ACE\textsuperscript{+} EMPs.

**Treatments with inhibitors**

We used HPMECs at passage 4 for the examinations. We plated 1.0 x 10\textsuperscript{5} cells onto each well of a 6-well cell culture plate (Corning). We used 2 ml culture media (Endothelial Cell Growth Medium MV 2; PromoCells; containing 2% FCS) for each well. We cultured the cells at 37 °C in 5% CO\textsubscript{2}. We changed the media once 24 h after plating the cells. At 72 h after plating, the endothelial cells were 80 % confluent. Then, we removed the culture supernatant and washed the cells with media twice. We then replaced the 2ml culture media with media containing DMSO (20 μl; vehicle control), SB203580 (10 μM, AdoQ Bioscience, Irvine, CA, USA), Z-VAD-FMK (50 μM, AdoQ Bioscience) or Necrostatin-1 (20 μM, Enzo life sciences, Farmingdale, NY, USA) followed by incubation for pre-treatment. After one hour incubation, TNF-α (25 ng/ml) or PBS (20 μl; vehicle control) was added. After 24 h, the culture supernatants were harvested to examine the EMPs. The cells were also harvested to examine apoptotic cells by flow cytometric analyses with annexin V.

**Flow cytometric analyses with annexin V to identify apoptotic cells.**

Harvested HPMECs were washed twice with PBS and then resuspended in 100 μl of Annexin V Binding Buffer (Biolegend). Both 5 μl of APC-conjugated Annexin V (Biolegend) and 5 μl of 7-AAD (Biolegend) were added to the cell suspension. The samples were incubated for 15 min at room temperature in the dark. After incubation, 400 μl of Annexin V Binding Buffer were added. The samples were analysed by flow cytometry (BD LSRFortessa).
Flow cytometric analyses with annexin V to identify phosphatidylserine-positive EMPs.

Ten µl of the MP-rich fluid derived from each culture supernatant were incubated with human APC-conjugated anti-human ACE antibody and mouse FITC-conjugated anti-human CD31 antibody for 30 min in the dark at room temperature. After incubation, the sample was diluted with 90 µl of Annexin V Binding Buffer. 5 µl of APC-conjugated Annexin V were then added and incubated for 15 min at room temperature in the dark. After incubation, 200 µl of Annexin V Binding Buffer were added. The samples were analysed by flow cytometry (BD LSRFortessa). The sample without annexin V staining was used for the negative staining control.

Animal models of acute lung injury

All experimental protocols were approved by the Animal Care Committee at the Tohoku University School of Medicine. Eight- to twelve-week-old C57BL/6J male mice (Charles River Laboratories Japan, Yokohama, Japan) were used for the two acute lung injury model mouse groups and a control group. Lung injury was induced by cecal ligation and puncture (CLP) for the acute lung injury model induced by indirect injury or by intratracheal LPS administration for the acute lung injury model induced by direct injury.

CLP surgery was performed as described previously, with some modifications [2, 3]. Briefly, mice were subjected to the inhalation of isoflurane for anaesthesia. A small abdominal midline incision was made to expose the cecum, which was ligated 1.5 cm from the blind extremity of the cecum and punctured twice with an 18-G needle.
The cecum was replaced in the peritoneal cavity and the abdomen was closed. A sham operation was performed using the same procedure but without ligation and puncture. After the operation, 1 ml of 0.9% saline was administered for fluid resuscitation. We prepared 12 mice for each experimental group.

For the acute lung injury model induced by direct injury, lung injury was induced by the intratracheal instillation of 50 µl of LPS (100 µg/mouse) solution in anesthetized mice under isoflurane as described previously [4, 5]. Briefly, following exposure of the trachea through a ventral incision in the neck, the mice were given a single intratracheal injection through a 27-G needle. For the control group, we administered an equal amount of PBS. We prepared 12 mice for each experimental group.

Measurement of histological evidence of tissue injury in the mouse lung injury models.

We evaluated the histological evidence of tissue injury in the mouse lung injury models according to An Official American Thoracic Society Workshop Report: Features and Measurements of Experimental Acute Lung Injury in Animals [6]. Briefly, lung tissues were harvested 24 h after treatment, inflated and fixed with formalin at a transpulmonary pressure of 20 cmH₂O, and embedded in paraffin. Five-micrometre sections were stained with hematoxylin and eosin. Twenty random high-power fields (400X total magnification) were independently scored in a blinded fashion for each section according to LUNG INJURY SCORING SYSTEM [6]. The assessment was independently performed by two investigators in a blinded fashion.
Wet-to-dry lung ratio

The Wet-to-dry lung ratio was measured as previously reported [7]. Briefly, the isolated lungs were weighted (wet weight). Then, the lungs were dried in an oven at 60°C for 48 h and weighed (dry weight). The wet and dry weights were measured to calculate the lung wet-to-dry ratio (W/D ratio).

Analyses for circulating EMPs and ACE+ EMPs in the mouse lung injury models

Twenty-four hours after treatment, blood samples for the EMP analysis were obtained via inferior vena cava puncture from all mice, followed by centrifugation to purify the serum. Blood was kept for 60 min at room temperature and centrifuged at 3000 g for 15 min to obtain the serum. Serum samples were stored at -80 °C until use.

EMPs in serum were examined by flow cytometry (online supplementary figure 4). Ten μl of mouse serum were incubated with rat FITC-conjugated anti-mouse CD31 antibody (clone 390, Biolegend, San Diego, CA, USA), rat PE-conjugated anti-mouse CD41 (clone MWReg30, Biolegend) and rat Alexa Fluor 647-conjugated anti-mouse ACE (CD143) antibody (clone 230214, Novus Biologicals, Littleton, CO, USA) for 30 min in the dark at room temperature. We used rat FITC-conjugated IgG2a κ isotype control antibody (clone RTK2758, Biolegend), rat PE-conjugated anti-mouse IgG1 κ isotype (clone RTK2071, Biolegend) and rat APC-conjugated anti-mouse IgG2a isotype (clone 54447, Novus Biologicals) for staining the control (online supplementary figure 4 b-g). The samples were diluted to 300 μl with 0.9% saline solution and 100 μl of the diluted samples were added to an equal amount of Flow-Count Fluorospheres solution (Beckman Coulter). The samples were analysed by a flow cytometer to count the numbers of EMPs and ACE+ EMPs.
**Immunohistochemical staining for mouse ACE**

Immunohistochemical staining for ACE was performed in sections of lungs from the CLP and Sham experimental groups. Paraffin-embedded lungs were deparaffinised by xylene and rehydrated in ethanol. Antigen retrieval was performed by autoclaving sections (121°C, 5min) in Antigen retrieval HIAR (pH9; Nichirei Bioscience Inc.). For blocking, the sections were incubated with 1% FCS containing PBS for 2 h. The sections were incubated with the rabbit anti-mouse ACE (CD143) antibody (1:300; Bioss Antibodies, Boston, MA, USA) overnight at 4°C with gentle agitation. The sections were washed with PBS three times and incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Histofine® Simple Stain™ MAX PO; Nichirei Bioscience Inc., Tokyo, Japan) for 40 minutes at room temperature. The sections were washed with PBS three times and incubated with Peroxidase Chromogen/Substrate Solution, DAB (Nichirei Bioscience Inc.). The sections were counterstained with hematoxylin.

**Immunofluorescent staining for mouse CD31 and ACE**

Paraffin-embedded lungs were deparaffinised by xylene and rehydrated in ethanol. Then, antigen retrieval was performed by autoclaving sections (121°C, 5min) in Antigen retrieval HIAR (pH9; Nichirei Bioscience Inc.). For blocking, the sections were incubated with PBS containing 10% donkey serum and 0.3% Triton X-100 for 30 minutes at room temperature. The sections were incubated with rat-anti mouse CD31 (final concentration: 10 μg/ml; clone SZ31; Dianova GmbH, Hamburg, Germany) and the rabbit anti-mouse ACE (CD143) antibody (final concentration: 20 μg/ml; Bioss
Antibodies, Boston, MA, USA) overnight at 4°C. The sections were washed with PBS three times and then incubated with Alexa 647-conjugated donkey anti-rat secondary antibody and Alexa 555-conjugated donkey anti-rabbit for 1 hour at room temperature. The sections were washed with PBS three times. ProLong Gold Antifade Mountant with DAPI (Thermo Fischer Scientific) was used for mountants.

The preparation of single lung cells from whole lung and flow cytometric cell sorting of lung endothelial cells

The preparation of single lung cells from mouse whole lungs and flow cytometric cell sorting of mouse lung endothelial cells were performed as we described previously [8]. Briefly, mice received an overdose of inhaled halothane and their lungs were perfused with PBS via the right ventricles. The PBS-perfused lungs were isolated with other mediastinal organs. The Dispase II solution (Roche Applied Science, Mannheim, Germany; final concentration, 2.0 U/ml) was instilled into the lungs through the trachea, which was then ligated with a silk suture. After incubation at 37°C for 50 min, the lungs were separated from the other mediastinal organs. The lungs were then thoroughly minced and digested in PBS containing 0.1% collagenase (Roche Applied Science) and 0.01% deoxyribonuclease I (Sigma-Aldrich, St. Luis, MO) at 37°C for 20 min. The cells were then suspended in red blood cell lysis buffer to remove red blood cells (Sigma-Aldrich) and were subsequently washed with PBS. The cells were then centrifuged and re-suspended in PBS. We used the following antibodies: Alexa Fluor 647-conjugated anti-mouse EpCAM antibody (clone G8.8, Biolegend, San Diego, CA); phycoerythrin (PE)-conjugated anti-mouse VE-cadherin antibody (clone VECD1, Biolegend); and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD45
antibody (clone 30-F11, Biolegend). To discriminate between live and dead cells, we used 7-amino actinomycin D (7-AAD; eBioscience, San Diego, CA). The antibodies were incubated with the samples for 30 min at 4°C and the samples were then washed. We re-suspended the cells in 2% FBS/ PBS and labelled dead cells with 7-AAD. We sorted live and single-cell-gated subpopulations based on their staining patterns using EpCAM, VE-cadherin and CD45 with a FACS Aria II Cell Sorter (BD Biosciences, San Jose, CA). The sorted endothelial (CD45⁻, VE-cadherin⁺ cells) were collected in DMEM/10% FCS for further analyses.

**Western blotting for the detection of mouse ACE.**

The mouse lung, liver or kidney were mechanically homogenized in Cell Lysis Buffer (Cell Signalling Technology, Danvers, MA, USA). The sorted mouse lung endothelial cells were washed with PBS and lysed by Cell Lysis Buffer. The mouse lung homogenates or cell lysates of the sorted lung endothelial cells were mixed with 3X Blue Loading Buffer and dithiothreitol (Bio-Rad Laboratories, Hercules, CA, USA). The samples were denatured for 5 minutes at 95°C. The protein in the samples was quantified using a bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (20 μg for tissue samples or 5 μg for sorted lung endothelial cells) were loaded and separated by electrophoresis on a Novex™ 4-20% Tris-Glycine Mini Gels, WedgeWell™ format (Thermo Fischer). After electrophoresis, the separated proteins were transferred to a PVDF membrane using the iBlot Dry Blotting System (Thermo Fischer). The membranes were blocked with 5% skim milk for 1 hour at room temperature. The following antibodies were used to detect the target proteins: rabbit anti-mouse ACE (CD143) antibody (1:1000 dilution, Bioss Antibodies,
Boston, MA, USA) or rabbit monoclonal anti-β-actin antibody (1:1000 dilution, Cell Signalling Technology). The bound antibodies were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK) with a chemiluminescence imaging system (LAS-4000 mini; Fujifilm, Tokyo, Japan). The band intensity was quantified by densitometry (Quantity One software, Bio-Rad). We calculated the normalized ACE levels by dividing the band intensity for ACE by the intensity for β-actin.

The study protocol for measuring ACE⁺ EMPs in septic patients.

The prospective cohort study was performed to investigate whether ACE⁺ EMPs could serve as a prognostic indicator of ARDS development in septic patients, or circulating ACE⁺ EMPs in septic patients admitted to the intensive care unit or medical emergency centre at Tohoku University hospital between August 2015 and January 2018. This study was approved by the Tohoku University School of Medicine Ethics Committee (Registration Number: 2015-1-279). Patients older than 20 years with sepsis/septic shock who were admitted to the intensive care unit or medical emergency centre at Tohoku University Hospital were included. Written informed consent was obtained from the patient’s next of kin before recruitment. Blood samples were drawn to prepare serum at the time of recruitment.

Until publication of the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) in February 2016, sepsis was defined as systemic inflammatory response syndrome (SIRS) combined with an infectious episode [9]. Severe sepsis was characterized by dysfunction of at least one organ, but septic shock
was characterized by persistent hypotension despite adequate volume resuscitation, based on the American College of Chest Physicians /Society of Critical Care Medicine consensus conference [9]. After the publication of Sepsis-3, the definition of sepsis was established as life-threatening organ dysfunction caused by a dysregulated host response to infection and diagnosed as organ dysfunction identified as an acute change in the total SOFA score by 2 points due to the infection [10]. Septic shock is sepsis with persistent hypotension requiring vasopressors to maintain an adequate blood pressure and a serum lactate level > 2 mmol/L despite adequate volume resuscitation [10]. To equilibrate the entry criteria, the diagnoses of sepsis among the subjects before Sepsis-3 were re-evaluated based on Sepsis-3.

The exclusion criteria were as follows: 1) failure to obtain informed consent from the patient’s next of kin; 2) supported by extracorporeal membrane oxygenation (ECMO); 3) the initiation of renal replacement therapy (RRT) before the entry; and 4) prolonged infection for more than 7 days before recruitment, or infection with unknown onset.

The clinical data from the septic patients were recorded to determine whether the patients fulfilled the Berlin criteria for ARDS [11]: 1) acute onset within 1 week of a known clinical insult of new or worsening respiratory symptoms, 2) bilateral opacities that are not fully explained by effusions, lobar/lung collapse, or nodules, 3) respiratory failure that is not fully explained by cardiac failure or fluid overload, and 4) severe oxygenation disorder (PaO$_2$/FiO$_2$ ≤ 300 mmHg, with PEEP or CPAP ≥ 5 cmH$_2$O). Patients were followed for 5 days for the development of ARDS using a two-physician review of chest radiographs and clinical data. All of the septic patients who developed ARDS fulfilled the Berlin criteria during Day 1. We also collected serum samples from
the septic patients who developed ARDS on days 2, 3, 5 and 7 to examine the time-course of serum EMPs and ACE\(^+\) EMPs.

We performed a subgroup analysis based on the source of injury. The patients developing ARDS due to pneumonia or aspiration were categorized as direct ARDS patients (n = 8). The patients developing ARDS due to non-pulmonary sepsis were categorized as indirect ARDS patients (n = 13).

We also performed another analysis to compare the septic patients developing ARDS with the septic patients with hydrostatic pulmonary edema (n = 7; online supplementary table S1).

### Analyses for circulating EMPs and ACE\(^+\) EMPs in human subjects

After the collection of whole blood in evacuated tubes, the blood was kept for 30 min at room temperature. Clots were removed by centrifugation at 2000 g for 5 min and the isolated supernatant was centrifuged at 1500 g for 20 min to obtain serum. Serum was stored at -80 °C until use. A flow cytometer (BD LSRFortessa) was used to characterize EMPs from serum after multiple fluorescent immunostaining (online supplementary figure 6). Ten µl of human serum were incubated with mouse FITC-conjugated anti-human CD31 antibody (Clone WM59, BD bioscience), mouse PE-conjugated anti-human CD41 (Clone HIP8, BD bioscience) and APC-conjugated anti-human ACE (CD143) IgG\(_1\) antibody (clone REA522, Miltenyi Biotec) for 30 min in the dark at room temperature. We used mouse FITC-conjugated isotype control antibody (Clone MOPC-21, BD bioscience), mouse PE-conjugated isotype control antibody (clone MOPC-21, BD bioscience), and human APC-conjugated isotype control antibody (REA Control (S) antibody, clone REA293, Miltenyi Biotec) for staining control (online
supplementary figure S6b-h). We utilized staining with an isotype control antibody to determine the boundary fluorescent value for distinguishing whether microparticles were positive for marker proteins. The boundary fluorescent value was set when the fluorescence of more than 99% of the total cell or microparticle events in the staining with isotype control antibody was less than the boundary value. To enumerate the number of microparticles, positive events with isotype controls were subtracted from the positive events identified with the corresponding antibodies. For further characterization of EMPs, mouse APC-conjugated anti-human CD9 antibody (Clone HI9a, Biolegend), mouse Brilliant Violet 421 (BV421) anti-human CD45 antibody (Clone HI30, Biolegend) and mouse BV 421-conjugated isotype control antibody (clone MOPC-21, Biolegend) were also used for staining. The samples were diluted to 300 μl with 0.9% saline solution, and 100 μl of the diluted samples were added to an equal amount of Flow-Count Fluorospheres solution (Beckman Coulter). The samples were analysed by flow cytometry to determine the numbers of EMPs and ACE⁺ EMPs.

**Triton X-100 treatment**

To confirm that the endothelial microparticles detected by flow cytometry were lipid membrane-bound vesicles rather than immune complexes or debris, we utilized a lipid-solubilizing detergent, Triton X-100. For the pre-treatment before flow cytometric staining, we added Triton X-100 to MP-rich fluids derived from the culture supernatant or human serum samples. The final concentration of Triton X-100 in the samples was 0.5%. We then vortexed the samples for 30 sec and incubated them at 4 °C for 1 hour. We then performed staining for flow cytometry and analysis for comparison between the same sample with treatment and without treatment.
Cell Membrane labelling with PKH26

To confirm that the endothelial microparticles detected by flow cytometry were membrane-bound vesicles, we performed membrane labelling with PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) in MP-rich fluids derived from the culture supernatant. 100 μl of MP-rich fluids were diluted with Diluent C to adjust the total volume to 1 ml. Immediately prior to staining, we prepared a 2x dye solution (4x10⁻⁶ M) in Diluent C by adding 4 μl of the PKH26 ethanolic dye solution to 1 ml of Diluent C. We rapidly added the 1 ml MP suspension to 1 ml of 2x dye solution and immediately mixed the sample by pipetting and then incubated the MP/dye suspension for 5 minutes. We stopped the staining by adding an equal volume of 1% BSA followed by incubation for 1 minute to allow binding of the excess dye. Then the samples were centrifuged at 21,000 g for 45 min. The supernatants were discarded and the samples resuspended with PBS for washing. The suspension was centrifuged at 21,000 g for 45 min again and the precipitated MPs were then resuspended with 100 μl of 0.2 μm filtered PBS. We then performed additional staining with surface markers for detecting EMPs and analysis by flow cytometry.

Statistical analysis

The data are expressed as medians with interquartile ranges as indicated. Statistical tests were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) or JMP Pro 13.2.1 (SAS, Cary, NC, USA). Statistical analyses were assessed by Wilcoxon rank sum tests or Student’s t-test (n = 3 for each group) to compare variables between two groups. For comparisons among more than 3 groups, the Kruskal-Wallis
test was used, followed by Dunn’s test. Logistic regression was performed to determine the risk factors that predicted in-hospital mortality. Statistical correlation analyses were performed using Spearman’s test. Probability values less than 0.05 were considered significant.
Supplementary Figure Legends

FIGURE S1 The levels of ACE cell-surface expression on human pulmonary or dermal microvascular endothelial cells, or human pulmonary artery endothelial cells and changes after the administration of inflammatory stimuli. a) Representative images of ACE cell-surface expression on cultured primary human endothelial cells at passage 4 analysed by flow cytometry. b) The percentage of ACE-positive cells in the HPMEC, HDBEC or HPAEC populations at passage 4. c) The geometric mean of the ACE expression levels normalised by the corresponding isotype control Ab staining on HPMECs, HDBECs or HPAECs at passage 4. n = 3 for each type of endothelial cell. Each bar indicates the mean of each group. Each dot shows the value of each sample. *: p < 0.05 versus HPMECs, †: p < 0.05 versus HDBECs. d) Cell viability of HPMECs or HDBECs at 24 h after stimulation with vehicle control (PBS), LPS (100 ng/ml) or TNF-α (25 ng/ml). n = 6 for each experimental group. Each bar indicates the mean of each group. Each dot indicates the value of each sample. *: p < 0.05 versus each control. d) Representative images of ACE cell-surface expression analysed by flow cytometry. HPMECs (upper layer) or HDBECs (lower layer) were stimulated with vehicle control (PBS, left), LPS (middle) or TNF-α (right). ACE expression on the cells was examined 24 h after incubation. e) The percentage of ACE-positive cells in the HPMEC or HDBEC populations 24 h after incubation with each stimulation. f) The geometric mean of the ACE expression levels normalised by the corresponding isotype control Ab staining on HPMECs or HDBECs. n = 6 for each experimental group. Box and whisker plots show the first and third quartiles (bottom and top of the box), the median (the band inside the box), and the minimum and maximum (the ends of the whiskers). *: p < 0.05 versus HDBEC, †: p < 0.05 versus each control. ACE: angiotensin-converting enzyme;
HPMECs: human pulmonary microvascular endothelial cells; HDBECs: human dermal blood endothelial cells; HPAECs: human pulmonary artery endothelial cells.

**FIGURE S2** The staining strategy of the flow cytometric analyses for EMPs and ACE⁺ EMPs in the culture supernatant of human endothelial cells. a) Setting the regions around each singlet bead population using a mix of fluorescent beads of various diameters (Megamix Plus SSC™). b) The gate for MPs is defined as the rectangle with a green line based on the peaks and the distribution of 0.16 (purple), 0.20 (blue), 0.24 (light green) and 0.5 μm (red) bead singlets according to the manufacturer’s instructions. The gate for MPs cover a major part of the theoretical MP size range (0.1 to 1.0 μm). c) A FSC/SSC dot plot of the culture supernatant of HPMECs (HPMEC-sup). SSC threshold is set at the lowest boundary of the gate for MPs. The gate for MPs is shown as a rectangle with a green line. d-g) Staining strategy for the detection of ACE⁺ EMPs in HPMEC-sup. d) A dot-plot of staining with the FITC-conjugated isotype control antibody. e) A dot-plot of staining with the FITC-conjugated anti-human CD31 antibody. f) A dot-plot of staining with the FITC-conjugated anti-human CD31 antibody and APC-conjugated isotype control antibody. g) A dot-plot of staining with the FITC-conjugated anti-human CD31 antibody and APC-conjugated anti-human ACE antibody. Note that the EMPs are identified as CD31-positive MPs (within the blue line square). ACE⁺ EMPs are identified as CD31-positive and ACE-positive MPs (within the red dot-line square). ACE: angiotensin-converting enzyme; APC: allophycocyanin; EMPs: endothelial microparticles; FITC: fluorescein isothiocyanate; FSC: forward scatter; HPMECs: human pulmonary microvascular endothelial cells; HDBECs: human dermal blood endothelial cells; MP: microparticles; SSC: side scatter; Sup: supernatant.
FIGURE S3 Caspases may be involved in the TNF-α-induced release of EMPs from human pulmonary microvascular endothelial cells. a) Representative images of flow cytometric analyses with annexin V to identify apoptotic cells in HPMECs 24 h after stimulation with vehicle control (PBS), or TNF-α (25 ng/ml) after pre-treatment with DMSO (vehicle control), SB203580 (10 μM), Z-VAD-FMK (50 μM) or Necrostatin-1 (20 μM) for 1 h. b) The percentage of annexin V-positive cells in the HPMEC populations 24 h after incubation with each stimulation and each pre-treatment. Note the pre-treatment with Z-VAD-FMK attenuated the increase in annexin V-positive cells induced by TNF-α. c) Representative images of flow cytometric analyses with annexin V to identify PS-positive CD31+ EMPs in the culture supernatant of HPMECs 24 h after stimulation with vehicle control (PBS), or TNF-α with pre-treatment with DMSO, SB203580, Z-VAD-FMK or Necrostatin-1 for 1 h. The sample without annexin V staining was used for negative staining control. d) The counts of EMPs. e) The percentage of annexin V-positive EMPs in total EMPs. f) The counts of annexin V-positive EMPs. g) The counts of ACE+ EMP. h) ACE+ EMP/EMP. Note the pre-treatment with Z-VAD-FMK attenuated the increases in both total EMPs and annexin V-positive EMPs induced by TNF-α. n = 6 for each experimental group. Data are presented as mean ± SD. *: p < 0.05 vs. vehicle DMSO control. †: p < 0.05 versus each TNF-α DMSO experimental group. ACE: angiotensin-converting enzyme; EMPs: endothelial microparticles; HPMECs: human pulmonary microvascular endothelial cells. PS: phosphatidylserine.

FIGURE S4 Mouse lung injury models induced by CLP or intratracheal LPS
administration. a-d) Representative histological images of mouse lung paraffin-embedded sections stained with hematoxylin-eosin. a) Sham-operated mice (experimental control for CLP mice), b) CLP-operated mice, c) intratracheal PBS-administered mice (experimental control for intratracheal LPS-administered mice), and d) intratracheal LPS-administered mice. The bar indicates 50 μm. e) Lung injury scores in each experimental group (n = 6). f) The wet-to-dry lung ratio (W/D ratio) in each experimental group (n = 6). Box and whisker plots show the first and third quartiles (bottom and top of the box), the median (the band inside the box), and the minimum and maximum (the ends of the whiskers). *: p < 0.05 vs. experimental control. CLP: cecal ligation and puncture; PBS-IT: intratracheal PBS-administered; LPS-IT: intratracheal LPS-administered; W/D ratio: wet-to-dry lung ratio.

**FIGURE S5** CD31-positive mouse lung alveolar endothelial cells co-express angiotensin-converting enzyme. Representative images of immunofluorescent staining for both CD31 and ACE on a histological section of the lung of a sham-operated mouse. Lower panels present negative staining controls that were stained only with secondary antibodies. CD31-positive mouse lung alveolar endothelial cells co-expresses ACE (upper panels). Auto-fluorescence of RBCs in alveolar capillaries were also detected by the detector of a confocal microscopy for Alexa 555. Scale bar: 20 μm.

**FIGURE S6** The staining strategy of the flow cytometric analyses for EMPs and ACE\(^+\) EMPs in human serum. a) A FSC/SSC dot plot of the human serum sample. SSC threshold is set at the lowest boundary of the gate for MPs. The gate for MPs is shown as a rectangle with a green line. The gate for MPs covers a major part of the theoretical
MP size range (0.1 to 1.0 μm). b-f) Staining strategy for the detection of EMPs in human serum. b) A dot-plot of staining with FITC-conjugated isotype control antibody. c) A dot-plot of staining with FITC-conjugated anti-human CD31 antibody. d) A dot-plot of staining with PE-conjugated isotype control antibody. e) A dot-plot of staining with PE-conjugated anti-human CD41 antibody. f) A dot-plot of staining with FITC-conjugated anti-human CD31 antibody and PE-conjugated anti-human CD41 antibody. Note that EMPs are identified as CD31 (a surface marker for both endothelial cells and platelets)-positive and CD41 (a surface marker for platelets)-negative MPs (within the blue line square). Platelet microparticles are identified as CD41-positive microparticles (within the yellow line square). g-h) Staining strategy for the detection of ACE⁺ EMPs in human serum. g) A dot-plot of staining with APC-conjugated isotype control antibody on EMPs. h) A dot-plot of staining with APC-conjugated anti-human ACE on EMPs. Note that ACE⁺ EMPs are identified as CD31⁺ CD41⁻ ACE⁺ MPs (within the red line square). ACE: angiotensin-converting enzyme; APC: allophycocyanin; EMPs: endothelial microparticles; FITC: fluorescein isothiocyanate; FSC: forward scatter; MP: microparticles; PE: phycoerythrin; SSC: side scatter; YG: yellow-green laser excited.

FIGURE S7 Characterization of endothelial microparticles in the culture supernatant and the serum. a) Representative images of the flow cytometric analyses for EMPs in both the culture supernatant samples of HPMECs (upper) and the serum samples (lower) after the pre-treatment with or without 0.5 % Triton X-100. Note EMP events were not detected in the samples after the pre-treatment with 0.5 % Triton X-100. b) Representative images of flow cytometric analyses for staining with PKH26 (a cell
membrane labelling agent) in CD31$^+$ EMPs in the culture supernatant of HPMECs. Note almost all CD31$^+$ EMPs were labelled with PKH26. c) Representative images of the flow cytometric analyses for CD9 on EMPs in both the culture supernatant samples of HPMECs (upper) and the serum samples (lower). Note some CD31$^+$ EMPs in the culture supernatant of HPMECs were positive for CD9, whereas almost all of CD31$^+$ CD41$^-$ EMPs in the serum samples were negative for CD9. d) Representative images of flow cytometric analyses for CD45 on EMPs in both the culture supernatant samples of HPMECs (upper right) and the serum samples (lower). The culture supernatant of THP-1 (a human monocyctic cell line; upper left) was used as the positive staining control for CD45. Note there were both CD45$^+$ CD31$^-$ and CD45$^+$ CD31$^+$ microparticles but not CD45$^-$ CD31$^+$ microparticles in the culture supernatant of THP-1, whereas no CD45$^+$ microparticles were detected in the culture supernatant samples of HPMECs. Almost all of the CD31$^+$ CD41$^-$ EMPs in the serum samples were negative for CD45.

HPMEC: human pulmonary microvascular endothelial cells. Sup: supernatant; PE: phycoerythrin; YG: yellow-green laser excited; APC: allophycocyanin; EMPs: endothelial microparticles; FITC: fluorescein isothiocyanate; FSC: forward scatter; BV 421: Brilliant Violet 421.

**FIGURE S8** Receiver-operating characteristic (ROC) curves for the counts of ACE$^+$ EMPs (a) or the ratio of ACE$^+$ EMPs to total circulating EMPs (b) for predicting ARDS in septic patients. ACE: angiotensin-converting enzyme; EMPs: endothelial microparticles.

**FIGURE S9** No significant difference in the circulating EMPs, ACE$^+$ EMPs and the
ratio of the ACE⁺ EMP/EMP ratio between the in-hospital survivors and non-survivors of the septic patients with ARDS. The number of EMPs (a), ACE⁺ EMPs (b) and the ACE⁺ EMP/EMP ratio (c) in the serum of survivors (white; n = 11) or non-survivors (gray; n = 10) in hospital. ACE: angiotensin-converting enzyme; ARDS: acute respiratory distress syndrome; EMPs: endothelial microparticles.

FIGURE S10 The time course of the counts of circulating EMPs (a), ACE⁺ EMPs (b), and the ACE⁺ EMP/EMP ratio (c) in the serum of ARDS patients. Red circles indicate the counts in ARDS patients who were survivors in hospital. Blue squares indicate the counts in non-surviving ARDS patients. Note the counts of ACE⁺ EMPs significantly decreased on day 5, and the ACE⁺ EMP/EMP ratio significantly decreased on day 5 and 7 compared with day 1 (on admission). Number of survivors or non-survivors whose serum samples were continuously collected (on admission, day2, 3, 5, and 7) is six or four respectively. *p < 0.05 vs. day 1. ACE: angiotensin-converting enzyme. EMPs: endothelial microparticles.

Figure S11 The time course of the counts of circulating EMPs (a, b), ACE⁺ EMPs (c, d), and the ACE⁺ EMP/EMP ratio (e, f) in the serum of survivors and non-survivors of ARDS patients. Left panels show the data in ARDS patients who were survivors in hospital. Right panels show the data in non-survivors of ARDS patients. Note the ACE⁺ EMP/EMP ratio significantly decreased on day 5 both in survivors and non-survivors, whereas the ratio decreased on day 7 only in survivors. Number of survivors or non-survivors whose serum samples were continually collected (on admission, day2, 3, 5, and 7) was six or four, respectively. *p < 0.05 vs. day 1. ACE: angiotensin-converting
enzyme; EMPs: endothelial microparticles.
Supplementary Table S1 Summary of the septic patients with hydrostatic pulmonary edema.

<table>
<thead>
<tr>
<th>Case</th>
<th>Cause of sepsis</th>
<th>Cause of hydrostatic edema</th>
<th>PaO$_2$/FiO$_2$</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Severe acute pancreatitis</td>
<td>Alcoholic cardiomyopathy, fluid overload</td>
<td>139</td>
<td>Survived</td>
</tr>
<tr>
<td>2</td>
<td>Traumatic pancreatitis</td>
<td>Left ventricle diastolic dysfunction</td>
<td>196</td>
<td>Survived</td>
</tr>
<tr>
<td>3</td>
<td>Critical Limb Ischemia and infection</td>
<td>Acute heart failure, sepsis-induced cardiomyopathy</td>
<td>120</td>
<td>In-hospital death</td>
</tr>
<tr>
<td>4</td>
<td>Infective endocarditis</td>
<td>Infective endocarditis, acute aortic valve regurgitation</td>
<td>92</td>
<td>Survived</td>
</tr>
<tr>
<td>5</td>
<td>Pneumonia</td>
<td>Ischemic heart disease, chronic heart failure</td>
<td>171</td>
<td>In-hospital death</td>
</tr>
<tr>
<td>6</td>
<td>Intra-abdominal infection</td>
<td>Hypertrophic cardiomyopathy, Left ventricle diastolic dysfunction, mitral valve regurgitation</td>
<td>110</td>
<td>Survived</td>
</tr>
<tr>
<td>7</td>
<td>Pneumonia</td>
<td>Acute heart failure, sepsis-induced cardiomyopathy</td>
<td>204</td>
<td>In-hospital death</td>
</tr>
</tbody>
</table>

Median (interquartile ranges) 139 (118 – 177)
Supplementary Table S2 Logistic regression analysis to determine the factors influencing the in-hospital mortality in the septic patients who developed ARDS.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Odds ratio (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (Male)</td>
<td>1.21 (0.04-32.63)</td>
<td>0.90</td>
</tr>
<tr>
<td>Age (year)</td>
<td>0.94 (0.80-1.10)</td>
<td>0.48</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>1.33 (1.00-1.84)</td>
<td>0.04</td>
</tr>
<tr>
<td>Lactate (mmol/L)</td>
<td>0.84 (0.48-1.49)</td>
<td>0.55</td>
</tr>
<tr>
<td>PaO₂/FiO₂ ratio (mmHg)</td>
<td>1.00 (0.97-1.03)</td>
<td>0.83</td>
</tr>
<tr>
<td>EMPs (/μl)</td>
<td>1.00 (0.99-1.01)</td>
<td>0.77</td>
</tr>
<tr>
<td>ACE⁺ EMPs (/μl)</td>
<td>0.99 (0.91-1.07)</td>
<td>0.80</td>
</tr>
<tr>
<td>ACE⁺ EMP/EMP ratio (%)</td>
<td>0.89 (0.21-3.68)</td>
<td>0.87</td>
</tr>
</tbody>
</table>

*: A unit odds ratio (odds per one unit change in each factor) is shown. ACE: angiotensin-converting enzyme; APACHE: Acute Physiology and Chronic Health Evaluation; EMPs: endothelial microparticles.
References


7. Lan CC, Peng CK, Tang SE, Lin HJ, Yang SS, Wu CP, Huang KL. Inhibition of


Supplementary Figure 2

a) Megamix-Plus SSC

FITC-H

SSC-H

b) Megamix-Plus SSC

FITC-H

SSC-H

c) HPMEC-sup

SSC-H

MP gate

0.24 μm

0.20 μm

0.16 μm beads

0.50 μm

d) HPMEC-sup

unstained

APC-H

FITC-H

isotype control

0.00%

0.00%

98.97%

1.03%

e) HPMEC-sup

unstained

APC-H

FITC-H

CD31

0.00%

0.00%

97.03%

2.97%

f) HPMEC-sup

isotype control

APC-H

FITC-H

CD31

88.76%

1.14%

96.40%

8.65%

g) HPMEC-sup

ACE+ EMPs

ACE

FITC-H

CD31

EMPs

88.76%

1.14%

6.65%

1.46%
Supplementary Figure 3

**a)**

<table>
<thead>
<tr>
<th></th>
<th>HPMECs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>vehicle control</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td>annexin V-APC</td>
<td>3.8%</td>
</tr>
<tr>
<td>counts</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>Z-VAD-FMK</td>
</tr>
<tr>
<td>counts</td>
<td>9.3%</td>
</tr>
</tbody>
</table>

**b)**

![Bar graph showing % annexin V positive cells](image)

**c)**

**EMPs in HPMEC culture supernatants**

<table>
<thead>
<tr>
<th></th>
<th>vehicle control</th>
<th>TNF-α</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>DMSO</td>
<td>SB203580</td>
</tr>
<tr>
<td>unstained</td>
<td>0.92%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSC-H</td>
<td>42.4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>24.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α DMSO</td>
<td>67.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSC-H</td>
<td>72.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α SB203580</td>
<td>38.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSC-H</td>
<td>62.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>annexin V-APC</td>
<td>74.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV 421</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>annexin V</td>
<td>3.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV 421</td>
<td>24.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSC-H</td>
<td>67.9%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>annexin V-APC</td>
<td>72.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV 421</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**d)**

![Bar graph showing average EMPs](image)

**e)**

![Bar graph showing annexin V+ EMPs](image)

**f)**

![Bar graph showing annexin V+ EMPs](image)

**g)**

![Bar graph showing ACE+ EMPs](image)

**h)**

![Bar graph showing ACE+ EMPs](image)
Supplementary Figure 4

a) sham-operated

b) CLP-operated

c) PBS-administrated

d) LPS-administrated

e) Lung injury score

f) W/D ratio

[Graphs showing the lung injury score and W/D ratio for Sham, CLP, PBS-IT, and LPS-IT conditions]
Supplementary Figure 5

Anti-CD31 Ab & Alexa 647-2nd Ab

Anti-ACE Ab & Alexa 555-2nd Ab

DAPI

Merged

Alexa 647-2nd Ab only

Alexa 555-2nd Ab only

DAPI

Merged
Supplementary Figure 6

a) human-serum

SSC-H

FSC-H

MP gate

g) isotype control

APC-H

d) human-serum

isotype control

YG-PE-H

FITC-H

unstained

b) human-serum

unstained

YG-PE-H

FITC-H

isotype control

e) human-serum

unstained

YG-PE-H

FITC-H

human-serum

f) human-serum

CD41

YG-PE-H

FITC-H

CD31

human-serum

PMPs

EMPs

h) human-serum

ACE

YG-PE-H

APC-H

FITC-H

CD31

ACE+ EMPs

EMPs
Supplementary Figure 8

a) ACE*EMPs
- cut-off: 56.0/μL
- AUC: 0.969
- sensitivity: 100%
- specificity: 81.6%

b) ACE*EMPs/EMPs
- cut-off: 3.7%
- AUC: 0.979
- sensitivity: 94.1%
- specificity: 95.9%
Supplementary Figure 9

(a) EMPs (/μl)
(b) ACE+ EMPs (/μl)
(c) ACE+ EMPs/EMPs (%)

Survivor vs. Non-survivor comparison for EMPs and ACE+ EMPs.
Supplementary Figure 10

(a) 

![Graph showing EMPs over days 1 to 7 for different conditions.]

(b) 

![Graph showing ACE+ EMPs over days 1 to 7 for different conditions.]

(c) 

![Graph showing ACE+EMP% over days 1 to 7 for different conditions.]

Legend: 
- Red circles: Survivors
- Blue squares: Non-survivors

* indicates statistical significance.
**Supplementary Figure 11**

**a)** survivors

- EMPs ($\mu$L)
- ACE+ EMPs/EMPs (%)
- Days: day1, day2, day3, day5, day7

**b)** non-survivors

- EMPs ($\mu$L)
- ACE+ EMPs/EMPs (%)
- Days: day1, day2, day3, day5, day7

**c)**

- EMPs ($\mu$L)
- ACE+ EMPs/EMPs (%)
- Days: day1, day2, day3, day5, day7

**d)**

- EMPs ($\mu$L)
- ACE+ EMPs/EMPs (%)
- Days: day1, day2, day3, day5, day7

**e)**

- EMPs ($\mu$L)
- ACE+ EMPs/EMPs (%)
- Days: day1, day2, day3, day5, day7

**f)**

- EMPs ($\mu$L)
- ACE+ EMPs/EMPs (%)
- Days: day1, day2, day3, day5, day7