Increased levels of systemic LPS-positive bacterial extracellular vesicles in patients with intestinal barrier dysfunction

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SUPPLEMENTARY MATERIAL AND METHODS

Blood and fecal samples

Venous blood from patients and healthy volunteers was collected using Venosafe-citrate tubes (VF-054SBCS07, Terumo Europe, Leuven, Belgium). Collection of blood samples was according to Ethical Committee of Ghent University Hospital approval EC/2014/0655 and EC/2017/0882 and in accordance to relevant guidelines. Participants gave written informed consent. Within 120 min after collection, whole blood was centrifuged 15 min at 2500 g and room temperature, resulting in platelet poor plasma (PPP). To obtain platelet free plasma (PFP), PPP was centrifuged 15 min at 2500 g and room temperature. Plasma (PFP) was stored by -80 °C until further use. The total time between collection and -80°C storage was not more than 160 min. Collection of fecal samples was according to Ethical Committee of Ghent University Hospital approval EC/2006/377 and in accordance to relevant guidelines. Participants gave written informed consent.

PBMC isolation

PBMC were isolated from venous blood of healthy volunteers using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to manufacturer's protocol.

Bacterial EV isolation from plasma

A combination of size exclusion chromatography (SEC) and OptiPrep density gradient (DG) centrifugation was used to isolate bacterial EV from plasma. Sepharose CL-2B (GE Healthcare, Uppsala, Sweden) was washed 3 times with endotoxin-free PBS (Merck Millipore, Billerica, Massachusetts, USA) containing 0.32 % trisodiumcitrate dihydrate (ChemCruz, Dallas, Texas, USA). For preparation of the SEC column, nylon filter with 20 µm pore size (NY2002500, Merck Millipore, Billerica, Massachusetts, USA) was placed on bottom of a 10 ml syringe (BD Biosciences, San Jose, California, USA), followed by stacking of 10 ml Sepharose CL-2B. On top of the SEC column, 2 ml plasma was loaded and fractions of 1 ml eluate were collected. SEC fractions 4, 5 and 6 were pooled and concentrated to 1 ml using 10 kDa centrifugal filter (Amicon Ultra-2mL, Merck Millipore, Billerica, Massachusetts, USA).[1] The resulting 1 ml sample was loaded on top of a DG. This discontinuous iodixanol gradient was prepared by layering 4 ml of 40 %, 4 ml of 20 %, 4 ml of 10 % and 3.5 ml of 5 % iodixanol in a 16.8 ml open top polyallomer tube (Beckman Coulter).[2] The DG was centrifuged 18 h at 100,000 g and 4 °C using SW 32.1 Ti rotor (Beckman Coulter). Fractions of 1 ml were collected and pooled (1-5, 6-7, 8-9, 10-11, 12-13, 14-16) to obtain DG fractions 1-6. An additional SEC was performed on the pooled DG fractions to remove iodixanol.[1] SEC fractions 4-7 were pooled and concentrated to 100 µl and stored at -80 °C until further use.

Bacterial EV isolation from cell culture

Escherichia coli Nissle 1917 *(EcN)* (Ardeypharm, Herdecke, Germany) were grown overnight at 37 °C in 250 ml Luria-Bertani broth (LB) with constant rotation (150 rpm). Growth was monitored by measuring the optical density at 600 nm. Bacterial cells were pelleted by centrifugation at 8000 g for 15 min at 4 °C and the obtained supernatant was filtered through a 0.22 µm pore size filter (Whatman, Dassel, Germany) to remove residual bacteria. 10 kDa Centricon Plus-70 centrifugal units (Merck Millipore, Billerica, Massachusetts, USA) were used to concentrate the filtered supernatant at 4 °C to 667 µl. A discontinuous iodixanol gradient was prepared by layering 4 ml of 50 %, 4 ml of 40 %, 4 ml of 20 %, 3.5 ml of 10 % iodixanol and 1 ml of PBS in a 16.8 ml open top polyallomer tube (Beckman Coulter). The 50 % layer was obtained by mixing 667 µl of the sample with 3.33 ml OptiPrep. The DG was centrifuged 18 h at 100,000 g and 4 °C using SW 32.1 Ti rotor (Beckman Coulter). DG fractions of 1 ml were collected and DG fractions 8-9 pooled and diluted to 15 ml with PBS, followed by 3 h ultracentrifugation at 100,000 g and 4 °C using SW 32.1 Ti rotor (Beckman Coulter, Fullerton, California, USA). Resulting pellet was resuspended in 100 µl PBS and stored at -80 °C until further use.

Bacterial EV isolation from human feces

50 g feces were dissolved in 250 ml PBS and centrifuged twice at 8000 g and 4 °C for 15 min. The obtained supernatant was filtered through a 0.22 µm pore size filter (Whatman, Dassel, Germany). 10 kDa Centricon Plus-70 centrifugal units (Merck Millipore, Billerica, Massachusetts, USA) were used to concentrate the filtered supernatant at 4 °C to 667 µl. Similar to bacterial EV isolation from cell culture, a discontinuous iodixanol gradient was prepared by layering 4 ml of 50 %, 4 ml of 40 %, 4 ml of 20 %, 3.5 ml of 10 % iodixanol and 1 ml of PBS in a 16.8 ml open top polyallomer tube (Beckman Coulter). The 50 % layer was obtained by mixing 667 µl of the sample with 3.33 ml OptiPrep. The DG was centrifuged 18 h at 100,000 g and 4 °C using SW 32.1 Ti rotor (Beckman Coulter, Fullerton, California, USA). DG fractions of 1 ml were collected, DG fractions 8-9 pooled and SEC was performed

(similar to bacterial EV isolation from plasma). SEC fractions 4-7 were pooled and concentrated to 100 µl and stored at -80 °C until further use.

Transwell system

Caco-2 cells were grown in DMEM medium supplemented with 10 % FCS, 0.01 % nonessential amino acids (100X), 1 % (w/v) L-Glutamine 200 mM, 100 IU/ml Penicillin and 100 µg/ml Streptomycin and were cultivated on a permeable filter support (diameter 12 mm, membrane pore size 0.4 µm, Merck KGaA, Darmstadt, Germany) for 21 days to form a monolayer, as described before.[3] Cell monolayers in transwell systems were incubated for 24 h with different concentrations of dextran sulfate sodium salt (DSS, MW =40000, Merck KGaA, Darmstadt, Germany) in DMEM to the apical side. After removing the apical medium, 1.5 x 10^{11} feces bacterial EV were placed to the apical side and basolateral bacterial EV were isolated after 24 h (see 'bacterial EV isolation from cell culture'). Cell viability was assessed by performing a trypan blue viability test. A 1:1 dilution of the cell suspension was prepared by using a 0.4 % trypan blue solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and cell viability was calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer.

Western blotting

All fractions and pellets were dissolved in reducing sample buffer (0.5M Tris-HCl (pH 6.8), 40 % glycerol, 9.2 % SDS, 3 % 2-mercaptoethanol, 0.005 % bromophenol blue) and boiled at 95 °C during 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, California, USA). After blocking the membranes, blots were incubated overnight with primary antibodies. Antibodies against E. coli LPS (1:1000, 2D7/1, Abcam, Cambridge, UK), OmpA (1:5000, 111120, Antibody Research Corporation, Missouri, USA), Alix (1:1000, 3A9, Cell Signaling Technology, Beverly, Massachusetts, USA), Flotillin-1 (1:1000, 610820, BD Biosciences, Franklin Lakes, New Jersey, USA) and CD9 (1:1000, D3H4P, Cell Signaling Technology, Beverly, Massachusetts, USA) were used. Incubation with secondary antibodies was performed after extensive washing of the membranes in PBS with 0.5 % Tween20. After final extensive washing, chemiluminescence substrate (WesternBright Sirius, Advansta, Menlo Park, California, USA) was added and imaging was performed using Proxima 2850 Imager (IsoGen Life Sciences, De Meern, The Netherlands).

Coomassie brilliant blue staining

The bacterial EV pellet was dissolved in reducing sample buffer (1M Tris-HCl (pH 6.8), 30 % glycerol, 6 % SDS, 3 % 2-mercaptoethanol, 0.005 % bromophenol blue) and boiled at 95 °C during 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis. Gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, California, USA) during 1 h followed by destaining overnight at 4 °C with Coomassie Brilliant Blue destaining solution (Bio-Rad).

Nanoparticle tracking analysis

Aliquots of isolated particles were used for Nanoparticle Tracking Analysis (NTA) using NanoSight LM10 microscope (NanoSight Ltd, Amesbury, UK) equipped with 405 nm laser. For each sample, three videos of 60 s were recorded and analyzed with camera level 13 and detection threshold 3. Temperature was monitored during recording. Recorded videos were analyzed with NTA Software

version 3.0. For optimal measurements, samples were diluted with PBS until particle concentration was within the concentration range of NTA Software (3 x 10^8-10^9 particles/ml).

Confocal fluorescence microscopy

Caco-2 cell monolayers were fixed in 3 % paraformaldehyde for 20 min at room temperature. Following permeabilization with 0.2 % (w/v) Triton X-100 for 5 min, sections were blocked with 5 % BSA and tight junctions labeling was performed with a primary anti-ZO-1 mouse monoclonal antibody (Invitrogen, Rockford, USA) 2.5 µg/ml and a secondary Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen, Rockford, USA) 1 µg/ml for 1h at room temperature. Nuclei were stained with 0.4 µg/ml DAPI. Imaging was performed using a Leica DMI60000 microscope (40X objective lens) coupled to an Andor DSD2 confocal scanner and a Zyla5.5 CMOS camera. High-throughput analysis of the intensity of ZO-1 staining was performed by using ImageJ.

Electron microscopy

Samples were deposited on Formvar carbon coated, glow-discharged grids. After 20 min, the grids were incubated in a blocking serum containing 1 % BSA in PBS. Antibodies and gold conjugates were diluted in 1 % BSA in PBS. In case of immunostaining, the grids were exposed to the primary mouse anti-E. coli LPS antibody (10 mg/ml) for 20 min, followed by rabbit anti-mouse secondary antibody (Zymed) for 20 min and protein A-gold complex (10 nm size) (Center for Molecular Medicine, Utrecht, the Netherlands) for 20 min. The blocking efficiency was controlled by performing the labelling procedure in the absence of primary antibody. The grids were stained with neutral uranylacetate and embedded in methylcellulose/uranyl acetate and examined in a Tecnai Spirit transmission electron microscope (Thermo Fisher Scientific FEI). Images were captured by Quemesa charge-coupled device camera (Olympus Soft Imaging Solutions).

LC-MS/MS analysis

Samples were processed for LC-MS/MS by filter-aided sample preparation (FASP). Lysates were prepared by mixing samples with SDT-lysis buffer (2 % SDS, 500 mM Tris-HCL (pH 7.6), 0.5 M DTT) at a 4:1 sample to buffer ratio and incubated at 95 °C for 5 min. After clarification of lysates by centrifugation (16,000 g for 5 min), samples were mixed with 300 µl UA (8 M urea, 0.1 M Tris-HCl (pH 8.5)) in a Microcon YM-10 centrifugal filter device (Merck KGaA, Darmstadt, Germany). Filters were centrifuged twice (14,000 g for 40 min at 20 °C) with the addition of 200 µl UA in between. Proteins were alkylated by addition of 100 µl IAA solution (0.05 M iodoacetamide in UA buffer) and incubated for 30 min at room temperature, followed by centrifugation. This was followed twice by addition of 100 µl UA and twice by addition of 100 µl DB buffer (1 M urea, 0.1 M Tris-HCl (pH 8.5), with centrifugation in between. Filter units were transferred to new collection tubes and proteins were resuspended in 40 µL DB with Trypsin/Lys-C mix (Promega, Madison, Wisconsin, USA) for overnight proteolytic digestion at 37 °C. Digests were collected by addition of 100 µl DB and centrifugation for 15 min at 14,000 g. This step was repeated once. Collected peptides were acidified with 1 % trifluoroacetic acid to a pH of 2-3, followed by desalting with C18-StageTips (C18 Empore Disks, 3M, St. Paul, Minnesota, USA).

Desalted peptides were vacuum dried, dissolved in 0.1 % formic acid and analyzed by LC-MS/MS. Equal amounts of peptides of each sample (300 ng) were loaded on a nanoflow HPLC system (Easy-

nLC1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA) coupled to a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with a nano-electrospray ionization source. The mobile phase consisted of 0.1 % formic acid (solvent A) and acetonitrile/water (95:5 (v/v)) with 0.1 % formic acid (solvent B). The peptides were separated with a 50 min gradient from 8 to 35 % of solvent B. Before the end of the run, the percentage of solvent B was raised to 100 % in 5 min and kept there for 5 min. Full MS scan over the mass-to-charge (m/z) range of 300-1750 with a resolution of 120,000, followed by data dependent acquisition with an isolation window of 2.0 m/z and a dynamic exclusion time of 30 s was performed. The top 12 ions were fragmented by higher energy collisional dissociation (HCD) with a normalized collision energy of 27 % and scanned over the m/z range of 200-2000 with a resolution of 15,000. After the MS2 scan for each of the top 12 ions had been obtained, a new full mass spectrum scan was acquired and the process repeated until the end of the 60-min run. Three repeated runs per sample were performed.

Tandem mass spectra were searched using the MaxQuant software (version 1.5.2.8) against a database containing both reviewed (SwissProt) and unreviewed (TrEMBL) sequences of homo sapiens and common gut microbes (Prevotella, Bacteroides, Clostridiales and Mitsuokella; based on the *frequent microbe proteins* dataset provided by Qin et al.), including different isoforms, of UniProtKB release 2018_07.[4] Peptide-spectrum-match- and protein-level false discovery rates were set at 0.01. Carbamidomethyl (C), as a fixed modification, and oxidation (M) and acetylation of the protein N-terminus as dynamic modifications were included. A maximum of two missed cleavages was allowed. The LC-MS profiles were aligned, and the identifications were transferred to non-sequenced or non-identified MS features in other LC-MS runs (matching between runs). The protein was determined as detected in the sample if its identification had been derived from at least two unique peptide identifications. Filtering for contaminating proteins, reverse identification and identification by site was used.

LPS, ApoA1, ApoB, ZO-1 and cytokine/chemokine measurements

Quantification of ApoA1, ApoB and ZO-1 was performed using Human Apolipoprotein A-I Quantikine ELISA Kit (R&D Systems, Minneapolis, USA), Apolipoprotein B (APOB) Human SimpleStep ELISA Kit (Abcam, Cambridge, UK) and Human Zonulin ELISA Kit (Biomatik, Cambridge, Canada) respectively according to manufacturer's protocol. LPS activity levels were measured using the Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod, Massachusetts, USA) according to manufacturer's protocol. The human cytokine/chemokine luminex array 65-plex panel (Eve Technologies, Calgary, Canada) was used to determine the chemokine/cytokine concentration in the samples.

Quantification of TLR4 agonist activity

HEK-Blue-hTLR4 reporter cell line was obtained from InvivoGen (Toulouse, France). Isolated bacterial EV were added to the reporter cell line and incubated with HEK-Blue detection medium according to manufacturer's protocol.

Statistical data analysis/illustrations

The experimental data were analyzed with GraphPad Prism 7. The body of the box plots represents the first and third quartiles of the distribution, and the median line. The whiskers comprise the minimum and maximum values. Mann-Whitney U tests were performed to compare non-normally distributed continuous variables. Pearson r correlation was calculated to measure the degree of relationship between linearly related variables. Spearman rank correlation was calculated to measure the degree of association between two variables. ANOVA was used for comparison between multiple groups. A p value <0.05 was defined as significant (*p<0.05, **p<0.01, ***p<0.001). Illustrations were made in Adobe Illustrator CS6.

EV-TRACK

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV180059).[5]

SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1 Evaluation of quantitative and qualitative methods to characterize LPS-positive bacterial EV from *Escherichia coli* Nissle 1917 (*EcN*), a nonpathogenic gram-negative strain. (A) Size distribution of bacterial EV obtained by nanoparticle tracking analysis. (B) SDS polyacrylamide gel electrophoresis and coomassie blue staining of bacterial EV identified protein bands characteristic of outer membrane protein A (OmpA) and other porins. (C) Western blot analysis of OmpA and LPS in bacterial EV or bacterial cell lysate. (D-E) The Limulus Amebocyte Lysate (LAL) and TLR4 reporter assay quantify LPS and TLR4 agonistic activity, respectively. TLR4 agonists stimulate the HEK-Blue-hTLR4 reporter cell line, which induces a colorimetric reaction (optical density (OD) measurement at 630 nm by UV-Vis spectrophotometry). Within the detection range of the assay, a linear relationship exists between LPS (EU/ml) or TLR4 activity (OD 630 nm) and bacterial EV concentration (three technical replicates, LAL: Pearson's r=0.9994; TLR4 reporter: Pearson's r=0.9814). (F) Immunoelectron microscopy images of purified bacterial EV using gold-conjugated protein A to detect secondary antibodies against primary antibodies recognizing lipopolysaccharide (LPS) (scale bar = 200 nm).

Supplementary figure 2 Two-dimensional fractionation of plasma (size and density) separates bacterial EV-associated LPS from other LPS products. (A) A combination of size exclusion chromatography (SEC) and density gradient (DG) centrifugation is used to isolate bacterial EV from plasma. (B) 2 ml plasma, spiked with 1 x 10^{10} *EcN* bacterial EV, was applied on top of the SEC column and fractions of 1 ml were collected. Bacterial EV elute in SEC fractions 4-6 as visualized by western blot and LPS activity levels (EU/ml). SEC fractions 4-6 were concentrated to 1 ml and applied on top of a DG. During centrifugation at 100,000 g for 18 h, bacterial EV mainly float to DG fraction 5 (1.141-1.186 g/ml). (C) 2 ml plasma of a breast cancer patient with chemotherapy-induced intestinal mucositis was applied on top of the SEC column and fractions of 1 ml were collected. Bacterial EV elute in SEC fractions 4-6 as visualized by LPS activity levels (EU/ml). SEC fractions 4-6 were concentrated to 1 ml and applied on top of a DG. DG centrifugation at 100,000 g for 18 h revealed enrichment of bacterial EV-associated LPS in DG fraction 5 (1.141-1.186 g/ml) compared to respective control. An enzyme-linked immunosorbent assay (ELISA) for apolipoprotein A1 and B identified HDL and LDL in the lowest DG fractions (1.041-1.079 g/ml), indicating that these fractions

contain other LPS products such as lipoprotein-associated LPS. Of note, eukaryotic EV are enriched in DG fraction 3 (1.086-1.103 g/ml).[1,2]

Supplementary figure 3 Schematic size and density indication of soluble LPS, lipoproteins, eukaryotic EV and bacterial EV. Note that only the hotspot location for each biological component is visualized.

Supplementary figure 4 Quantitative assessment of LPS products in lower density fractions (1.041-1.068 g/ml) in plasma of healthy volunteers and patients with intestinal barrier dysfunction. Both LAL (A) and TLR4 reporter assays (B) of the lower density fractions revealed no significant difference (Mann-Whitney U test, p=0.8315 and p=0.9372, respectively).

Supplementary figure 5 Paracellular translocation of bacterial EV across a Caco-2 monolayer. (A) A Caco-2 monolayer was grown on a permeable transwell filter support and challenged with dextran sulfate sodium (DSS) to mimic colitis. 1.5 x 10^{11} bacterial EV isolated from feces (supplementary figure 6) were applied to the apical side and translocation was investigated by isolating and quantifying the bacterial EV present in the basolateral medium. (B) After basolateral bacterial EV isolation, LPS activity was measured as a direct marker for bacterial EV translocation using the LAL assay. (C) Caco-2 monolayers were fixed, tight junctions were stained with anti-ZO-1 antibodies (green) and nuclei with DAPI (blue) (scale bar = 20 µm). (D) The tight junction integrity was assessed through high-throughput analysis of the intensity of ZO-1 staining and a clear DSS-induced tight junction disruption and barrier dysfunction was evidenced (ANOVA, ***p<0.001). (E) Cell viability was not affected during incubation with DSS (ANOVA, p=0.4691).

Supplementary figure 6 Characterization of gut bacteria-derived EV isolated from healthy volunteer feces. (A) Western blot analysis after density gradient (DG) centrifugation revealed the presence of both eukaryotic and bacterial EV in fecal samples. Alix, Flotillin-1 and CD9 were used as eukaryotic EV markers and outer membrane protein A (OmpA) as bacterial EV marker. Note the cross-specificity of the OmpA antibody with all different forms of outer membrane proteins present in the heterogeneous bacterial EV fractions. (B) Size distribution of feces-derived bacterial EV obtained by nanoparticle tracking analysis. (C) Electron microscopy images of feces-derived bacterial EV (scale bar = 200 nm). (D) Calculation of the number of bacterial EV in the human gut based on Sender et al. (three biological replicates).[6] (E) Selected bacterial proteins identified in the proteomic analysis of feces-derived bacterial EV (PAMP are indicated in bold). (F) Stimulation of peripheral blood mononuclear cells (PBMC) indicated the immunogenicity of bacterial EV (data obtained by luminex assay were transformed into Z-scores).

SUPPLEMENTARY TABLE LEGEND

Supplementary table 1 Baseline clinical characteristics of the subjects included in this study.

SUPPLEMENTARY REFERENCES

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