

RESEARCH ARTICLE

Characterisation of LPS+ bacterial extracellular vesicles along the gut-hepatic portal vein-liver axis

Heetanshi Jain¹ | Ashish Kumar¹ | Sameh Almousa¹ | Shalini Mishra¹ |
Kendall L. Langsten¹ | Susy Kim¹ | Mitu Sharma¹ | Yixin Su¹ | Sangeeta Singh¹ |
Bethany A. Kerr^{1,2} | Gagan Deep^{1,2,3,4} 

¹Department of Cancer Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA

²Atrium Health Wake Forest Baptist Comprehensive Cancer Center, Winston-Salem, North Carolina, USA

³Department of Internal Medicine-Gerontology and Geriatric Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA

⁴Sticht Center for Healthy Aging and Alzheimer's Prevention, Wake Forest University School of Medicine, Winston-Salem, North Carolina, USA

Correspondence

Gagan Deep, Wake Forest University School of Medicine, Biotech Place, 3E-031, Winston-Salem, NC 27101, USA.

Email: gdeep@wakehealth.edu

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Abstract

Gut microbiome dysbiosis is a major contributing factor to several pathological conditions. However, the mechanistic understanding of the communication between gut microbiota and extra-intestinal organs remains largely elusive. Extracellular vesicles (EVs), secreted by almost every form of life, including bacteria, could play a critical role in this inter-kingdom crosstalk and are the focus of present study. Here, we present a novel approach for isolating lipopolysaccharide (LPS)+ bacterial extracellular vesicles (bEV^{LPS}) from complex biological samples, including faeces, plasma and the liver from lean and diet-induced obese (DIO) mice. bEV^{LPS} were extensively characterised using nanoparticle tracking analyses, immunogold labelling coupled with transmission electron microscopy, flow cytometry, super-resolution microscopy and 16S sequencing. In liver tissues, the protein expressions of TLR4 and a few macrophage-specific biomarkers were assessed by immunohistochemistry, and the gene expressions of inflammation-related cytokines and their receptors ($n = 89$ genes) were measured using a PCR array. Faecal samples from DIO mice revealed a remarkably lower concentration of total EVs but a significantly higher percentage of LPS+ EVs. Interestingly, DIO faecal bEV^{LPS} showed a higher abundance of *Proteobacteria* by 16S sequencing. Importantly, in DIO mice, a higher number of total EVs and bEV^{LPS} consistently entered the hepatic portal vein and subsequently reached the liver, associated with increased expression of TLR4, macrophage markers (F4/80, CD86 and CD206), cytokines and receptors (*Il1rn*, *Ccr1*, *Cxcl10*, *Il2rg* and *Ccr2*). Furthermore, a portion of bEV^{LPS} escaped liver and entered the peripheral circulation. In conclusion, bEV could be the key mediator orchestrating various well-established biological effects induced by gut bacteria on distant organs.

KEYWORDS

bacterial extracellular vesicles, dysbiosis, gut microbiome, inflammation, lipopolysaccharide

1 | INTRODUCTION

The human gastrointestinal tract is inhabited by a complex community of microbiota harbouring over 100 trillion microorganisms. These microbiota consist of bacteria, viruses, archaea, protozoa and fungi, mainly dominated by anaerobic bacteria

Heetanshi Jain and Ashish Kumar contributed equally to this work.

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belonging to Bacteroidetes and Firmicutes phyla. Gut microbiome is influenced by several environmental and lifestyle factors, and participates in a range of physiological functions, such as host immunity, protection against pathogens, development of the metabolic system, absorption and distribution of nutrients, and maintenance of intestinal barrier integrity. Apart from these physiological functions, gut microbiota also have an important role in maintaining communication between the gut and peripheral organs/ tissues like liver, lung, brain and other. Moreover, seminal studies have demonstrated the crucial role of gut microbiome in influencing several disease conditions (Bull & Plummer, 2014; Clemente et al., 2012; de Vos et al., 2022; Hou et al., 2022; Li et al., 2008; Vijay & Valdes, 2022). The imbalance of the gut microbiota, referred to as gut dysbiosis, has been associated with biological effects in distant organs, leading to the identification of microbiota-gut-brain, microbiota-gut-lung, microbiota-gut-liver and microbiota-gut-skin axes (Giridharan et al., 2022; Kang et al., 2023; Varela-Trinidad et al., 2022). These gut-organ axes are bi- or multi-directional, or multi-channel communications that allow the gut and extra-intestinal organs to communicate with one another and dictate the effect of the gut microbiome on human health.

Even though the gut-organ axes are well appreciated, the mediators of these communications are not well recognised with some sparse studies shedding light on gut-brain and gut-immune cells interactions (Carabotti et al., 2015; Kasarello et al., 2023). The production of bile acids, choline, short chain fatty acids, endotoxins, DNA and other metabolites are proposed to mediate the connection between the gut and other organs (Ahlawat et al., 2021; Nicholson et al., 2012). Although the short half-life of metabolites and possible degradation of other bacterial biomolecules (such as proteins and/or nucleic acids) by host enzymes do not fully justify the dependence of this communication solely on these bacterial biomolecules being secreted without getting loaded in a protective vehicle.

Recently, extracellular vesicles (EVs), lipid bound vesicles spontaneously secreted by all cell types in the body, have emerged as significant players in inter-cellular/organ/tissue communication carrying variety of biomolecules as cargos. Many recent studies have shown that bacterial EVs (bEVs) play a crucial role in physiology and pathogenesis by mediating detoxification mechanisms, horizontal gene transfer and cell to cell communication messengers (Hosseini-Giv et al., 2022; Jahromi & Fuhrmann, 2021). The bEVs can be divided into distinct categories based on their secretory pathways as outer membrane vesicles (OMVs), outer-inner membrane vesicles, cytoplasmic membrane vesicles and tube-shaped membranous structures (Toyofuku et al., 2019). The OMVs are derived from gram-negative bacteria, and hence, their outer leaflet consists of lipopolysaccharide (LPS) along with bacterial outer membrane proteins (Avila-Calderón et al., 2021). These bEVs, like EVs secreted by eukaryotic cells, carry diverse cargos such as proteins, enzymes, DNA, RNA, peptidoglycans and lipids, to maintain intra- and inter-kingdom communication (Chronopoulos & Kalluri, 2020; Ha et al., 2020). Numerous studies have characterised EVs derived from bacterial culture, faeces or plasma (Díaz-Garrido et al., 2021; Ellis et al., 2010; Fizanne et al., 2023; Liang et al., 2022; Ou et al., 2023; Tulkens et al., 2020); however, no study has in-depth investigated these bEVs along the entrance into the peripheral circulation from gut and their biodistribution to the extra-intestinal organs/tissues. Here, we focused on isolating and characterising LPS-positive bEV (bEV^{LPS}) along the gut-hepatic portal vein-liver axis using a well-established diet-induced obesity gut dysbiosis mouse model and show the presence of these bEVs in the peripheral circulation as a mode of communication between the gut microbiome and extra-intestinal tissues.

2 | MATERIALS AND METHODS

2.1 | Animal experiment

Lean and diet-induced obese (DIO) C57BL/6 male mice were purchased from Jackson Laboratory (Bar Harbor, Maine) at 20 weeks of age. Male mice were selected for this study as these mice in general have high susceptibility to weight gain (Casimiro et al., 2021; Maric et al., 2022). The lean mice were fed a low-fat (10% kCal) diet (D12450B, Research Diet, New Brunswick, NJ), and the DIO mice were fed a high-fat (60% kCal) diet (D12492, Research Diet, New Brunswick, NJ). For faecal sample collection, each mouse was housed individually for 20 min in a fresh cage with no bedding and faeces were collected and quickly stored at -80°C . Mice were sacrificed at ~ 28 weeks of age to collect blood from the hepatic portal vein and other tissues (intestine and liver). Cardiac blood was used from another cohort of lean and DIO mice (aged ~ 59 weeks).

2.2 | EV isolation

2.2.1 | Faeces

Frozen faecal samples, from ~ 28 weeks old lean and DIO mice, were resuspended in HBSS supplemented with HEPES and homogenised into faecal slurries before sequential centrifugation at 300, 2000 and $10,000 \times g$, all for 10 min at 4°C . The supernatant was collected and passed through a $0.45 \mu\text{m}$ filter. The filtrate was then ultra-centrifuged at $100,000 \times g$ in a Ti-70.1 rotor

for 75 min at 4°C, and the collected pellet underwent a second ultracentrifugation step. Then pellet that is, total faecal EVs (faecal-EV^{total}), was resuspended in 0.1 µm filtered PBS.

Faecal-bEV^{LPS} from faecal-EV^{total} were isolated by immunoprecipitation method as reported by us earlier (Kumar et al., 2023; Kumar et al., 2022; Kumar et al., 2021). Briefly, LPS polyclonal antibody (Catalogue no. PA1-73178, ThermoFisher) was biotin-tagged using FluoReporter Mini-Biotin-XX Protein labelling kit (Catalogue no. F6347, ThermoFisher). Next, faecal-EV^{total} were incubated with biotin-tagged LPS antibody overnight at 4°C. After incubation, streptavidin-tagged agarose beads (Catalogue no. 20359, ThermoFisher) were added, and the samples were incubated for 2 h at room temperature (RT). The samples were then centrifuged at 2500 × g to remove the supernatant. Beads were washed twice with isolation buffer (0.1% BSA in PBS). Finally, beads were resuspended in 100 µL IgG elution buffer to elute LPS-positive EVs (faecal-bEV^{LPS}). Beads were removed by centrifugation, and the collected supernatant containing faecal-bEV^{LPS} were transferred to a new tube containing 10% v/v freshly prepared 1 M Tris (pH = 9) to neutralise the pH.

2.2.2 | Plasma (hepatic portal vein and cardiac plasma)

Total EVs (plasma-EV^{total}) from hepatic portal vein and cardiac plasma were isolated by modified precipitation method as described previously (Kumar et al., 2021; Kumar et al., 2022; Kumar et al., 2023). Briefly, PBS (without Ca²⁺ and Mg²⁺) was added to the hepatic portal vein and cardiac plasma collected from lean and DIO mice and centrifuged at 500 and 2000 × g both for 5 min at RT followed by 10,000 × g for 30 min at 4°C. Thromboplastin-D (Catalogue no. 176065, ThermoFisher) was added to the collected supernatant and incubated for 60 min at RT. After incubation, PBS with protease and phosphatase inhibitor was added and centrifuged at 1500 × g for 20 min at RT. ExoQuick™ was added to the collected supernatant and incubated for 60 min at 4°C. The samples were then centrifuged at 1500 × g for 30 min at 4°C. Then, the pellet that is total EV (plasma-EV^{total}), was resuspended in 0.1 µm filtered PBS. LPS-positive EVs were isolated from plasma following similar immunoprecipitation method as described above.

2.2.3 | Liver tissue

Total EVs (liver-EV^{total}) were isolated from liver tissue following previous methods (Crescitelli et al., 2021; Vella et al., 2017). Briefly, frozen liver tissues were cut into smaller pieces (~1 mm³) and added to a tube containing digestion buffer (75U/mL of collagenase type 3 in RPMI media; 1 mL/50 mg of tissue) and incubated in a shaking water bath at 37°C for 20 min. Next, 1X halt protease and phosphatase inhibitor was added, and digested tissue solution was strained using a 70 µm cell strainer. Next, liver-EV^{total} were isolated from this liver tissue digest by sucrose cushion method. The liver tissue digest was centrifuged at 300 × g for 5 min and 2000 × g for 10 min at 4°C to remove cellular debris. The supernatant was then centrifuged at 10,000 × g for 30 min at 4°C. The volume of supernatant was made up to 30 mL with PBS and then filtered with 0.2 µm syringe filter. 3 mL of 30% sucrose solution (in D₂O) was added carefully to the bottom of the tube with the help of a pasture pipette and centrifuged at 100,000 × g for 90 min at 4°C in SW32Ti rotor (Beckman Coulter, California, USA). 2 mL of sucrose fraction was taken from the bottom of tube and 6 mL of 0.1 µm filtered PBS was added. EVs were pelleted by ultracentrifuging at 100,000 × g for 90 min at 4°C in 70.1Ti rotor. Pellet was resuspended in 0.1 µm filtered PBS and stored at 4°C.

2.3 | Nanoparticle tracking analysis (NTA)

The size and concentration of EVs were analysed with NTA using Nanosight NS300 (Malvern Instruments, UK), as described earlier (Kumar et al., 2023). Five videos of 30 s each were recorded for every sample, and the average of five videos was presented as the final size and concentration count for each sample. For the machine calibration, standard polystyrene beads of known sizes (100 and 200 nm) were used.

2.4 | Immunogold labelling

For immunogold labelling, EV samples were fixed with 2% paraformaldehyde in PBS buffer (pH 7.4), then adsorbed for 1 h to a carbon-coated grid. Samples were incubated with anti-bacteria LPS (Catalogue no. PA1-73178, ThermoFisher) primary antibody. A secondary antibody tagged with 10 nm gold particles (Catalogue no. ab41496, Abcam) was further used, and images were captured on a Tecnai T12 transmission electron microscope. Samples treated with gold-labelled secondary antibody, but without primary antibody, were used as negative control.

2.5 | Flow cytometry

Antibodies specific to surface markers, LPS and OmpC, were conjugated to fluorophores using Alexa Fluor 647 Conjugation Kit (Fast)—Lightning-Link (Catalogue no. ab269823, Abcam) or PE/R-Phycoerythrin Conjugation Kit—Lightning-Link (Catalogue no. ab102918, Abcam). EVs were labelled and acquired as reported previously (Kumar et al., 2022; Kumar et al., 2023). Briefly, EVs were incubated with respective antibodies and co-labelled with membrane labelling dye CellBrite 488 (Catalogue no. 30106, Biotium). EVs without the membrane labelling dye were used to set the gate for dye-positive events, and EVs without antibodies (but with dye) were used to set the gate for antibody-positive events. All samples were acquired on CytoFlex (Beckman Coulter Life Science) for 60 s at a low flow rate. Samples were acquired by triggering with violet side scatter at threshold cutoff at 2000 to remove machine noise and smaller particles.

2.6 | Super-resolution microscopy

25 μ L EV sample was incubated with 0.6 μ L of AF-647 tagged anti-LPS antibody for 90 min in dark. AF-550 tagged lipid membrane dye was then diluted at 1:200, and 60 μ L of the diluted dye was added to the EV sample and incubated for 20 min at RT in the dark. Then 4% paraformaldehyde was added and incubated for 20 min at RT in dark. For imaging, a slide was prepared by diluting the stained EV sample to 1:100, from which 10 μ L of the sample was added to 1.5 mm thick coverslip. Once the sample was dried, the coverslip was transferred to chamlide chamber and blinking buffer was added and imaged immediately utilising Vutara VXL super-resolution microscope (Bruker).

2.7 | 16S sequencing

16S sequencing was performed by Microbiome Insights Inc (Richmond, British Columbia, Canada) or LC Sciences (Houston, Texas). 16S rRNA sequencing was performed on Illumina MiSeq platform. Bioinformatics was performed by LC Sciences (Houston, Texas), including merging reads, chimera filtering, data quality control and operational taxonomic units (OTUs) clustering. A Divisive Amplicon Denoising Algorithm (DADA2) was used for acquiring taxonomic compositions of samples and performing diversity analyses (Callahan et al., 2016). Taxonomic compositions were also validated using Mothur open-source software packages (Batut et al., 2018; Hiltmann et al., 2023).

2.8 | Immunohistochemistry (IHC) staining

IHC analysis on liver and intestine tissue was performed as previously reported (Foster et al., 2021). The primary antibody utilised was TLR4 (Catalogue no. 710185, Invitrogen). Images were captured using Olympus VS120 Slide Scanner and quantified using VisioPharm digital pathology analysis software. As mentioned, immunoreactivity to TLR4 was also subjectively scored by a board-certified veterinary anatomic pathologist.

2.9 | Immunofluorescence (IF) staining

Expression of macrophage markers was investigated in liver sections of lean and DIO mice by IF staining. Briefly, slides were deparaffinised and re-hydrated using serial changes of xylene (3 times, the first time for 10 min followed by next two times for 5 min each), 100% ethanol (ETOH), 90% ETOH, 80% ETOH, 70% ETOH, 50% ETOH and DI H₂O for 5 min each. Antigen retrieval was performed using 10 mM sodium citrate solution (pH 6.0) at sub-boiling temperature for 1 h. Slides were washed thrice with 1X PBS. The slides were incubated with 2.5% normal horse serum for 30 min at RT in a humidified chamber. After overnight incubation with primary antibodies for F4/80 (Catalogue no. 30325S, Cell Signalling Technology), CD86 (Catalogue no. MA1-10299, Invitrogen) or CD206 (Catalogue no. PA5-46994, Invitrogen) at 4°C, slides were washed in PBS-Tween 20 (0.2%) and incubated with suitable Alexa Fluor® conjugate secondary antibody for 2 h in the dark in a humidified chamber. Slides were washed thrice with PBS-Tween 20 (0.2%). Slides were mounted using VECTASHIELD® Antifade Mounting Medium with DAPI (4',6-diamidino-2-phenylindole). The slides were imaged using Keyence All-in-One fluorescence microscope (BZ-X700) at 10X magnification. The images (25-30 images/group) were quantified using ImageJ software for mean intensity expression of these markers.

2.10 | PCR array for inflammatory cytokines and receptors

Total RNA was isolated from liver tissue of lean and DIO mice by homogenising the tissue with 500 μ L of Trizol. The tissue lysate was spun at $2000 \times g$ for 3 min, and the supernatant was collected. The supernatant was mixed with 100 μ L of chloroform and following 2 min incubation at RT, samples were centrifuged at $12,000 \times g$ for 15 min at 4°C. The top clear phase was collected in a fresh tube and mixed with one volume 70% ethanol. The samples were then passed through RNeasy mini (Qiagen) columns. The washing and elution of RNA was performed according to the manufacturer's instructions provided with the kit. The cDNA was synthesised using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Subsequently, the analysis of inflammatory cytokines & receptors (in total 89 genes, including 5 housekeeping genes) was performed using RT² profiler PCR array, specifically the mouse inflammatory cytokines and receptors assay plates (Catalogue no. 330231, Qiagen), following the manufacturer's recommendations. A volcano plot was made using VolcanoR software showing the overexpressed genes in DIO mice.

2.11 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. Unless otherwise mentioned, an unpaired t-test was used for statistical analysis. Bar diagrams are presented as mean $\pm$ SEM (standard error of the mean). For 16S sequencing data analysis, a two-way ANOVA with Sidak's multiple comparisons test was utilised for analysis of significance; whilst a two-way ANOVA with Tukey's multiple comparisons test was utilised for analysis of significance for the bar graphs representing the expression of macrophage markers in lean and DIO mice liver tissue. Statistical significance was set at $p \leq 0.05$.

3 | RESULTS

3.1 | Isolation and characterisation of faecal EVs

The DIO mice possessed significantly higher body weight (~ 1.5 fold) compared to lean mice at the time of collection of the faecal samples (~ 28 week) (Figure 1a). First, we isolated and analysed total EVs from the faecal samples (faecal-EV^{total}) of lean and obese mice by double ultracentrifugation method. We observed a significant decrease in faecal-EV^{total} concentration and size in obese mice (Figure 1b,c). We then confirmed the expression of LPS on the surface of faecal-EV^{total} through immunogold labelling coupled with transmission electron microscopy (Figure 1d) and super-resolution microscopy (Figure 1e). Interestingly, we observed a significant higher percentage of LPS-positive population in faecal-EV^{total} from obese mice compared to lean mice using flow cytometry (Figure 1f) which was further supported by the observed increase in OmpC+ EV population (Figure 1g). Furthermore, we isolated faecal-bEV^{LPS} from the faecal-EV^{total} by immunoprecipitation using an LPS-biotin-tagged antibody and streptavidin-coated agarose beads as reported by us (Kumar et al., 2023; Kumar et al., 2022; Mishra et al., 2023). We noticed a slight increase in the concentration of bEV^{LPS} from DIO mice ($p = 0.058$) (Figure 1h), although no difference in their size was observed (Figure 1i). The relatively smaller observed differences between the two groups for bEV^{LPS} concentration could be due to relatively low yield of immunoprecipitation method. Lastly, 16S sequencing analysis of faecal-bEV^{LPS} revealed a significant increase in Proteobacteria ($p = 0.0497$) in the DIO group (Figure 1j).

3.2 | DIO mice showed differential TLR4 expression in the intestine

The potential pathogenic bacteria and their products interact with toll-like receptors (TLRs) present on the membrane of the gut immune cells, as well as on the cellular membrane of the gut epithelium, to turn on the body's defence mechanism by activation of the innate immune system. LPS derived from the outer membrane of gram-negative bacteria (and hence present on bEV surface) binds to TLR4 and activate pro-inflammatory signalling. Since, bEVs were identified to have LPS on their surface, we analysed the expression of TLR4 in the small and large intestine of lean and DIO mice (Figure 2). We observed a significantly lower expression of TLR4 in the small intestine (Figure 2a), whilst statistically non-significant higher expression was observed in large intestine of DIO mice compared to the lean mice (Figure 2b). These results suggested a variable TLR4 expression in the intestine of DIO mice.

3.3 | DIO mice showed higher levels of bEV^{LPS} in the hepatic portal vein blood

Considering the possibility that the reduced concentration of faecal-EV^{total} in DIO mice could result from their increased transport to the hepatic portal vein via paracellular and/or transcellular transport or "leaky gut," we isolated total EVs from hepatic

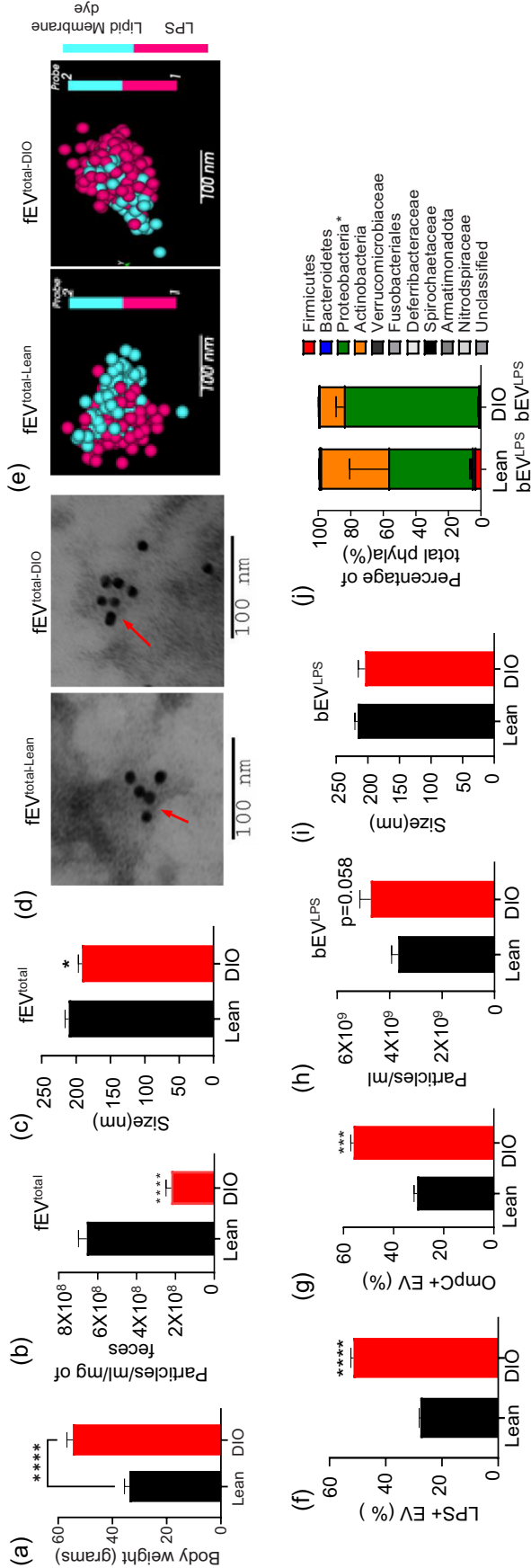


FIGURE 1 Characterisation of bacterial extracellular vesicles (bEV) isolated from faeces. (a) Bar graph representing the average body weight (mean $\pm$ SEM) of lean and diet induced obese (DIO) mice ($n = 5/\text{group}$) at 28 weeks of age. **** $p \leq 0.0001$ (b) Bar graph representing the concentration of faecal EV total (fEV^{total}) from lean and DIO mice as estimated by nanoparticle tracking analysis (NTA) ($n = 5/\text{group}$). Concentration (particles/mL) was normalised with the corresponding weight of faecal matter. **** $p \leq 0.0001$ (c) Bar graph representing the size (nm) of fEV^{total} from lean and DIO mice as estimated by NTA ($n = 5/\text{group}$). * $p = 0.03$ (d) Representative micrograph showing the size and surface expression of LPS on lean and DIO fEV^{total} by immunogold labelling coupled with transmission electron microscopy ($n = 3/\text{group}$) (scale bar: 100 nm). (e) Representative micrograph showing the size and surface expression of LPS on lean and DIO fEV^{total} by super-resolution microscopy ($n = 3/\text{group}$) (scale bar: 100 nm). (f) Bar graph showing expression of LPS-positive EV in lean and DIO fEV^{total} as estimated by flow cytometry ($n = 3/\text{group}$). **** $p \leq 0.0001$ (g) Bar graph showing expression of OmpC-positive EV in lean and DIO fEV^{total} as estimated by flow cytometry ($n = 3/\text{group}$). *** $p = 0.001$ (h and i) Bar graphs representing the concentration ($p = 0.058$) and size (nm) of bEV^{LPS} in lean and DIO mice faeces as estimated by NTA ($n = 4-5/\text{group}$). (j) Bar graph showing 16S rRNA sequencing data representing the percentage of total phyla in lean and DIO bEV^{LPS} ($n = 3/\text{group}$, * $p = 0.0497$).

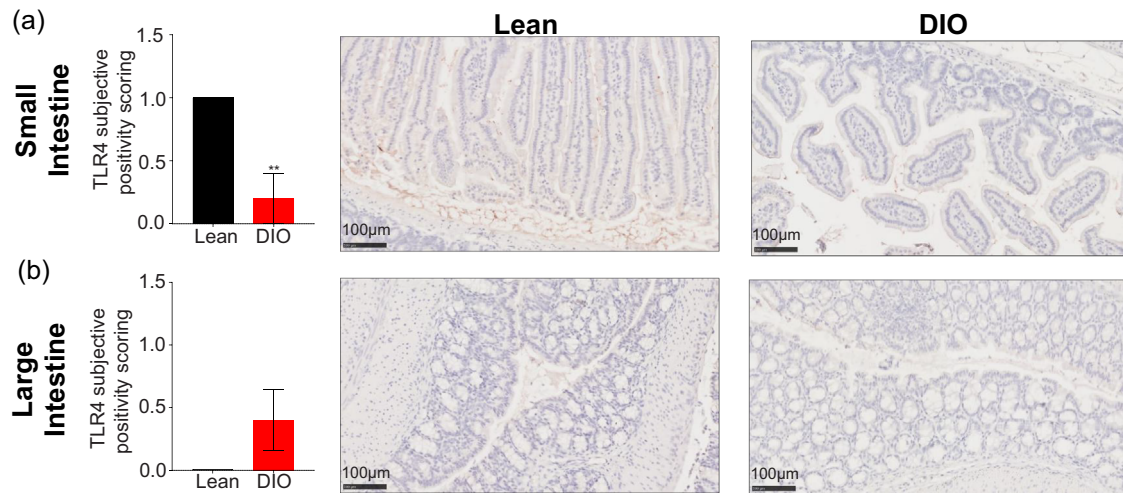


FIGURE 2 TLR4 expression in the intestine. TLR4 expression in the small (a) and large (b) intestinal tissue in lean and DIO mice ($n = 5/\text{group}$) was measured by immunohistochemistry. Images were captured using Olympus VS120 Slide Scanner and quantified by subjectively scoring them. The bar graph in the left panel represents TLR4 positivity (%), whilst the right panel shows representative images (20X magnification, scale bar: 100 μm). ** $p = 0.003$.

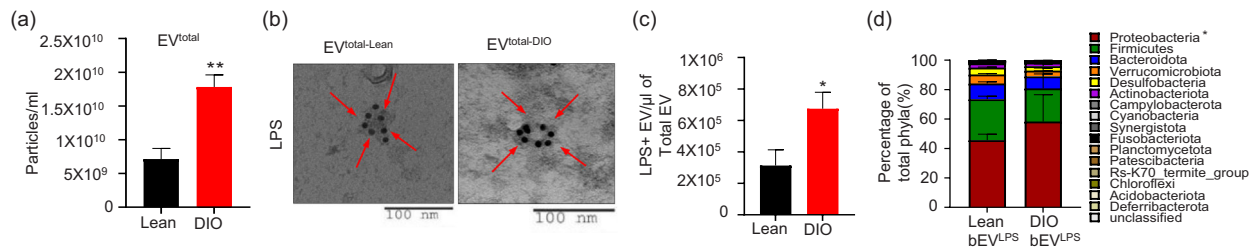


FIGURE 3 Characterisation of total EVs isolated from hepatic portal vein. (a) Bar graph representing the concentration of EV^{total} from lean and DIO mice in hepatic portal vein plasma as estimated by NTA ($n = 5/\text{group}$). ** $p = 0.002$ (b) Representative micrograph captured at 98,000X magnification showing the size and surface expression of LPS on lean and DIO hepatic portal vein plasma- EV^{total} by immunogold labelling coupled with transmission electron microscopy ($n = 3/\text{group}$) (scale bar: 100 nm). (c) Bar graph showing the number of LPS+ EV, normalised by volume, in lean and DIO EV^{total} isolated from hepatic portal vein plasma by flow cytometry ($n = 5/\text{group}$). * $p = 0.03$ (d) Bar graph showing 16S rRNA sequencing data representing the percentage of total phyla in lean ($n = 5$) and DIO ($n = 3$) bEV^{LPS} from hepatic portal vein plasma (* $p = 0.040$).

portal vein plasma (plasma- EV^{total}). Interestingly, we observed a significant increase in plasma- EV^{total} concentration in DIO mice (Figure 3a), with no significant change in their size (data not shown). We further characterised the presence of bEV^{LPS} in the hepatic portal vein plasma (plasma- bEV^{LPS}) by examining the surface expression of LPS on lean and DIO plasma- EV^{total} by immunogold labelling coupled with transmission electron microscopy (Figure 3b) and flow cytometry (Figure 3c). Although the percentage of LPS+ EVs in plasma- EV^{total} was similar between lean and DIO mice (data not shown), we observed a significantly higher number of LPS+ EVs in the hepatic portal vein of DIO mice (Figure 3c). Furthermore, similar to faecal- bEV^{LPS} , 16S sequencing analysis of plasma- bEV^{LPS} also showed a significant increase in Proteobacteria ($p = 0.040$) (Figure 3d).

3.4 | LPS+ EVs and inflammation in the liver of DIO mice

Since the hepatic portal vein connects gut to the liver, we next collected total EVs from liver tissue (liver- EV^{total}) by sucrose cushion-based double ultracentrifugation method. We confirmed the expression of LPS on their surface by immunogold labelling coupled with transmission electron microscopy (Figure 4a) and flow cytometry (Figure 4b), and OmpC expression by flow cytometry (Figure 4c). We observed a trend towards a higher concentration of LPS+ bEV in the liver of DIO mice, though statistical significance could not be achieved ($p = 0.081$) (Figure 4b). Concomitantly, we noted a significantly higher expression of TLR4 in the liver tissue of DIO mice ($p = 0.0079$) (Figure 4d). Furthermore, as LPS is known to induce pro-inflammatory effects, we analysed the expression of macrophage markers in liver tissues and observed a significant increase in F4/80 ($p < 0.0001$), CD86 ($p < 0.0001$) and CD206 ($p < 0.0001$) in DIO mice (Figure 4e).

To further understand the pathways associated with inflammation, we analysed cytokines and receptors in the liver by a PCR array (Figure 4f-left panel). We observed an upregulation in the expression of 51 of 89 genes involved in mediating inflammatory

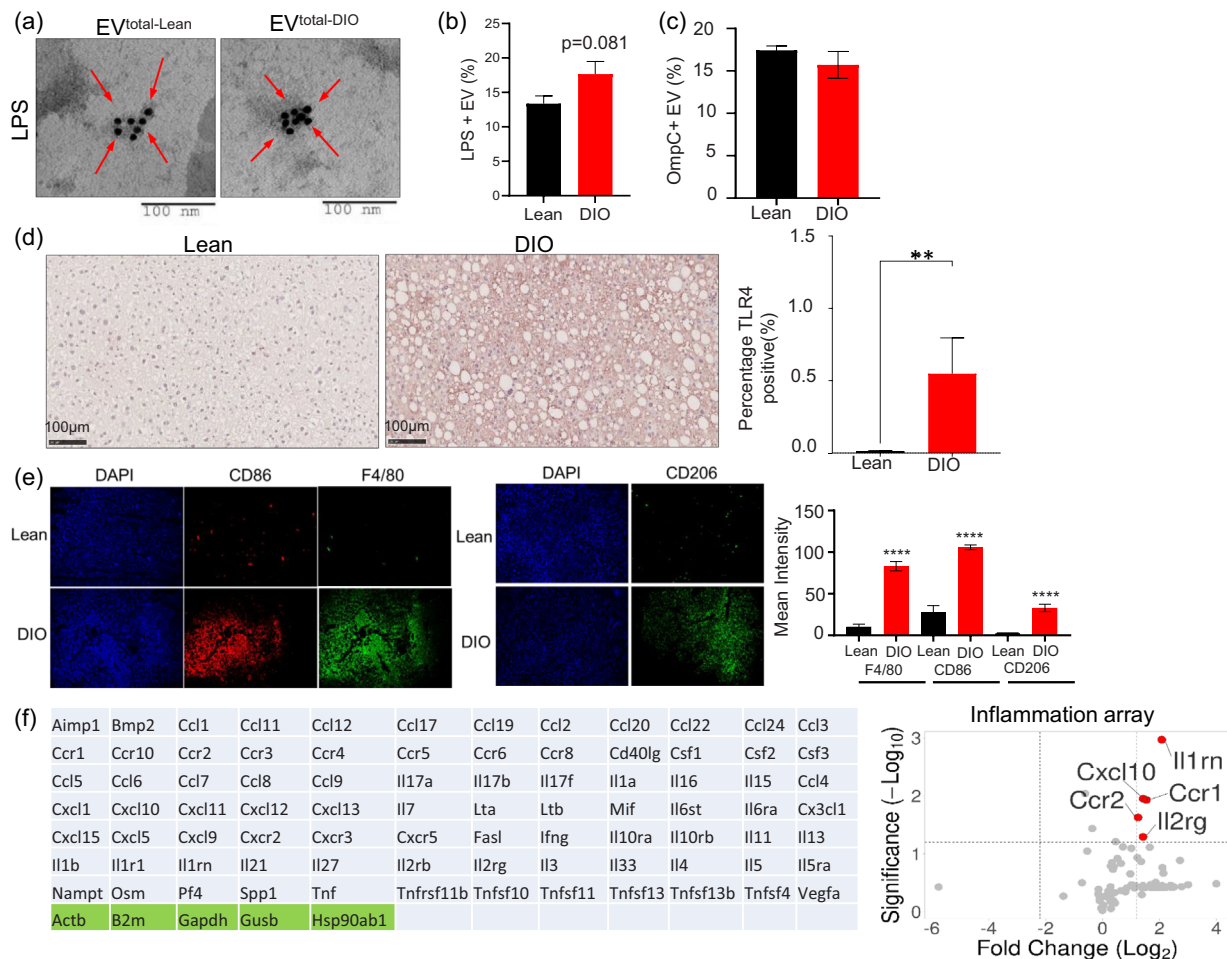


FIGURE 4 Isolation and characterisation of total EVs from liver tissue. (a) Representative micrograph captured at 98,000X magnification showing the size and surface expression of LPS in lean and DIO liver EV^{total} by immunogold labelling coupled with transmission electron microscopy ($n = 3/\text{group}$) (scale bar: 100 nm). (b) Bar graph showing expression of LPS-positive EVs in lean and DIO liver-EV^{total} as estimated by flow cytometry ($n = 5/\text{group}$). $p = 0.081$ (c) Bar graph showing expression of OmpC-positive EV in lean and DIO liver EV^{total} as estimated by flow cytometry ($n = 3/\text{group}$). (d) TLR4 expression in the liver tissues of lean and DIO mice ($n = 5/\text{group}$) was measured by immunohistochemistry. Images were captured using Olympus VS120 Slide Scanner and quantified using VisioPharm digital pathology analysis software. The bar graph in the right panel represents TLR4 positivity (%), whilst the left panel shows representative images (at 20X magnification, scale bar: 100 μ m). Non-parametric Kolmogorov-Smirnov test was utilised for analysis of significance. $**p = 0.0079$ (e) Immunofluorescence staining for macrophage markers F4/80, CD86 and CD206 in lean and DIO mice liver tissue ($n = 3/\text{group}$). The left side shows representative images (10X magnification) of expression of macrophage markers (CD86, F4/80 and CD206) and the nuclei stain (DAPI) whilst the bar graph on the right side represents the mean intensity of expression of F4/80, CD86 and CD206 respectively. $****p < 0.0001$ (f) Left panel: All genes analysed by PCR array are tabulated. The housekeeping genes are highlighted in green. Right panel: Volcano plot representing the differential changes in the genes related to mouse inflammatory cytokines and receptors in the liver tissues of lean and DIO mice ($n = 3/\text{group}$). The dotted line on the x-axis represents the cutoff at 1.5-fold. The dotted line on the y-axis represents the p -value of 0.05. Genes showing a statistically significant change in lean and DIO mice liver tissue are shown in red.

responses (Figure 4f-right panel). Importantly, we identified a statistically significant overexpression of *Il1rn* (4.23 fold, $p = 0.001$), *Ccr1* (2.91 fold, $p = 0.011$), *Cxcl10* (2.69 fold, $p = 0.011$), *Il2rg* (2.68 fold, $p = 0.05$) and *Ccr2* (2.37 fold, $p = 0.02$) genes in the liver of DIO mice (Figure 4f-right panel).

3.5 | bEV can be detected beyond the gut-liver axis

Lastly, to identify whether bEVs can escape liver and be detected in the peripheral circulation to affect other organs, we isolated EVs from the plasma of cardiac blood (cardiac-EV^{total}). We did not observe any changes in their concentration and size (Figure 5a,b). LPS+ EVs were detected in cardiac-EV^{total} by flow cytometry, and a significant decrease was observed in DIO mice (Figure 5c). The immunocaptured LPS+ EVs from cardiac-EV^{total} (cardiac-bEV^{LPS}) showed no significant change in their size and concentration (Figure 5d,e). LPS expression on cardiac-bEV^{LPS} was confirmed through immunogold labelling coupled with transmission electron microscopy (Figure 5f) and super-resolution microscopy (Figure 5g).

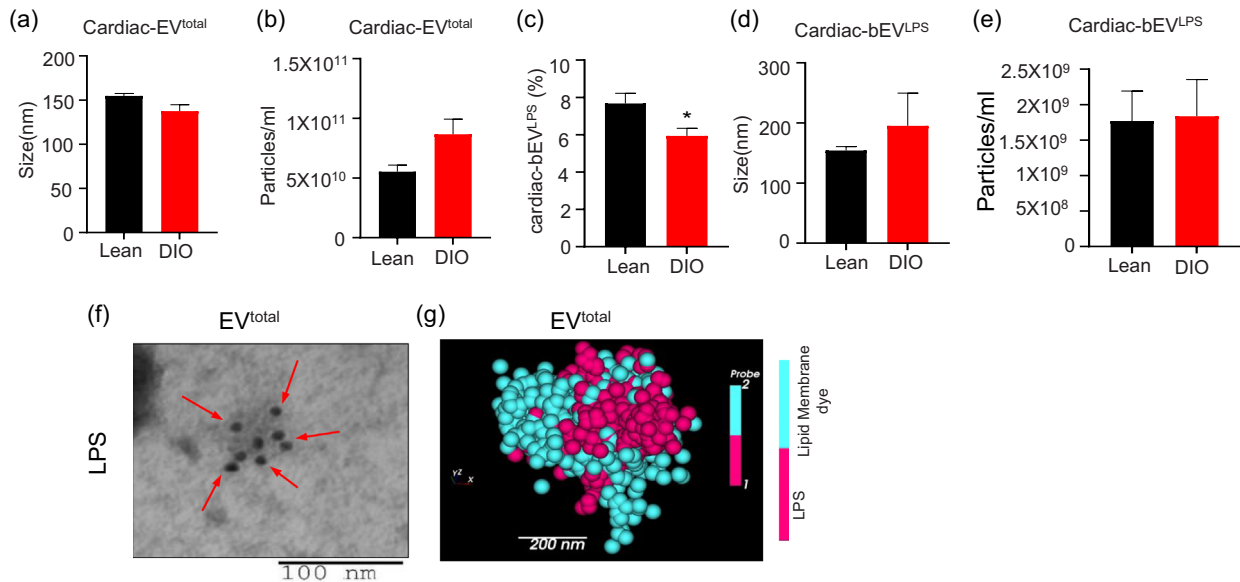


FIGURE 5 Characterisation of bEV in cardiac plasma from lean and DIO mice. (a and b) Bar graphs representing the size (nm) and concentration (particles/mL) of EV^{total} in cardiac plasma from lean and DIO mice as estimated by NTA ($n = 5/\text{group}$). (c) Bar graph showing expression of LPS-positive EVs in lean and DIO mice cardiac plasma EV^{total} as estimated by flow cytometry ($n = 5/\text{group}$). * $p = 0.03$ (d and e) Bar graphs representing the size (nm) and concentration (particles/mL) of bEV^{LPS} in lean and DIO mice cardiac plasma as estimated by NTA ($n = 5/\text{group}$). (f) Representative micrograph captured at 98,000X magnification showing the size and surface expression of LPS on lean and DIO mice plasma EV^{total} by IG/TEM ($n = 3/\text{group}$) (scale bar: 100 nm). (g) Representative micrograph showing the size and surface expression of LPS on lean and DIO mice plasma EV^{total} by super-resolution microscopy ($n = 3/\text{group}$) (scale bar: 100 nm). * $p \leq 0.05$.

4 | DISCUSSION

The mucosal covered host intestine curtails the ability of both pathogenic and non-pathogenic bacteria to enter circulation and their direct interaction with host tissues. However, bEVs are well recognised to carry diverse bioactive molecules, including proteins, RNA, DNA, and metabolites and are considered as an efficient mean to establish the communication between the gut microbiome and host organs. Moreover, bEVs are an excellent part of the immune evasion strategy of bacterial pathogens to resist host antimicrobial molecules. However, the direct evidence that bEVs can cross the mucosal barrier of the intestine and enter the circulation are lacking. In this study we provide compelling evidence that bEVs can enter the circulation, accumulate in the liver and potentially induce inflammation by inducing TLR4-mediated signalling.

Altered gut microbial composition has been implicated in mucosal barrier dysfunction and inflammatory responses, which predispose the host animals to systemic diseases including obesity and inflammation (Levy et al., 2017; Tarantino, 2014; Turnbaugh et al., 2006). Similarly, in obesity, the altered microbiota can instigate the gut leakiness by reducing tight junctions (Mishra et al., 2023) and facilitating relatively easy access of bacterial biomolecules, including EVs, across the intestine. Bacterial secretions could directly affect host physiology and health (Vidal-Veuthey et al., 2022) but without the protecting membrane(s), as in bEV, these biomolecules will probably have a shorter half-life and are prone to be targeted and cleared by the host immune system. Therefore, bEV could play a critical role in gut microbiota and host interaction as well as inform about the dynamic changes in the gut microbiome. We observed significant decrease in faecal-EV^{total} in obese mice, which could be associated with reduced bacterial diversity associated with obesity-related gut dysbiosis (Cuevas-Sierra et al., 2019) and also with gut leakiness, resulting in increased accessibility of these EVs to the peripheral circulation. In line with this observation, we noticed a significant increase in the total EVs isolated from hepatic portal vein blood with a significant contribution from the bacterial LPS+EVs. The reduced concentration of faecal EVs and concomitant increase of bEVs in the hepatic portal vein blood also correlate with gut dysbiosis in DIO mice. However, surprisingly, we observed a differential expression of TLR4 along the intestine with a significant decrease observed in the small intestine and a trend towards increased expression in large intestine. An earlier study reported an increase in TLR4 expression in the intestine of C57BL/6 mice after administration of high fat diet at day 7 before observing a gradual reduction at day 9 (Wang et al., 2013). Another study also reported elevated TLR4 expression in the colon of DIO mice (Kim et al., 2012); though both the studies employed different methodologies (Western blotting and/or PCR) to assess TLR4 expression and mice were fed high-fat diet for a shorter duration compared to the current study. Moreover, with immuno-staining, it is difficult to precisely assess whether the TLR4 expression is from immune cell pockets or epithelial cell surfaces. TLR4 has been demonstrated to be involved in promoting macrophage activation, and TLR4 knockout mice on a high fat diet displayed an attenuated adipose tissue inflammation and concomitant shift in macrophage polarisation to an alternatively

activated state (Orr et al., 2012). Further, TLR4 is crucial in controlling intestinal epithelial homeostasis (Inoue et al., 2017) and the reduced expression of TLR4 in small intestine could affect barrier and absorptive functions. However, further studies are needed to better understand the observed decrease in TLR4 expression in the small intestine of DIO mice.

Further, analysis of EVs isolated from the liver tissue revealed a slight increase in LPS+ EV in DIO mice compared to lean mice. Though, we did not observe similar difference in the OmpC+ EV and its relative percentage was also lower than LPS+ EV. Therefore, there could be heterogenous expression of OmpC and LPS on EVs' surface in liver tissue. The higher abundance of LPS+ EV in the liver tissue of DIO mice also supports that the liver, along with the intestine, may play a crucial role in regulating the infiltration of bEV into the circulation. In both the lean and DIO mice, we detected LPS+ EV in the liver tissue, though it is possible that, under healthy conditions, LPS+ EV act to prime immune cells and keep the immune system 'battle ready,' whereas in the gut dysbiosis (obesity) condition, LPS+ EV, due to higher concentration and unique cargo, puts a stiff challenge to immune cells and creates a pro-inflammatory condition ('real battle'). Supporting this hypothesis, we observed an increase in TLR4 expression and a higher number of pro-inflammatory macrophages in the liver tissue of obese mice. Furthermore, the observed increased expressions of chemokines, interleukins and chemokine receptors (*Il1rn*, *Ccr1*, *Cxcl10*, *Il2rg* and *Ccr2*) in the liver tissue of obese mice have previously been reported to mediate inflammation in the liver (Bartneck et al., 2021; Hintermann et al., 2010; Li et al., 2024; Pihlajamäki et al., 2012). In addition, our data suggested that a percentage of bEV^{LPS} escape from the liver, which likely increases with the duration and extent of gut dysbiosis and liver damage, and these vesicles, carrying various cargo such as metabolites, proteins and nucleic acid, could potentially reach any part of the body, resulting in several known distant effects of the gut microbiome.

It is important to consider that the bEV can be taken up by the host cells and also the LPS secreted by bacteria or LPS or OmpC released following uptake of bEV by the host cell, could be presented on the EVs secreted by the host cell. This could affect the estimation of pure bEV in the circulation. Also, the presented nano-flow cytometry results for bEV could be influenced by factors such as fluorophore used to label antibodies and its free form, EV concentration and purity, and inherent heterogeneity in the size of EV/bEV. Furthermore, the analysed bEV may also be contributed by the circulating bacteria and/or the resident bacteria of the other tissues, though their contribution is expected to be significantly smaller compared to bacteria present in colon (Sender et al., 2016). Despite these limitations, overall, these results demonstrate the existence of bEV^{LPS} in the gut, hepatic portal vein and liver, as well as significant changes in their biodistribution and pro-inflammatory effects in DIO mice. The tools and techniques described here could have a tremendous impact in enhancing our understanding of the role of the gut microbiome in various pathologies. Specifically, bEV^{LPS} characterisation and analysis of their protein and metabolites cargos, could offer valuable insights into the communication between the gut bacteria and spatially distant organs.

AUTHORS CONTRIBUTION

Heetanshi Jain: Conceptualization; data curation; formal analysis; methodology; writing—original draft; writing—review and editing. **Ashish Kumar:** Conceptualization; data curation; formal analysis; methodology; validation, visualization; writing—original draft; writing—review and editing. **Sameh Almousa:** Conceptualization; data curation; formal analysis; methodology; validation; writing—original draft; writing—review and editing. **Shalini Mishra:** Investigation; methodology; writing—original draft; writing—review and editing. **Kendall L. Langsten:** Formal analysis, methodology; resources; writing—original draft; writing—review and editing. **Susy Kim:** Investigation, methodology; validation; writing—original draft, writing—review and editing. **Mitu Sharma:** Data curation; methodology; writing—original draft; writing—review and editing. **Yixin Su:** Data curation; investigation; methodology; writing—original draft; writing—review and editing. **Sangeeta Singh:** Data curation; investigation; methodology; writing—original draft, writing—review and editing. **Bethany A. Kerr:** Methodology; resources; supervision; writing—original draft; writing—review and editing. **Gagan Deep:** Conceptualisation; formal analysis; funding acquisition; methodology; project administration; resources; supervision; writing—original draft; writing—review and editing.

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CONFLICTS OF INTEREST STATEMENT

GD is the founder of LiBiCo that has no influence or contribution to the work presented in this manuscript.

DATA AVAILABILITY STATEMENT

All data, analytic methods and study materials are included in the manuscript. Further details or any material needed could be obtained from the corresponding authors without any restrictions.

ORCID

Gagan Deep  <https://orcid.org/0000-0003-4445-8796>

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