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Bacterial outer membrane vesicles from dextran sulfate sodium-induced colitis differentially regulate intestinal UDP-glucuronosyltransferase 1A1 partially through TLR4/MAPK/PI3K pathway

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Running Title:

Bacterial outer membrane vesicles regulate intestinal UGT1A1

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Abbreviations:

UGT1A1, UDP-glucuronosyltransferase 1A1; DMEs, drug-metabolizing enzymes; DSS, dextran sulfate sodium; FMT, fecal microbiota transplantation; NRs, nuclear xenobiotic receptors; PXR, pregnane X receptor; CAR, constitutive and rostane/active receptor; PPARs, peroxisome proliferator activation receptors; OMVs, bacterial outer membrane vesicles; TLR, toll like receptor; IBD, inflammatory bowel disease; UC, ulcerative colitis; DOC, deoxycholate**.**

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Abstract

UDP-glucuronosyltransferase 1A1 (UGT1A1) constitutes an important part of intestinal epithelial barrier and catalyzes glucuronidation of many endogenous compounds and drugs. Down-regulation of UGT1A1 in inflammation has been reported, while the association with gut dysbiosis is poorly defined. This study verified the involvement of gut microbiota in intestinal UGT1A1 regulation using dextran sulfate sodium (DSS)-induced rat colitis model plus fecal microbiota transplantation (FMT). Generally, both DSS induction and colitis-to-normal FMT suppressed mRNA and protein expressions of UGT1A1 and nuclear xenobiotic receptors (NRs) in colon, but enhanced mRNA and decreased protein of rUGT1A1/rNRs in small intestine. Normal-to-colitis FMT alleviated DSS-induced changes. Bacterial outer membrane vesicles (OMVs) from colitis rats and rats receiving colitis feces reduced both mRNA and protein of hUGT1A1/hNRs in Caco-2 cells. Interestingly, using deoxycholate to reduce LPS, normal OMVs up-regulated hUGT1A1/hNRs, while colitis OMVs decreased, indicating the involvement of other OMVs components in UGT1A1 regulation. The 10-50 kD fractions from both normal and colitis OMVs down-regulated hUGT1A1, hPXR and hPPAR-γ, while >50 kD fractions from normal rats up-regulated hUGT1A1 and hCAR. Additionally, the conditioned medium from OMVs-stimulated rat primary macrophages also reduced hUGT1A1/hNRs expression. Both toll like receptor 2 (TLR2) and TLR4 were activated by DSS, colitis-to-normal FMT and the opposite, while only TLR4 was increased in OMVs-treated cells. TLR4 siRNA blocked hUGT1A1/hNRs down-regulation and PI3K/Akt, ERK and NF-κB phosphorylation evoked by bacterial OMVs. Taken together, this study demonstrated that gut microbiota regulate intestinal UGT1A1

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partially through secreting OMVs which interact intestinal epithelial cells directly or *via* activating macrophage.

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Introduction

UDP-glucuronosyltransferase 1A1 (UGT1A1), one of the major intestinal drug-metabolizing enzymes (DMEs), catalyzes glucuronidation of many potentially harmful compounds and drugs. Inhibition of UGT1A1 may bring increased risks of drug-drug interactions and cause bilirubin-related diseases (Strauss et al., 2006). Existing data indicate an involvement of gut microbiota in regulating UGT1A1 or its upstream regulators, the nuclear xenobiotic receptors (NRs) pregnane X receptor (PXR), constitutive androstane/active receptor (CAR) and peroxisome proliferator activation receptors (PPARs). Exposure to lipopolysaccharide (LPS) or Citrobacter rodentium infection down-regulated hepatic UGT1A1 in mice (Richardson et al., 2006). Indole 3-propionic acid produced by commensal *Clostridium sporogenes* promoted PXR mRNA expression in $Nr1i2^{-/-}Tlr4^{-/-}$ mice (Venkatesh et al., 2014). However, the contribution of gut microbiota community as a whole to UGT1A1 regulation in intestinal epithelial cells remains to be addressed.

Ulcerative colitis (UC), a form of inflammatory bowel disease (IBD), is characterized with chronic local inflammatory responses and microbial imbalance. Commensal bacteria or their products are drivers of dysregulated immunity in IBD. Alterations of Gram-negative bacteria dominated gut dysbiosis in UC (Vigsnaes et al., 2012). Gram-negative bacteria interact with the host by producing secretory nano-sized outer membrane vesicles (OMVs) to deliver cohort of soluble and insoluble components into host cells (Ellis and Kuehn, 2010). LPS, one of the most abundant components of OMVs, is considered as the primary initiator for the pathogenic activities of OMVs (Beutler and Rietschel, 2003). LPS has been demonstrated to be involved in regulation of DMEs and transporters (Lu et al., 2008; Morgan

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et al., 2008). OMVs mediated the cytosolic localization of LPS (Vanaja et al., 2016). OMVs also contain other components (proteins, peptidoglycan, DNA, RNA, *etc*) that can elicit inflammatory responses (Kaparakis-Liaskos and Ferrero, 2015). *Neisseria meningitides* OMVs showed more potent proinflammatory properties than LPS in human whole blood (Mirlashari et al., 2001). Thus, OMVs is more suitable than LPS as the materials to investigate Gram-negative bacteria-host interactions.

Diminished drug metabolic capability is associated with inflammation (Christensen and Hermann, 2012). Recent advances indicate a key role for innate immunity in colonic inflammation (Marks and Segal, 2008). A large population of macrophages inhabit in intestine and steer innate immune response. Contacting with invading microorganisms lead to polarization of macrophages to M1 type (classically activated) or M2 type (alternatively activated), causing intestinal tissue damage or maintain intestinal homeostasis by secreting pro- and anti- inflammatory cytokines, respectively (Nakata et al., 2013). A significant increase of M2 macrophages has been observed in UC patients (Cosin-Roger et al., 2013). In colitis animals, DSS drives the macrophage phenotype towards the M2 lineage (Kono et al., 2016). Macrophage colony-stimulating factor–deficient (op/op) mice, which are not able to develop mature macrophages, show decreased susceptibility to DSS-induced colitis (Ghia et al., 2008). Moreover, OMVs from *N. meningitidis* could promote macrophage polarization (Tavano et al., 2009). Macrophages sensing both LPS and protein components of *Pseudomonas aeruginosa* OMVs contribute to bacterial strain-specific inflammatory responses (Ellis et al., 2010). To our best knowledge, there is no report linking macrophage polarization with intestinal UGT1A1 regulation.

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Toll-like receptors (TLRs) are key participants in innate immune responses. Among 11 human TLRs, TLR2 and TLR4 could recognize structurally diverse molecules from microbe. Bacterial OMVs could elicit biological effects and inflammatory responses through activating TLR2 and/or TLR4. *P. aeruginosa* OMVs induced lung inflammation partly through activating both TLR2 and TLR4 (Park et al., 2013). *Escherichia coli* OMVs up-regulated cell adhesion molecules in human microvascular endothelial cells *via* NF-κB and TLR4-dependent pathways (Kim et al., 2013).

Fecal microbiota transplantation (FMT) is arising as a promising therapeutic strategy for some gut dysbiosis related diseases through transplanting healthy fecal bacteria into the gut lumen of a patient. It has been demonstrated successful in colitis and *Clostridium difficile* infection (CDI) (Borody et al., 2013; van Nood et al., 2013). Transplantation of fecal microbiota, specific bacterial strains or combinations is widely adopted in basic research to assess their roles in disease etiology, gene regulation, drug interventions (Li et al., 2015).

In this study, the involvement of gut microbiota in intestinal UGT1A1 regulation was first examined by measuring intestinal UGT1A1 and major NRs in dextran sulfate sodium (DSS)-induced experimental colitis rat model and rats receiving FMT. The discriminative alterations of hUGT1A1 and hNRs in Caco-2 cells treated by OMVs (complete, different molecular-weight fractions, LPS-reducing) from normal and colitis rats, or conditioned medium from OMVs-stimulated macrophage were characterized to delineate the contributions of OMVs and the role of macrophage polarization. At last, the involvement of TLR4/MAPK/PI3K was assessed using TLR4 inhibition or TLR4 siRNA transfection in Caco-2 cells.

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Materials and Methods

Reagents

DSS (MW: 36,000-50,000) was purchased from MP Biomedicals (Santa Cruz, CA, USA) and dissolved in deionized water to make a solution of 5% (w/v). Enzyme-linked immunosorbent assay (ELISA) kits for measuring rat TNF-α, IL-1β, IL-6, IL-4, IL-10, IFN-γ, TGF-β1 and MCP-1 were purchased from Excell Biological Co. Ltd (Shanghai, China). The bicinchoninic acid (BCA) assay kit, Lipofectamine 2000 reagent, Reverse transcription (RT) kit, Pierce limulus amebocyte lysate (LAL) chromogenic endotoxin quantitation kit, Trizol and sodium deoxycholate detergent were supplied by Thermo Fisher Scientific Inc (Waltham, MA, USA). SYBR Premix Ex Taq (Perfect Real-time) PCR kit and primers were obtained from TaKaRa (Guangzhou, China). TLR4 siRNA was purchased from GenePharma Co. Ltd (Shanghai, China). Anti-phospho-Akt (Ser473) antibody (#9271), Phospho-NF-κB p65 (Ser536) antibody (#3031), HRP conjugated anti-rabbit and anti-mouse IgG antibodies were supplied by Cell Signaling Technology, Inc (Shanghai, China). Enhanced chemiluminescence (ECL) Plus Western blotting detection reagent was purchased from Beyotime Institute of Biotechnology (Nanjing, China). Antibodies for PXR (ab192579), PPAR-γ (ab209350), CAR (ab62590), UGT1A1 (ab194697), TLR2 (ab108998), TLR4 (ab22048) were purchased from Abcam, Inc (Abcam, Cambridge, UK) and those for p-ERK 1/2 (sc-136521) and p-PI3k (Tyr 467) (sc-293115) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) and ultrafiltration membranes were purchased from Merck Millipore (Billerica, MA, USA). TAK-242 (resatorvid) was purchased from ApexBio

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Technology (Houston, USA). DMEM, fetal bovine serum (FBS), phosphate buffered solution (PBS) and non-essential amino acids (NEAA) were products of Gibco (Waltham, MA, USA).

Animals

Male Sprague-Dawley rats (250-300 g, 8 weeks) were provided by the Experimental Animal Facility of University of Macau (Macao, China) and housed in a temperature (24 °C) and humidity (45~55%) controlled room with a 12 h light/dark cycle in a specific-pathogen-free (SPF)-class laboratory. Rats were placed in a conditioning chamber and allowed to acclimate to the new environment for 4 days prior to experiments with access to standard chew and reverse osmosis (RO) water ad libitum. The chew was comprised of corn, fish meal, wheat flour, salt, vitamins, trace elements, amino acids, etc. The care and treatment of the rats followed a protocol (No.: UMAEC-2015-09) approved by the Animal Ethics Committee, University of Macau.

In vivo protocol

Rats were divided randomly into 5 groups (6 animals each). Rats in UC group received 5% DSS in drinking water for consecutive 7 days (day 0 – day 7). Rats receiving drinking water alone served as controls (Normal group). The fecal samples from Normal and UC groups were freshly collected daily at 10:00 am and portions were pooled at equal amount within group. One gram of pooled fecal samples were suspended in 10 ml of sterile 0.9% normal saline by vortexing, and then fecal suspension were orally administered to each rat of NN (normal rats received Normal feces) and NU (normal rats received UC feces) groups,

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respectively, at 1 g/kg by gavage daily for 7 days. Rats in UN group received 5% DSS in drinking water as well as oral administration of 1g/kg fecal suspension from Normal group by gavage for 7 days. Throughout the experimental period, rats were fed standard chow and bottles were refilled daily with fresh DSS solution (UC and UN groups) or RO water (Normal, NN and NU groups). The body weight and stool consistency of each rat from all groups were recorded on daily basis. Blood samples (200 μL each) were collected from orbital sinus on day 0, 3, 5 and 7. On the last day of experiment (day 7), rats were sacrificed by cervical dislocation. Small intestines and colons were collected, flushed with ice-cold phosphate-buffered saline to move food particles and then cut longitudinally into several segments. Intestinal and colonic mucosa was scraped from the smooth muscle using a glass microscope slide.

Assessment of clinical signs of colitis

Disease activity index (DAI), histological evaluation, cytokine determination and myeloperoxidase (MPO) assay were carried out as described previously (Huang et al., 2015) with minor modifications. Each rat was given a DAI score for weight loss, stool consistency and bloody stool. Colon segment (0.5 cm) from the distal end of the colon was removed and 4 μm sections were obtained, stained with hematoxylin-eosin to assess epithelial damage, architectural derangements, goblet cell depletion, edema/ulceration and inflammatory cell infiltrate using Olympus CX21 microscope and an Olympus SC100 camera. Serum levels of IL-1β, IL-4, IL-6, IL-10 and TNF-α were assayed using ELISA kits according to manufacturer's instruction. For MPO assay, colonic mucosa was homogenized in 50 mM

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phosphate buffer (pH 6.0) containing 0.5% HTAB. The supernatant (5 µL) was added to 20 µL of 2.5mM o-dianisidine hydrochloride and 75 µL of 0.00065% H_2O_2 and kept for 5 min to record absorbance at 470nm on Spectra Max M5 Multi-Mode Microplate Readers. Protein concentration was determined by BCA assay. One unit of MPO activity was defined as the amount of enzyme degrading 1 nM H_2O_2 per min at 25°C.

Preparation of complete OMVs, fractions and low-LPS OMVs

Fecal samples freshly collected on day 7 were pooled within group and homogenized (10%, w/v) in 0.1 M PBS followed by centrifugation at 200 × g for 10 min. The supernatant was collected and centrifuged at 4500 × g for 30 min. Microbial OMVs were prepared as described previously (Eddy et al., 2014). The resultant precipitate was resuspended with Luria-Bertani (LB) broth to obtain gut microbiota suspension at 2 mg/ml and incubated at 37°C for 24 h. Bacterial cultures were pelleted at 6,000 × g for 20 min. The supernatant was filtered through a 0.45 µm vacuum filter and the filtrate was further filtered through a 0.22 µm vacuum filter to remove any remaining cells and then ultracentrifuged at 200,000 × g for 4 h at 4°C. The pellets (OMVs) were suspended in PBS for experiments. The protein content was determined by BCA assay.

OMVs preparations (50 µg/ml, 10 ml) were further fractionated by ultrafiltration with 3-, 10-, 30-, 50- kD ultrafiltration membranes according to the manufacturer's protocol to obtain fractions as follows: <3 kD (F<3), 3-10 kD (F3-10), 10-30 kD (F10-30), 30-50 kD (F30-50), >50 kD (F>50). Each fraction obtained was made up to 10 ml to maintain the same proportion in the OMVs.

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Low-LPS OMVs were prepared according to previous reports (Claassen et al., 1996; Zariri et al., 2016) with minor modifications. Briefly, the bacterial cultures were pelleted as described above and resuspended in 0.1 M Tris-10mM EDTA buffer. After incubation for 5 min at 4°C, deoxycholate (DOC, 100 g/L) was added to reduce LPS from OMVs (DOC : Tris-EDTA, 1:20,v/v) and then ultracentrifuged at 200,000 × g for 4 h at 4°C. The pellet was resuspended in 0.1 M Tris-10mM EDTA buffer containing 5 g/L DOC and then filtered through a 0.22 μm vacuum filter. The supernatant was then ultracentrifuged at 200,000 × g for 4 h at 4°C. The pellets (low-LPS OMVs) obtained were suspended in LPS-free PBS. The low-LPS OMVs were diluted and the amounts of LPS present in the samples were determined from the developed color intensity using a standard curve constructed with *E. coli* (011: B4)-derived LPS (concentration 0-1 EU/mL) using LAL chromogenic endotoxin quantitation kit according to manufacturer's protocol. The endotoxin-free water in the kit served as control. One Endotoxin Units of LPS per milliliter (EU/mL) equals 0.1 ng endotoxin/mL of solution.

Preparation of macrophages-derived conditioned medium

Primary macrophages were prepared according to a previous report (Liu et al., 2011) with minor modifications. In brief, untreated male SD rats (250-300 g, n=3) were sacrificed by cervical dislocation and 15 ml of PBS was injected intraperitoneally. After abdominal massage for 1 min, PBS containing peritoneal macrophages were collected, centrifuged at 500 × g for 5 min, and pellets were washed with PBS, and then cultured in 6-well plates (2×10^6/well) for 3 h. After washed twice with PBS, macrophages were cultured in serum-free DMEM, and stimulated with 50 μg/ml OMVs or OMV fractions from Normal or UC groups

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for 24 h. Cells were washed twice with PBS to remove OMVs or fractions and cultured in fresh serum-free DMEM for another 6h. The culture medium was centrifuged and the supernatant was collected as conditioned medium (CM) for experiments. Levels of TNF-α, IL-1β, IL-6, IL-4, IL-10, IFN-γ, TGF-β1 and MCP-1 in CM were assayed using ELISA kits according to manufacturer's instruction.

Caco-2 cell culture and treatments

Caco-2 cells at passage 19 were a gift from Dr. Jianqing Ruan at Department of Pharmaceutical Analysis of Soochow University (Suzhou, China) and cultured in DMEM supplemented with 10% FBS, 1% NEAA and streptomycin (100 U/ml) and penicillin (100 U/ml) under an atmosphere of 5% CO_2 and 95% humidified air at 37°C in 6-well plates. The medium was changed every 3 days until the cells were grown to confluence. Caco-2 cells were then challenged with CM for 24 h, or incubated in absence or presence of TAK-242 (1 μM) alone for 30 min followed by 50 μg/ml OMVs, different fractions, or Low-LPS OMVs for another 24 h. At the end of the experiments, cells were harvested for PCR or western blot analysis. Each assay was repeated at least 3 times.

Transfection of TLR4 siRNA into Caco-2 cells

Caco-2 cells were grown to 40 % confluence and transfected with TLR4 siRNA (5 nM) for 48 h using Lipofectamine 2000 reagent following the manufacturer's instructions. The positive control (PC) siRNA to human GAPDH was measured to monitor siRNA transfection efficiency by real-time RT-PCR. The negative siRNA control (NC) comprising of a 21-bp

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non-targeting sequence functions to distinguish sequence-specific silencing from non-specific effects. After transfection, Caco-2 cells were treated with 50 μg/ml OMVs or low-LPS OMVs from normal and colitis rats for 24 h, and cells were collected for western blot or PCR assay in triplicates. TLR4, GAPDH and NC siRNA sequences were as follows: TLR4-770, forward primer: 5'-GCUCACAAUCUUAUCCAAUTT-3', reverse primer: 5'-AUUGGAU AAGAUUGUGAGCTT-3'; TLR4-931, forward primer: 5'-CCUGAACCCUAUGAACUU UTT-3', reverse primer: 5'-AAAGUUCAUAGGGUUCAGGTT-3'; TLR4-1240, forward primer: 5'-CCUGGUGAGUGUGACUAUUTT-3', reverse primer: 5'-AAUAGUCACACU CACCAGGTT-3'; NC siRNA: forward primer: 5'-UUCUCCGAACGUGUCACGUTT-3', reverse primer: 5'-ACGUGACACGUUCGGAGAATT-3'; GAPDH siRNA: forward primer: 5'-UGACCUCAACUACAUGGUUTT-3', reverse primer: 5'-AACCAUGUAGUU GAGGUCATT-3'.

Western-Blot analysis

Small intestinal or colonic mucosa or Caco-2 cells were washed twice with ice-cold PBS and then lysed in Western-Blot lysis buffer (50 mM Tris–HCl, pH7.2, containing 1% sodium deoxycholate, 0.1% SDS, 0.15 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate) at 4°C for 30 min. The supernatant was then obtained by centrifugation at 12,000 rpm for 20 min at 4°C and the protein content was determined using BCA kits. The proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes by semi-dry electrophoretic transfer. The PVDF membranes were then blocked with 5% skim milk in TBST buffer (5 mM Tris–HCl, pH 7.6, 136 mM NaCl, 0.05% Tween-20) overnight at 4°C followed by incubating with

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primary antibody (1: 1000 dilution in TBST) at 4°C overnight. The PVDF membrane was washed three times with TBST buffer and incubated with the secondary antibody HRP-labeled anti-rabbit or anti-mouse IgG (1:2000 dilution in TBST) at room temperature for 1 h. The signals were detected by using ECL detection reagent and semi-quantified by densitometry with Image-Pro Plus software.

RT-QPCR assay

Small intestinal and colonic mucosa, Caco-2 cells or macrophages were homogenized and mRNA were extracted using TRIzol Plus. mRNA concentration was calculated from QuantiFluor™ RNA System. To generate complementary DNA (cDNA) from mRNA template, 500 ng total mRNA were dissolved in 20 μl reaction system (1 μl of $Oligo(dt)_{18}$, 4 μl of 5 × reaction buffer, 1 μl of RNase inhibitor, 2 μl of dNTP (10 mM) and 1 μl of reverse transcriptase). The mixture were degenerated at 42°C for 60 min and annealed at 70°C for 5 min. An aliquot of cDNA (4 μl of RT product) was dissolved in 50μl PCR reaction mixture (26μl of 1×SYBR Green Master Mix, 1μl of each primer (final concentration 0.2 μmol/L), 18μl of sterile water). The target gene primer sequences were provided in Table S1 and S2. The amplification profile consisted of an initial denaturation at 95°C for 30 s, 60 cycles of 95°C for 5 s and then 60°C for 34 s. The fluorescence data was collected by ViiA7 QPCR instrument at the end of the elongation step per each cycle. The PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method to determine the fold changes of relative abundance to internal control gene β-actin.

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Statistical analysis

All data were expressed as the mean ± standard deviation (S.D). Significance of the differences between groups was determined by one-way ANOVA with a Scheffe post hoc test using GraphPad Prism software. Differences were considered statistically significant when $p < 0.05$.

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Results

Effect of DSS and fecal microbiota transplantation on rat colitis

7-day DSS treatment decreased body weight with DAI increasing from day 1 to 7. DSS induction also caused colonic edema and ulcer, shortening and bleeding, leading to remarkable increase of the colon weight to length ratio in colitic rats. In addition, DSS induction significantly increased the activity of colonic MPO, and enhanced the production of both pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory (IL-4 and IL-10) cytokines from day 3 to 7 (Supplemental Figures S1 & S2).

FMT from UC to normal rats (NU) showed increased DAI from day 4 to 7, colonic edema, elevated MPO activity, and increased pro- and anti- inflammatory cytokine levels from day 3 to 7, although to much less extents than DSS induction. While the opposite (UN) significantly suppressed the increase of DAI from day 4 to 7 and colonic edema, shortening and bleeding, decreased MPO activity, and alleviated the production of both pro- and anti-inflammatory cytokines in NU group (Supplemental Figures S1 & S2). FMT from normal to normal rats (NN) showed insignificant changes on the above colitis measurements.

Overall, the tissue damage tended to the terminal colon and could be classified as mild to aggravated colitis (Supplemental Figure S3). DSS induction caused the loss of intestinal crypts with goblet cells, tissue damage on the epithelial layer and increasing of leukocyte infiltration. The NU rats showed similar colonic damage including increase of leukocyte infiltration and loss of intestinal crypts. FMT from normal to UC rats (UN) suppressed the colonic damage induced by DSS. Normal-to-normal FMT showed no colonic damage. The results indicated that DSS-induced gut dysbiosis could elicit colitis-like symptoms which

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could be abrogated by transplantation of normal fecal microbiota.

mRNA and protein expressions of UGT1A1 and NRs along small intestine and colon of rats

As shown in Figure 1A, 7-day DSS stimulation significantly increased mRNA expression of rUGT1A1 in small intestine of UC group. Correspondingly, DSS induction resulted in the up-regulation of rPXR, rCAR and rPPAR-γ in small intestine, while significant decreases of rPXR and rCAR in ileum were observed. Unlike the small intestine, colonic mRNA expression of UGT1A1 and NRs were all decreased in colitis rats.

Small intestinal and colonic mRNA expression of UGT1A1 and NRs were unaltered by transplantation of normal feces to normal rats (NN group) (Figure 1A). Colitis to normal FMT (NU group) unaltered small intestinal UGT1A1 expression, however, it caused more severe damage in colon, leading to diminished mRNA expression of rUGT1A1. While the opposite (UN group) could partially diminish DSS-induced changes (upregulation in small intestine and down-regulation in colon), although at less extents in most cases. Similarly, FMT from colitis rats to normal rats did not affect small intestinal NRs, except for an elevation of rPXR in duodenum, while the NRs expression in colon was reduced to an extent less than DSS insult. Oral administration of normal feces to colitis rats (UN group) could abrogate the changes of rPXR and rCAR in jejunum and ileum induced by DSS, but unaffect those in duodenum and colon. Neither did the rPPAR-γ expression in both small intestine and colon. The results indicated that gut dysbiosis induced by DSS alter intestinal UGT1A1 and NRs expression at mRNA levels with general elevation in small intestine and reduction in colon.

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When the protein expressions of UGT1A1 and NRs were measured, in contrast to the elevation at mRNA levels, DSS induction resulted in the down-regulation of rUGT1A1 proteins in duodenum and colon (Figure 1B). The down-regulation of rUGT1A1 and rNRs was also observed in the whole small intestine (data not shown). The protein expressions of rPXR, rCAR and rPPAR-γ were changed by DSS in the same direction. rUGT1A1 and rNRs proteins were unaltered in NN group. In contrast to the unaltered mRNA expression, significant reduction of the protein expressions of rUGT1A1 and rNRs in duodenum and colon were observed in NU group. Transplantation of normal feces to colitis rats (UN group) partly reversed DSS-induced rUGT1A1 and rNRs down-regulation. The results indicated that DSS-induced gut dysbiosis can lead to the down-regulation of intestinal UGT1A1 and NRs at protein levels which can be abolished by normal to colitis FMT.

mRNA and protein expressions of UGT1A1 and NRs in Caco-2 cells stimulated by microbial OMVs and DOC-treated OMVs

When the microbial OMVs obtained from fecal samples of each group were incubated with Caco-2 cells, both mRNA and protein expressions of hUGT1A1 were significantly decreased regardless of the origins of OMVs (Figure 2A, 2B). OMVs from colitis rats and normal rats receiving FMT from colitis rats reduced the expressions of hUGT1A1 in Caco-2 cells more significantly than those from their normal counterparts. FMT from normal to colitis rats diminished the effect of colitis OMVs on hUGT1A1 expression in Caco-2 cells. The expression of hPXR, hCAR and hPPAR-γ showed similar changes in Caco-2 cells, all down-regulated by OMVs stimulation. The inhibitory potency of OMVs on expressions of

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hUGT1A1 and hNRs is in the same descending order: UC > UN > NU > NN ≈ Normal.

As shown in Supplemental Figure S4, both Normal and colitis OMVs contain LPS with the LPS level in colitis OMVs significantly higher than that in normal OMVs. DOC treatment successfully reduced LPS from the OMVs preparations of both groups to control level (Control, 0.662 EU/ml; DOC-treated N_{OMVs}, 0.571 EU/ml; DOC-treated $Colitis_{OMVs}$, 0.497 EU/ml).

When DOC-treated OMVs preparations were incubated with Caco-2 cells, protein expressions of hUGT1A1 and hNRs were altered in opposite directions (Figure 3A): up-regulated by that of Normal, while down-regulated by that of colitis which were less potent than respective complete OMVs. The results indicate that the components other than LPS contribute significantly to the regulatory effects of OMVs on intestinal UGT1A1 and NRs expression.

mRNA expressions of UGT1A1 and NRs in Caco-2 cells treated with CM of OMVs-stimulated macrophages

As shown in Figure 4A, OMVs pretreatment evoked inflammatory responses in macrophages which resulted in over-production of both M1- (TNF-α, IL-1β, IL-6, MCP-1, IFN-γ) and M2-type (IL-4, IL-10, TGF-β1) cytokines and chemokines in CM, indicating activation of macrophages by bacterial OMVs. The effects of colitis OMVs were more potent than the normal OMVs.

Macrophages could steer intestinal immune responses through releasing cytokines and chemokines. In order to determine whether bacterial OMVs-stimulated macrophages

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contribute to regulation of UGTs/NRs in IECs, Caco-2 cells were treated with CM of macrophages stimulated with OMVs. Similar to the direct effects of OMVs, the mRNA expression of hUGT1A1 in Caco-2 cells was significantly suppressed accompanying with down-regulation of the hNRs (Figure 4B). The CM obtained from colitis OMVs treated macrophages was more potent than that treated by the Normal OMVs. The results indicated that OMVs could down-regulate UGT1A1 and NRs through evoking inflammation in IECs *via* promoting macrophage polarization.

Effect of different OMVs fractions on mRNA expression of UGT1A1 and NRs in Caco-2 cells

Bacterial OMVs were further fractionated into five fractions with different molecular weight ranges (<3 kD (F<3), 3~10 kD (F3-10), 10~30 kD (F10-30), 30~50 kD (F30~50), >50 kD (F>50)) which showed big differences in the protein contents between Normal and colitis groups (higher in <10kD fractions and lower in F10-30 and F>50 fractions of colitis group than the Normal group, Supplemental Figure S5), indicating that DSS induction caused marked changes of bacterial compositions and/or cellular components. The F<3 fractions of both Normal and colitis groups showed no effect on hUGT1A1/hNRs mRNA expressions (Figure 5A). F10-30 fractions significantly suppressed hUGT1A1 expression, colitis group showing more potent effects. The F30-50 fractions of both groups reduced hUGT1A1 expression to similar extents. Interestingly, a significant increase of hUGT1A1 was observed with the F>50 fraction of Normal group, while the fraction of colitis group decreased it significantly. Similarly, the F3-10 fraction of Normal group did not affect the mRNA

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expression of all three hNRs, while that of the colitis group significantly suppressed hPXR and hCAR expression (Figure 5A). The F10-30 and F30-50 fractions of both groups significantly down-regulated hPXR and hPPAR-γ with those of colitis group showing more potent effects. It's interesting to note that the F>50 fraction of Normal OMVs unaltered hPXR, enhanced hCAR, while suppressed hPPAR-γ expression. In contrast, that of colitis OMVs down-regulated all three NRs.

Those CM treated by >10 kD fractions (F10-30, F30-50 and F>50) of both Normal and colitis OMVs inhibited hUGT1A1 expressions with more potent effects observed with those treated by colitis OMVs (Figure 5B). The effects of CM on hNRs expression showed similar tendency and the suppressing effect generally increased with molecular weights of the OMVs fractions. The highest inhibition was observed with the CM treated by the F30-50 of the colitis OMVs which inhibited the mRNA expression of hPXR to one third of the control cells and half of the cells treated by its normal counterpart.

Expressions of TLR2, TLR4 and NF-κB in small intestine and colon from rats

As shown in Figure 6, DSS induction resulted in the up-regulation of protein levels of TLR2, TLR4 and NF-κB in small intestine and colon. Normal-to-normal FMT (NN group) unaffected the levels of the three proteins. Normal recipients of colitis fecal samples (NU group) showed significantly higher protein expression of these proteins than NN group. The FMT from normal to colitis rats partially suppressed the elevation induced by DSS, which, however, still significantly higher than Normal, NN and NU groups. The results indicated that DSS-induced gut dysbiosis can alter the protein expressions of TLR2, TLR4 and NF-κB in

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small intestine and colon and transplantation of normal fecal microbiota can diminish the changes.

Effects of TLR4 inhibition on mRNA expression of UGT1A1 and NRs in Caco-2 cells treated by OMVs and DOC-treated OMVs

As shown in Supplemental Figure S6, when treated by OMVs from Normal or colitis rats, TLR2 protein expression in Caco-2 cells was unaltered, while TLR4 protein level was elevated with colitis OMVs showing more potent effect. The results indicated that TLR4 might be the main signaling molecule mediating OMVs stimulation.

The presence of TAK-242, a specific inhibitor of TLR4, could partially reverse the down-regulation of hUGT1A1 and NRs by microbial OMVs from both Normal and colitis groups at the tested concentration (1 μM) of the inhibitor (Supplemental Figure S7). When Caco-2 cells were transfected with TLR4 siRNA, the down-regulation of hUGT1A1 and hNRs expression by OMVs from both groups was completely abrogated (Figure 7A). Knocking down TLR4 also abrogated the dysregulation of hUGT1A1 and hNRs expressions by DOC-treated OMVs from both normal and colitis feces (Figure. 3B). These results indicated that both complete OMVs and low-LPS OMVs regulate intestinal UGT1A1 and NRs mainly through TLR4 activation.

Effects of TLR4 inhibition on phosphorylation of PI3K/Akt and ERK1/2 in Caco-2 cells treated by OMVs

Knocking down TLR4 in Caco-2 cells resulted in a decrease of TLR4 protein expression

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as well as reduction of phosphorylation of PI3K/Akt, ERK1/2 and NF-κB (Figure 7B). When Caco-2 cells were stimulated by microbial OMVs, TLR4 protein expression was significantly elevated and so did the phosphorylated PI3K/Akt, ERK1/2 and NF-κB. The colitis OMVs showed more significant effects than the Normal OMVs. The TLR4 activation by microbial OMVs from both groups was successfully blocked by TLR4 siRNA transfection, leading to decreased TLR4 expression and diminished phosphorylation of PI3K/Akt, ERK1/2 and NF-κB. These results indicated that OMVs regulated intestinal UGT1A1 and NRs through activating TLR4 and PI3K/Akt and ERK1/2 phosphorylation which resulted in NF-κB activation.

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Discussion

UGT1A1 serves as an important constitute of intestinal epithelial barrier. Growing evidence links gut dysbiosis with UGT1A1 dysfunction. In this study, we verified the regulatory role of gut microbiota on intestinal UGT1A1 using DSS-induced colitis rat model plus FMT and pinpointed the main molecular events *in vitro* using bacterial OMVs. The main findings include: 1) DSS induced dysregulation of UGT1A1/NRs in rat intestine; 2) Colitis-to-normal FMT caused similar alterations of UGT1A1/NRs, while the opposite alleviated DSS-induced changes; 3) Complete OMVs from both normal and colitis rats down-regulated UGT1A1/NRs expressions in Caco-2 cells directly as well as *via* a macrophage-mediated mechanism; Low-LPS OMVs from normal rats elicited direct opposite effects to that from colitis rats; 4) Knocking down TLR4 blocked UGT1A1/NRs dysregulation evoked by OMVs and low-LPS OMVs.

DSS-induced rat colitis highly resembles human UC and is widely used in basic research and drug discovery. The microbial shifts in the experimental colitis are similar to those in UC patients (Giaffer et al., 1991; Munyaka et al., 2016). In this study, colitis-to-normal FMT caused similar clinical changes and microbial shifts (data not shown) to DSS induction, while FMT in opposite direction partially reversed DSS-induced changes, demonstrating the involvement of gut microbiota in colitis development and therapy.

We found that both UGT1A1 and its upstream transcriptional regulator NRs were changed by DSS-induced colitis, which can be alleviated by normal-to-colitis FMT, demonstrating a regulatory role of gut microbiota in intestinal UGT1A1/NRs. It's interestingly to note that normal-to-normal FMT unaffected UGT1A1 and NRs in all cases

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and colitis-to-normal FMT (NU group) only caused increase of PXR. These findings indicate that healthy gut microbiota are highly resistant to colonization of those microbial "foreigners" to maintain intestinal homeostasis (Lawley and Walker, 2013) and also have greater colonization capability than those from colitis rats.

Surprisingly, the mRNA of intestinal rUGT1A1 and rNRs were generally increased in small intestine and decreased in colon by DSS and normal-to-colitis FMT, while their proteins were all decreased in duodenum, whole small intestine and colon. The discrepancy between mRNA and protein levels of rUGT1A1/rNRs *in vivo* might be due to: 1) the existence of other compensatory mechanism in intestinal UGT1A1/NRs regulation; 2) more complicated post-transcriptional mechanisms involved in translating mRNA into proteins (Greenbaum et al., 2003); 3) the mRNA elevation is the consequence and the earlier event of the regulatory feedback of down-regulated proteins. Additionally, colon is the main colonization site of gut microbiota, explaining the more serious colonic mucosal injury. This may also account for the mRNA level discrepancy between small intestine and colon.

Nuclear xenobiotic receptors are essential regulators of drug-metabolizing enzymes and transporters (Ou et al., 2010). This study showed that intestinal rUGT1A1 mRNA was differentially regulated by gut microbiota through dysregulating the rNRs. The changes of rUGT1A1 mRNA were consistent with that of rPPAR-γ along small intestine, rPXR and rCAR in duodenum, and all three rNRs in colon, suggesting a tissue-specific regulation of UGT1A1 by NRs. UGT1A1 was relatively unaffected in small intestine, while down-regulated in colon in TNBS-treated rats (Zhou et al., 2013). The discrepancy could be due to different mechanisms involved in TNBS- and DSS- induced colitis which were

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believed to resemble human Crohn's disease and UC, respectively (Alex et al., 2009). They also observed decreased mRNA levels of PXR and PPAR-γ in both small intestine and colon, while CAR was unaffected (Zhou et al., 2013). Our study first reported the correlation between intestinal PPAR-γ and UGT1A1 at both mRNA and protein levels, indicating the involvement of PPAR-γ in regulating intestinal UGT1A1.

Gram-negative bacteria dominated microbial alterations of human UC (Vigsnaes et al., 2012; Kaparakis-Liaskos and Ferrero, 2015). We first addressed the role of Gram-negative bacterial OMVs in intestinal UGT1A1 regulation. hUGT1A1 and hNRs in Caco-2 cells were down-regulated at both mRNA and protein levels by all bacterial OMVs preparations, following same descending order of UC> UN > NU > NN ≈ Normal, regardless of their origins. These results agree with the protein level changes *in vivo*. The mRNA level *in vivo-in vitro* discrepancy could be attributed to: 1) other host factors involved in UGT1A1/NRs mRNA regulation; 2) stronger microbial invasion and pathogenic abilities *in vitro* than *in vivo* (Jandik et al., 2008); 3) regulatory feedback on mRNA expression did not occur *in vitro* due to shorter incubation (24h *vs* 7days) and/or simpler biological system (cells vs whole-organism). OMVs from colitis or NU group down-regulated UGT1A1/NRs in Caco-2 cells more potently. This should be attributed to different microbial compositional changes induced by DSS and FMT.

The intestine harbors largest population of macrophages which steer immune responses through releasing cytokines and chemokines (Nakata et al., 2013). In this study, both M1- and M2- type cytokines and chemokines were overproduced by OMVs-stimulated Caco-2 cells and colitis OMVs showed stronger effects, supporting that gut dysbiosis cause macrophage

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polarization and enhance proinflammatory responses in IECs. Incubation of Caco-2 cells with CM from OMVs-treated rat primary macrophages resulted in similar changes of UGT1A1/NRs which were inversely proportional to cytokines/chemokines production. Taken together, the *in vitro* data support that microbial OMVs regulate intestinal UGT1A1/NRs directly and *via* a macrophage-mediated mechanism.

We further tried to locate the major effector molecules of OMVs. In general, those >10 kD fractions from both normal and colitis OMVs significantly decreased hUGT1A1 and hNRs, and in most cases, colitis OMVs were more potent than the normal counterparts. Notably, the >50 kD fractions from normal OMVs up-regulated hUGT1A1 and hCAR comparing to a down-regulation by colitis OMVs. CM from macrophages treated by different OMVs fractions caused similar changes. The differential regulatory effects of normal and colitis OMVs and fractions on hUGT1A1 and hNRs should be a result of microbial compositional shifts and/or metabolic capability alterations induced by DSS. Even though we observed some correlations between the protein contents and the effects, for example, higher protein content of the >50 kD fractions of Normal group correlate with the their up-regulatory effects on hUGT1A1 and hCAR, we could not rule out the involvement of other components in the fraction and the constitute proteins may also vary with sample. However, the chemical complexity of microbial OMVs and the analytical bottleneck for complex biological samples hamper the identification of the molecular effectors.

TLR4 was significantly increased in IECs of UC patients (Cario and Podolsky, 2000). TLR4 pathway disturbance is implicated in UC development. TLR2 deficiency triggers early tight junction disruption which aggravates colonic inflammation (Cario et al., 2007). In this

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study, both TLR2 and TLR4 were up-regulated in small intestine and colon of colitis, NU and UN rats, and activated by total bacterial preparations from Normal and colitis groups (Supplemental Figure 8). However, only TLR4 was significantly activated by OMVs *in vitro*. These findings are in line with a previous report that TLR2 and TLR4 mainly sense Gram-positive and Gram-negative bacterial signals, respectively (Takeuchi et al., 1999). Knocking down TLR4 abrogated hUGT1A1/hNRs down-regulation by OMVs, confirming TLR4 as the main mediator of bacterial OMVs signaling. However, further study is needed to determine the role of TLR2 in intestinal UGT1A1/NRs regulation *in vivo* and whether it accounts for the mRNA level *in vivo-in vitro* differences.

PI3K/Akt and mitogen-activated protein kinase (MAPK) are two major downstream pathways of TLR4 and play critical roles in various cellular processes (Troutman et al., 2012; Peroval et al., 2013). NF-κB is a key mediator of inflammatory responses. TLR4 signaling leads to rapid activation of PI3K and phosphorylation of PI3K downstream targets Akt and ERK1/2, leading to NF-κB activation and proinflammatory cytokines production. MAPK signaling pathway activation also impacts NF-κB activation (Remels et al., 2009). NF-κB activation was shown to inhibit PXR, CAR and PPAR-γ, and vice versa, NF-κB inhibition could enhance these NRs activity (Shah et al., 2007; Necela et al., 2008; Chai et al., 2013). We also observed a negative correlation between NF-κB and the NRs. OMVs stimulated TLR4 resulting in NF-κB activation as well as PI3K/Akt and ERK1/2 phosphorylation in Caco-2 cells, which was abolished by TLR4 siRNA transfection, supporting that OMVs down-regulate intestinal UGT1A1 through decreasing NRs by TLR4 activation *via* MAPK/ERK and PI3K/Akt pathways.

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LPS was considered to be the major contributor to virulence and inflammatory responses of Gram-negative bacteria. To determine whether other components in OMVs contribute to intestinal UGT1A1/NRs regulation, we used DOC to prepare low-LPS OMVs (decreased from hundreds to <1 EU/mL). In contrast to the decreases of hUGT1A1/hNRs by complete OMVs, the low-LPS OMVs preparations of normal rats up-regulated hUGT1A1/NRs through TLR4. This might account for the up-regulation of hUGT1A1/hCAR by the normal F>50 fraction. The low-LPS colitis OMVs decreased hUGT1A1/hNRs expression, although less potent than respective complete OMVs. These data support the involvement of other OMVs components in intestinal UGT1A1/NRs regulation, demonstrating that OMVs is more suitable than LPS as the study materials for investigating host-bacteria interactions.

In conclusions, this study has demonstrated a regulatory role of gut microbiota on intestinal UGT1A1 and NRs. Gram-negative bacterial OMVs exhibited general down-regulation through directly interacting with host IECs *via* TLR4 activation and inducing macrophage polarization, offering new insights into intestinal UGT1A1 dysfunction in gut dysbiosis-related diseases.

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Authorship Contributions

Participated in research design: Gao and Yan*

Conducted experiments: Gao, Li, Wei, Yan, Hu, Huang, Han, Wei and Yang

Performed data analysis: Gao, Li and Yan*

Wrote or contributed to the writing of the manuscript: Gao and Yan*

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Footnotes

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Figure legends

Figure 1. mRNA and protein expressions of rUGT1A1 and rNRs along small intestine and colon of rats. Animals received water (Nor), 5% DSS (Colitis), feces from normal (NN) or colitis rats (NU), 5% DSS plus feces from normal rats (UN), respectively, for 7 days. Rats were sacrificed on the last day of experiment (day 7). Small intestines and colons were collected. (A) mRNA expressions of rUGT1A1 and rNRs in different small intestinal segments and colon; (B) Protein expressions of rUGT1A1 and rNRs in the duodenum and colon. mRNA expression was measured by real-time PCR. Protein expression was measured by Western-Blot. Data in bar charts were mean ± S.D. of 6 animals of each group, while representative Western-Blot result was presented. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. $^{*}p < 0.05$ *vs* Nor; $^{\#}p < 0.05$ between specific two groups compared. DSS: dextran sulfate sodium; rUGT1A1: rat UDP-glucuronosyltransferases 1A1; rNRs: rat nuclear xenobiotic receptors.

Figure 2. mRNA and protein expressions of hUGT1A1 and hNRs in Caco-2 cells stimulated by microbial OMVs. Cells were treated with or without 50 μg/ml OMVs from normal, colitis, NN, NU or UN rat feces for 24 h. (A) mRNA expression was measured by real-time PCR; (B) Protein expression was measured by Western-Blot. Data in bar charts were mean ± S.D. of triplicate determinations, while representative Western-Blot result was presented. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. $^{*}p < 0.05$ *vs* control; $^{\#}p < 0.05$ between specific two groups compared. OMVs:

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outer membrane vesicles; hUGT1A1: human UDP-glucuronosyltransferase 1A1; hNRs: human nuclear xenobiotic receptors.

Figure 3. Protein expressions of hUGT1A1 and hNRs in Caco-2 cells treated by DOC-treated OMVs. (A) Cells were treated with 50 μg/ml DOC-treated OMVs or (b) transfected with TLR4 siRNA followed by treatment of 50 μg/ml DOC-treated OMVs from normal and colitis feces for 24 h. Protein expressions were determined by Western-Blot. Data in bar charts were mean ± S.D. of triplicate determinations, while representative Western-Blot result was presented. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. $^{*}p < 0.05$ *vs* control; $^{\#}p < 0.05$ between specific two groups compared. OMVs: outer membrane vesicles; hUGT1A1: human UDP-glucuronosyltransferase 1A1; hNRs: human nuclear xenobiotic receptors; TLR: toll-like receptor; LPS: lipopolysaccharides; DOC: deoxycholate.

Figure 4. mRNA expressions of hUGT1A1 and hNRs in Caco-2 cells treated with conditioned medium of macrophages. Primary macrophages were treated with 50 μg/ml OMVs from Normal and colitis groups for 24 h. After incubation, macrophages were washed twice with PBS to remove OMVs and cultured in fresh serum free DMEM for another 6 h. The culture medium served as conditioned medium (CM) and collected for ELISA assay. Caco-2 cells were stimulated with CM which induced by OMVs in macrophages for 24 h. (A) M1-type and M2-type cytokines and chemokines produced in CM; (B) mRNA expressions of hUGT1A1 and hNRs in Caco-2 cells treated by CM. mRNA was measured by real-time PCR.

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Data were expressed as mean ± S.D. of triplicate determinations. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. *$p < 0.05$ *vs* control; $^{\#}p < 0.05$ between specific groups compared. OMVs: outer membrane vesicles; hUGT1A1: human UDP-glucuronosyltransferase 1A1; hNRs: human nuclear xenobiotic receptors; CM: conditioned medium.

Figure 5. mRNA expressions of hUGT1A1 and hNRs in Caco-2 cells treated by different OMVs fractions. 50 μg/ml OMVs preparations (10 ml) were further processed by ultrafiltration with 3-, 10-, 30-, 50- kD ultrafiltration membranes to obtain different fractions as follows: <3 kD (F<3), 3-10 kD (F3-10), 10-30 kD (F10-30), 30-50 kD (F30-50), >50 kD (F>50). Each fraction obtained was made up to 10 ml to maintain the same proportion in the OMVs. Cells were treated with different fractions from normal and colitis OMVs (A) or stimulated with CM of macrophages treated by different fractions of OMVs (B) for 24 h. mRNA expression was measured by real-time PCR. Data were expressed as mean ± S.D. of triplicate determinations. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. $^{*}p < 0.05$ *vs* control; $^{\#}p < 0.05$ between specific two groups compared. OMVs: outer membrane vesicles; hUGT1A1: human UDP-glucuronosyltransferase 1A1; hNRs: human nuclear xenobiotic receptors; CM: conditioned medium.

Figure 6. Protein expressions of TLR2, TLR4 and NF-κB in small intestine and colon of rats. Animals received water (Nor), 5% DSS (Colitis), feces from normal (NN) or colitis rats (NU), 5% DSS plus feces from normal rats (UN), respectively, for 7 days. Rats were

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sacrificed on the last day of experiment (day 7). Small intestines and colons were collected. Protein expression was measured by Western-Blot. Data in bar charts were mean ± S.D. of 6 animals of each group, while representative Western-Blot result was presented. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. $^{*}p < 0.05$ *vs* Nor; $^{\#}p < 0.05$ between specific two groups compared. DSS: dextran sulfate sodium; TLR: toll-like receptor; NF-κB: nuclear factor-κB.

Figure 7. mRNA expressions of hUGT1A1 and hNRs and protein expressions of phosphorylated PI3K/Akt, ERK1/2 and NF-κB in Caco-2 cells treated by TLR4 siRNA. Cells were transfected with TLR4 siRNA followed by treatment of 50 μg/ml OMVs for 24 h. (A) mRNA expressions of hUGT1A1 and hNRs were determined by real-time PCR; (B) Protein expressions of phosphorylated PI3K/Akt, ERK1/2 and NF-κB were determined by Western-Blot. Data in bar charts were mean ± S.D. of triplicate determinations, while representative Western-Blot result was presented. Significance of differences was determined using one-way ANOVA with a Scheffe post hoc test. $^{*}p < 0.05$ *vs* control; $^{\#}p < 0.05$ between specific two groups compared. OMVs: outer membrane vesicles; hUGT1A1: human UDP-glucuronosyltransferase 1A1; hNRs: human nuclear xenobiotic receptors; TLR: toll-like receptor.

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Figures

Figure 1

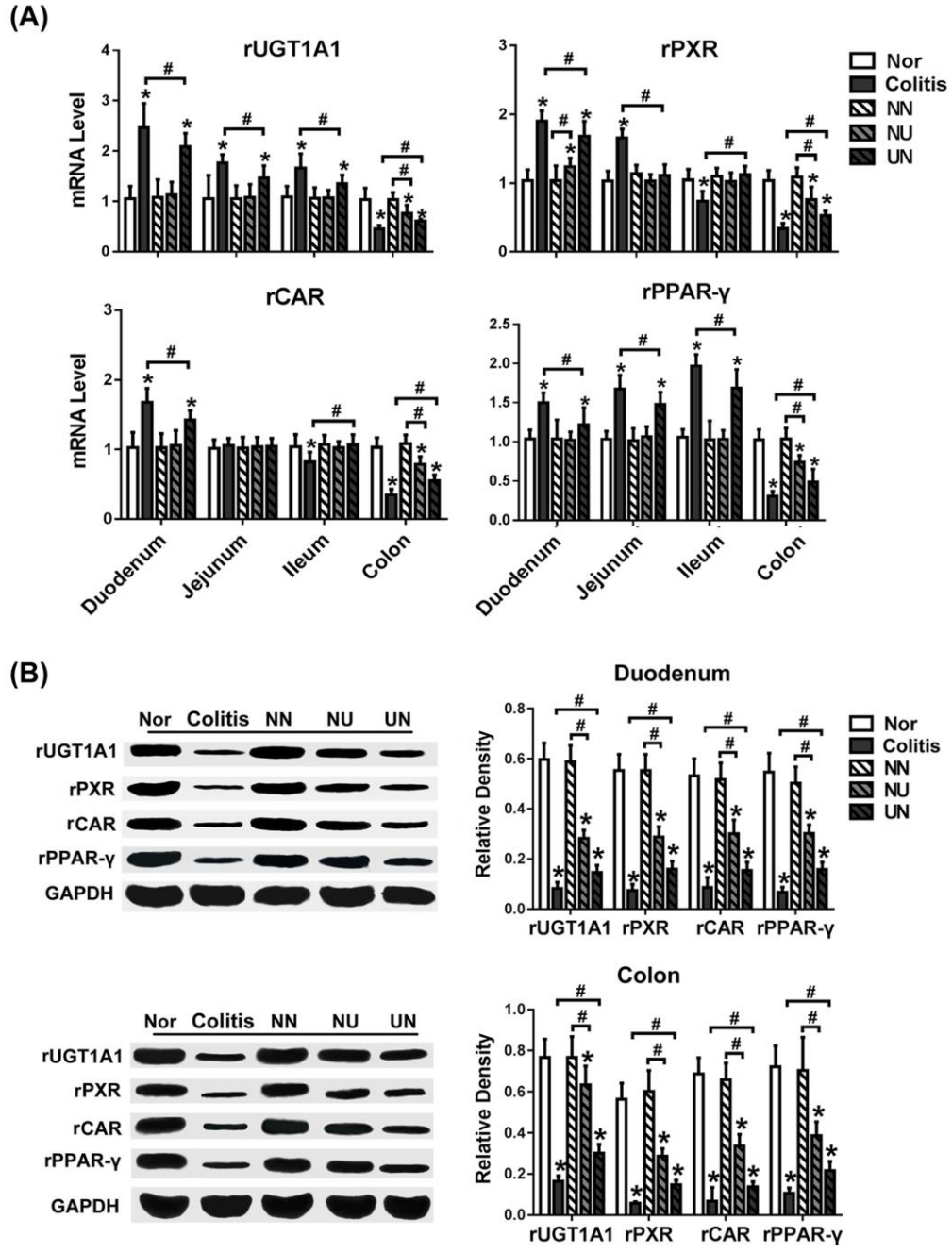

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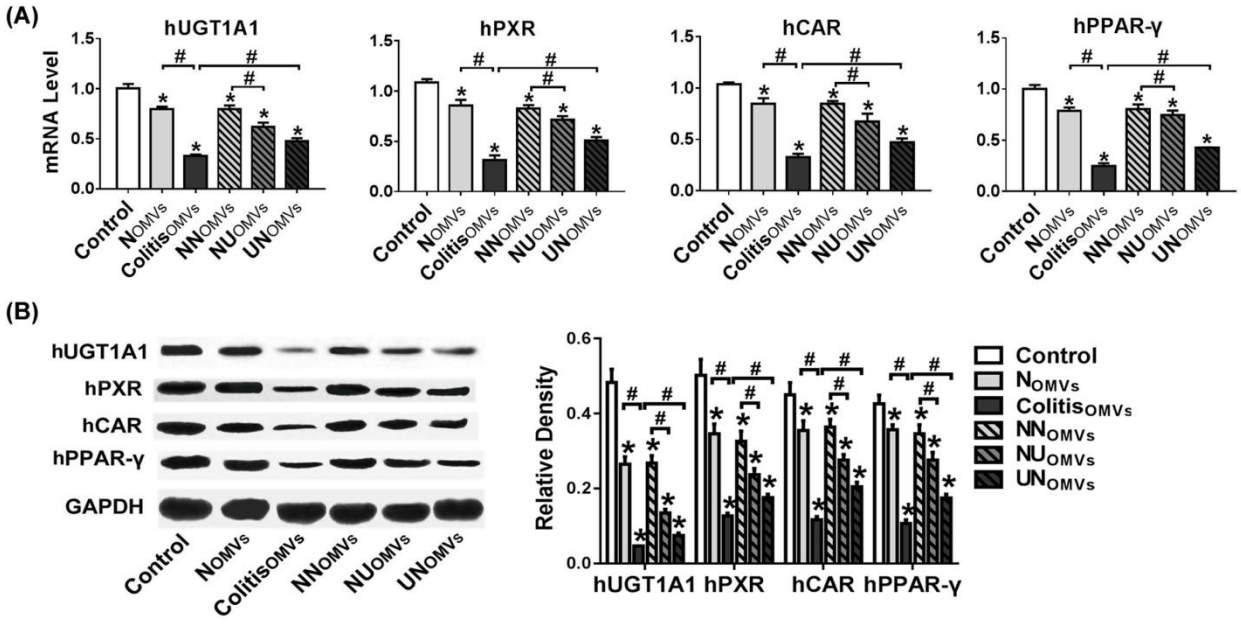
Figure 2



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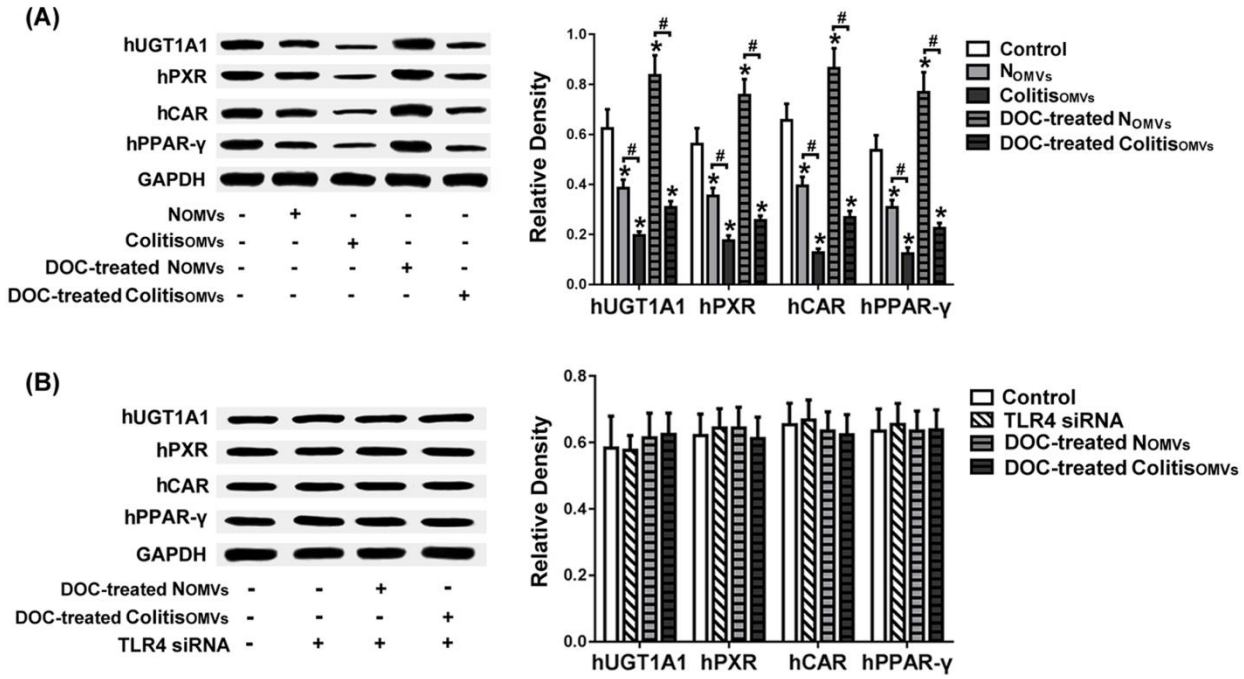

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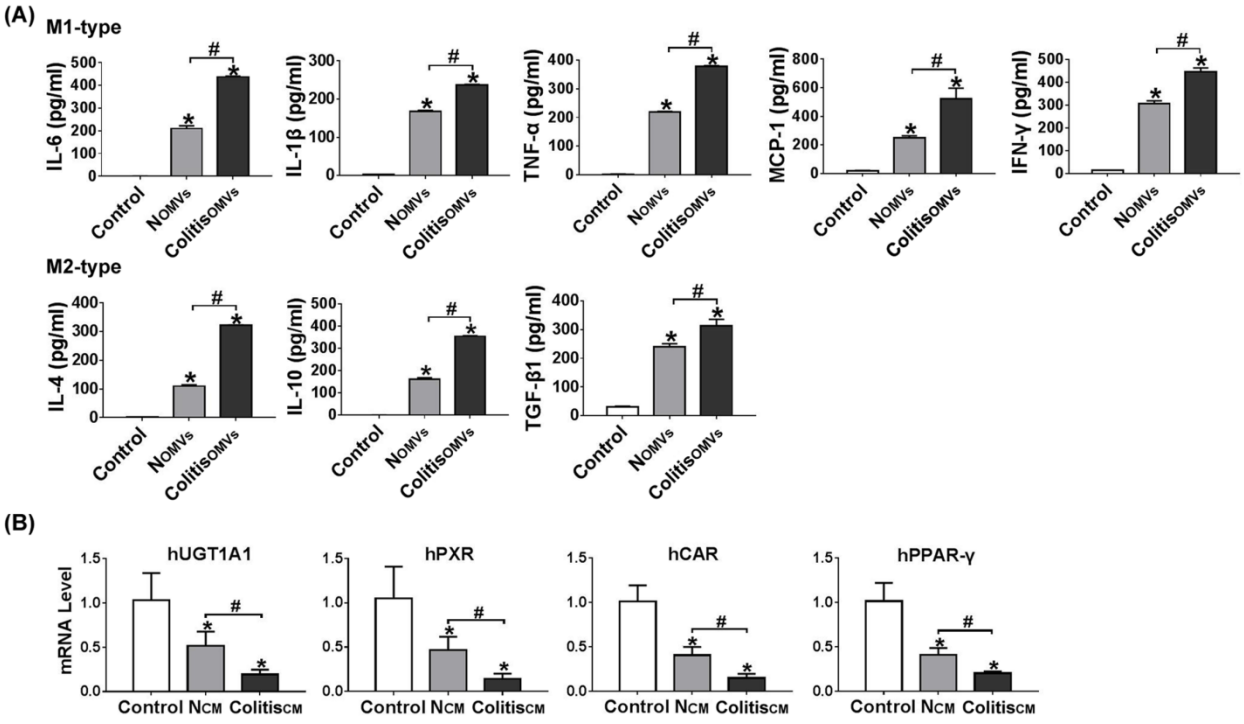

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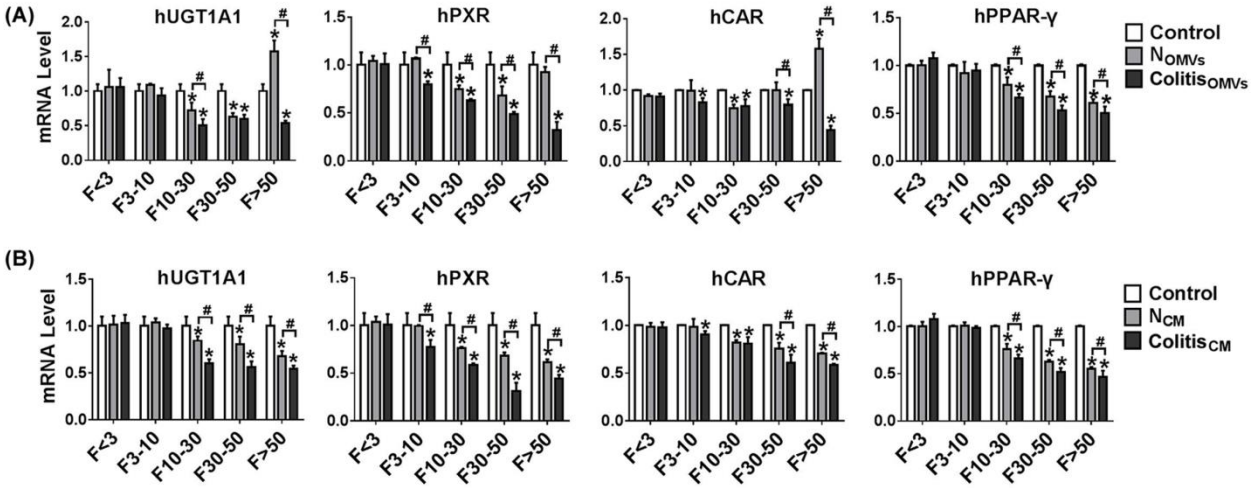

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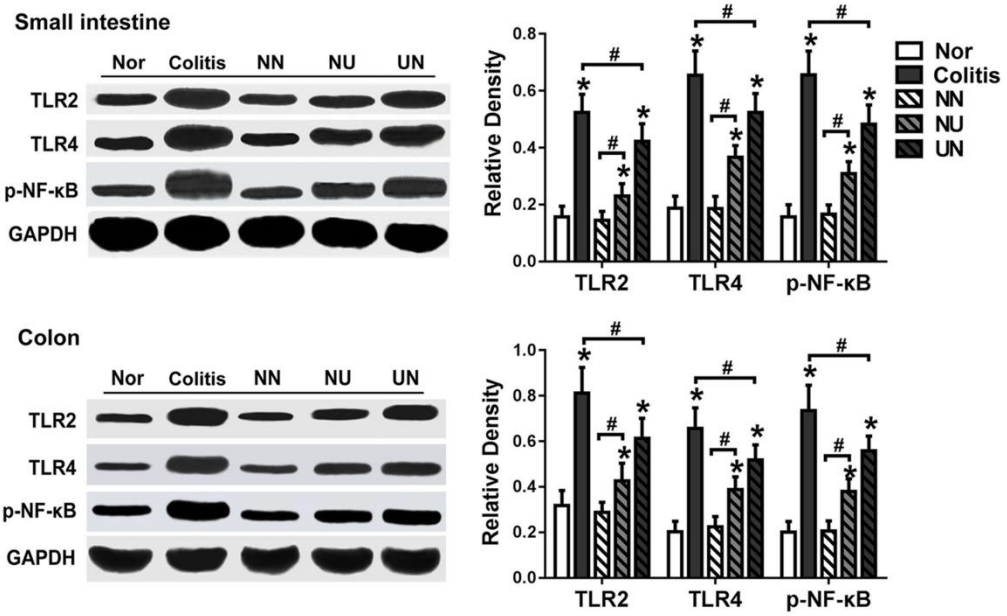
Figure 6



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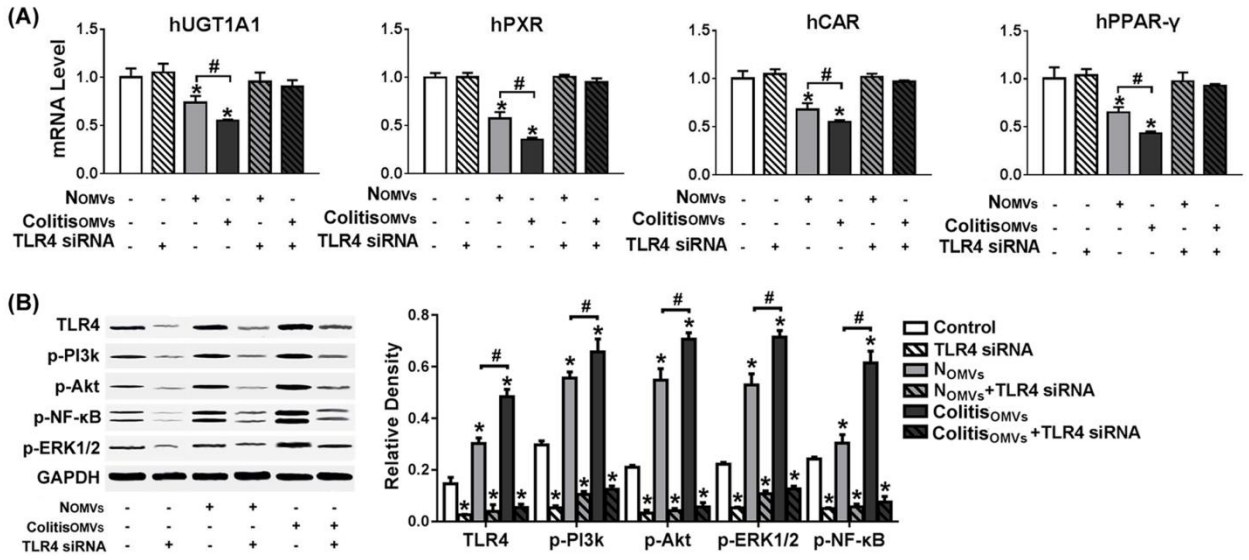

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