

The gut microbiota metabolite indole alleviates liver inflammation in mice

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ABSTRACT: The gut microbiota regulates key hepatic functions, notably through the production of bacterial metabolites that are transported *via* the portal circulation. We evaluated the effects of metabolites produced by the gut microbiota from aromatic amino acids (phenylacetate, benzoate, *p*-cresol, and indole) on liver inflammation induced by bacterial endotoxin. Precision-cut liver slices prepared from control mice, Kupffer cell (KC)-depleted mice, and obese mice (*ob/ob*) were treated with or without LPS and bacterial metabolites. We observed beneficial effects of indole that dose-dependently reduced the LPS-induced up-regulation of proinflammatory mediators at both mRNA and protein levels in precision-cut liver slices prepared from control or *ob/ob* mice. KC depletion partly prevented the antiinflammatory effects of indole, notably through a reduction of nucleotide-binding domain and leucine-rich repeat containing (NLR) family pyrin domain-containing 3 (NLRP3) pathway activation. *In vivo*, the oral administration of indole before an LPS injection reduced the expression of key proteins of the NF- κ B pathway and downstream proinflammatory gene up-regulation. Indole also prevented LPS-induced alterations of cholesterol metabolism through a transcriptional regulation associated with increased 4 β -hydroxycholesterol hepatic levels. In summary, indole appears as a bacterial metabolite produced from tryptophan that is able to counteract the detrimental effects of LPS in the liver. Indole could be a new target to develop innovative strategies to decrease hepatic inflammation.—Beaumont, M., Neyrinck, A. M., Olivares, M., Rodriguez, J., de Rocca Serra, A., Roumain, M., Bindels, L. B., Cani, P. D., Evenepoel, P., Muccioli, G. G., Demoulin, J.-B., Delzenne, N. M. The gut microbiota metabolite indole alleviates liver inflammation in mice. *FASEB J.* 32, 000–000 (2018). www.fasebj.org

KEY WORDS: gut–liver axis • LPS • Kupffer cells • cholesterol metabolism • PCLS

As a result of its anatomic position in the digestive system, the liver is constantly exposed to bacterial compounds coming from the gut (1). LPS, also known as endotoxins (components of gram-negative bacteria outer membrane), can translocate to the liver *via* the portal circulation,

especially when the gut barrier is impaired, as shown, for example, during diet-induced obesity or in genetic obese (*ob/ob*) mice (2–4). Although the hepatic immune system is tolerogenic in physiologic conditions, exposure to high levels of LPS induces the release of large amounts

ABBREVIATIONS: AAA, aromatic amino acids; AhR, aryl hydrocarbon receptor; ALAT, alanine transaminase; ASAT, aspartate transaminase; CCL2, chemokine ligand 2; CL, clodronate liposome; *Cyp1a1*, cytochrome P450, family 1, subfamily A, polypeptide 1; *Cyp1b1*, cytochrome P450, family 1, subfamily B, member 1; *Cyp2e1*, cytochrome P450, family 2, subfamily E, member 1; DEG, differentially expressed genes; I3S, indoxyl-3-sulfate; KC, Kupffer cells; LXR, liver X receptor; NAFLD, nonalcoholic fatty liver disease; NLR, nucleotide-binding domain and leucine-rich repeat containing; NLRP3, nucleotide-binding domain and leucine-rich repeat containing family pyrin domain-containing 3; NOX2, NADPH oxidase 2; PCLS, precision-cut liver slice; PTGS2, prostaglandin-endoperoxide synthase 2; ROS, reactive oxygen species; *Sult1a1*, sulfate-transfer implicating sulfotransferase family 1A, member 1; TBARS, thiobarbituric acid reactive substance; TBS-T, Tris-buffered saline–Tween 20; TLR4, Toll-like receptor 4

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of proinflammatory mediators, including upon activation of the resident macrophages–Kupffer cells (KC) (5). Endotoxemia and inflammation are common features of most chronic liver diseases such as nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis, and alcoholic hepatitis, and they contribute to their progression (6–8). Targeting liver inflammation therefore appears to be a promising strategy to support or restore hepatic homeostasis.

In the last decade, the gut microbiota has emerged as a key regulator of liver physiology through the gut–liver axis (4, 9). Liver inflammation has been associated with an alteration of the gut microbiota composition in patients with nonalcoholic steatohepatitis and alcoholic hepatitis (10, 11). The causal role of this dysbiosis has been demonstrated by transferring the microbiota of patients with severe alcoholic hepatitis into mice (10). Among the potential mechanisms involved, regulation of the gut barrier function by intestinal bacteria has been shown to play a central role in microbiota–liver interactions (12). In addition, microbiota-derived bioactive molecules are considered to be key regulators of immunity in organs at a distance from the gut, notably the liver (13). Indeed, except for the gut itself, the liver is the most exposed organ to metabolites produced by the gut microbiota (4). Bacterial products such as short-chain fatty acids, trimethylamine, ethanol, and secondary bile acids have been shown to regulate liver homeostasis (12, 14). However, only a few of the wide variety of microbiota-derived metabolites have been tested for their effects on liver inflammation.

Comparison of the metabolome of conventional and germ-free or antibiotic-treated mice revealed that the microbiota largely contributes to the peripheral plasma or urine metabolome, which are downstream the liver (15, 16). One of the most striking findings of these studies is that the liver is exposed to microbial metabolites produced from aromatic amino acids (AAA) such as phenylacetate, benzoate, *p*-cresol, and indole (17). Indeed, the concentrations of the corresponding host–microbiota cometabolites (*i.e.*, bacterial metabolites modified by host enzymes), namely phenylacetylglutamine, hippurate, *p*-cresyl sulfate, and indoxyl-3-sulfate (I3S), have been strongly linked to the presence of the gut microbiota (15, 16). However, the effects of these microbiota-derived compounds on liver inflammation have not been studied so far.

In this study, we first tested the effects of AAA-derived bacterial metabolites on liver inflammation induced by LPS using the model of precision-cut liver slices (PCLS). This tissue culture system preserves the interactions among the different types of hepatic cells in their original tissue matrix, providing a complex *ex vivo* model to study microbiota-derived metabolite–liver interactions (18). We identified the tryptophan-derived bacterial metabolite indole as a potent antiinflammatory molecule in the liver, and we confirmed this effect in PCLS prepared from genetically obese *ob/ob* mice, which we used as a model of NAFLD associated with chronic liver inflammation. KC depletion experiments allowed us to highlight the partial contribution of these cells to the antiinflammatory effects of indole. Finally, oral administration of indole in mice reduced the LPS-induced liver inflammation in association with a regulation of hepatic cholesterol metabolism, as revealed by transcriptome profiling.

MATERIALS AND METHODS

Animals

All mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and were maintained in a specific pathogen-free environment. Animals were housed in groups of 3 mice per cage in a controlled environment (12-h day/night light cycle) with free access to water and food (AIN-93M; Research Diets, New Brunswick, NJ, USA) for 1 wk before experiments. Housing conditions were as specified by the Belgian law of May 29, 2013, on Protection of Laboratory Animals (Agreement LA 1230314). Approval of the animal experiments performed in this study was provided by the local ethical committee (2017/UCL/MD/005).

Experiments

Experiment 1

Male C57BL/6J mice (12 wk old) were anesthetized with ketamine (100 mg/kg of body weight; Nimetek; Eurovet Animal Health, Bladel, The Netherlands) and xylazine (10 mg/kg of body weight; Rompun; Bayer, Leverkusen, Germany). The liver was collected and immediately processed for PCLS preparation or KC isolation. Mice were humanely killed by cervical dislocation. All PCLS experiments were performed with $n \geq 4$ mice in each condition.

Experiment 2

Male C57BL/6J mice (12 wk old) anesthetized with ketamine (50 mg/kg of body weight) and xylazine (5 mg/kg of body weight) and received a retroorbital intravenous injection of NaCl 0.9% (control, $n = 9$) or clodronate liposomes (CL; 10 mg/kg of body weight, $n = 8$ <http://www.clodronateliposomes.org>). Two days later, mice were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight). The liver was collected and immediately processed for PCLS preparation as described below. Mice were humanely killed by cervical dislocation.

Experiment 3

Male B6.V-Lep *ob/+* JRj ($n = 4$) and B6.V-Lep *ob/ob* JRj ($n = 6$) (5–6 wk old) were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight). The liver was collected and immediately processed for PCLS preparation as described below. Mice were humanely killed by cervical dislocation.

Experiment 4

Male C57BL/6J mice (12 wk old) that had had food withheld for 3 h received by gavage sterile ultrapure water (vehicle) or indole (MilliporeSigma, Burlington, MA, USA) dissolved in sterile ultrapure water warmed at 55°C to improve its solubility (3 μ mol/20 g of body weight in a volume of 200 μ l/20 g of body weight), based on a procedure previously described (19). Thirty minutes later, mice received an intraperitoneal injection of NaCl 0.9% (vehicle) or LPS (10 mg/kg of body weight, *Escherichia coli* O127:B8; MilliporeSigma). Mice were assigned to 1 of 3 groups: water + NaCl ($n = 4$), water + LPS ($n = 6$), or indole + LPS ($n = 6$). Four hours later, mice were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight). After removal of the gallbladder, the liver was freeze-clamped in liquid nitrogen. All samples were stored at -80°C until analysis. Mice were humanely killed by cervical dislocation.

Precision-cut liver slices

Immediately after liver collection, PCLS (~250 μ m thick) were prepared from liver tissue cores (5 mm diameter) in oxygenated ice-cold Krebs-Ringer solution (NaCl 144 mM, KCl 5.8 mM, KH_2PO_4 1.4 mM, MgSO_4 1.4 mM, NaHCO_3 0.2%, CaCl_2 2.6 mM, glucose 5.5 mM) using a Krumdieck slicer. PCLS were incubated in oxygenated Waymouth medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with insulin (100 U/ml; Actrapid), antibiotics (penicillin/streptomycin 100 U/ml; Thermo Fisher Scientific), and fatty acid-free bovine serum albumin (0.3%) at 4°C during 30 min and then at 37°C under agitation during 1 h. For treatment, PCLS were incubated with water (control) or LPS (100 μ g/ml, *Escherichia coli* O127:B8; MilliporeSigma) and DMSO 0.1% (vehicle) or bacterial metabolites (phenylacetate, benzoate, *p*-cresol, indole, and I3S; MilliporeSigma) in oxygenated Waymouth medium supplemented with insulin (100 U/ml), antibiotics (penicillin/streptomycin, 100 U/ml) and fatty acid-free bovine serum albumin (0.1%) at 37°C during 4 h under agitation (2 PCLS/flask containing 1 ml of incubation medium). After incubation, PCLS were rinsed with ice-cold NaCl 0.9% and frozen in dry ice. Incubation media were collected and frozen in dry ice. All samples were stored at -80°C until analysis.

KC isolation

KC were isolated from 4 mice using a previously described procedure (20). Briefly, liver tissue was minced before digestion in collagenase P (Roche, Basel, Switzerland) for 30 min at 37°C. After filtration (70 μ m), homogenates were centrifuged (50 g, 5 min, 4°C) to remove parenchymal cells. The supernatant was centrifuged (400 g, 10 min, 4°C), and the pellet containing nonparenchymal cells was washed in PBS before centrifugation (400 g, 10 min, 4°C). Nonparenchymal cells were resuspended in DMEM (Thermo Fisher Scientific) containing FCS 10% and penicillin/streptomycin 1% (Thermo Fisher Scientific). Cells (10⁷ cells per well) in 6-well plates were incubated for 2 h (CO₂ 5%, 37°C). After elimination of nonadherent cells, the adherent cells (KC) were washed twice with warm PBS. After an overnight incubation (CO₂ 5%, 37°C), isolated KC were treated with water (control) or LPS (100 μ g/ml) and DMSO 0.1% (vehicle) or indole (100 μ M) for 4 h.

Tissue mRNA analyses

Total RNA was isolated from liver tissues using the TriPure Isolation Reagent (Roche Diagnostics). cDNA was prepared by reverse transcription of 1 μ g of total RNA using the Reverse Transcription System (Promega, Madison, WI, USA). Real-time PCR was performed with a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using Mastermix Plus for SYBR Assay (Eurogentec, Liège, Belgium). Data were analyzed according to the 2^{- $\Delta\Delta C_t$} method. The purity of the amplified product was verified by analyzing the melt curve performed at the end of the amplification step. The ribosomal protein L19 (*Rpl19*) gene was chosen as a reference gene. The primer sequences of the targeted genes are listed in Supplemental Table 1.

Transcriptome analysis

For the *in vivo* experiment (experiment 4), hepatic transcriptome profiling was performed using the Clariom D mouse assay with the GeneChip WT Plus Reagent Kit (Thermo Fisher Scientific) according to the manufacturer's instructions (*n* = 5 in each group). All samples had a RNA integrity number of >8, as determined using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Western blot analysis

Liver samples (50 mg) were homogenized in 700 μ l ice-cold lysis buffer [20 mM Tris, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM β -glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 10% protease inhibitor cocktail (Roche)] using a TissueLyzer (Qiagen, Germantown, MD, USA). After centrifugation (10,000 *g*, 10 min, 4°C), protein concentration was measured in the supernatant using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Liver protein (40 μ g) were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) before blocking in Tris-buffered saline-Tween 20 (TBS-T) containing 5% nonfat dry milk (Bio-Rad) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies (dilution 1/1000 in 1% BSA TBS-T; Cell Signaling Technology, Danvers, MA, USA) detecting NF- κ B p65 (8242), phospho-NF- κ B p65 (Ser536) (3033), I κ B α (4814), and phospho-I κ B α (Ser32) (2859) or the loading control protein β -actin (ab6276, 1/10,000 in 1% nonfat milk TBS-T; Abcam, Cambridge, MA, USA). After membrane washing, horseradish peroxidase-linked secondary antibodies (7074 and 7076; 1/1000 in 1% nonfat milk TBS-T; Cell Signaling Technology) were incubated for 1 h at room temperature. Signals were revealed using the SuperSignal West Pico and Femto Chemiluminescent substrates (Thermo Fisher Scientific) and analyzed with the ImageQuant TL instrument and software v.8.1 (GE Healthcare, Waukesha, WI, USA).

Biochemical analysis

Alanine transaminase (ALAT) and aspartate transaminase (ASAT) activities were measured in the culture medium of PCLS using kits according to the manufacturer's instructions (Diasys, Waterbury, CT, USA). IL-1 β , chemokine ligand 2 (CCL2), and TNF were quantified in PCLS incubation medium (undiluted) using a Multiplex Immunoassay Kit (Bioplex; Bio-Rad) and measured using Luminex technology (Bioplex; Bio-Rad). Hepatic cholesterol was measured using commercial kits (Diasys) after chloroform-methanol extraction as previously described (21). Hepatic oxysterols were quantified by HPLC-MS as previously described (22). Reactive oxygen species (ROS and thiobarbituric acid reactive substances (TBARS) were measured in the liver tissue as previously described (23).

Statistical analyses and graphical representations

Statistical analyses were performed by R 3.4.3 software (2017; R Foundation for Statistical Computing, Vienna, Austria; <http://www.r-project.org/>) with the packages lme4, car, and lsmeans. For PCLS experiments, to take into account that several slices were prepared from the same mouse, a linear mixed model was used, followed by ANOVA (*lmer* and *Anova* functions), with the use of mouse as a random effect. The fixed effects used are indicated in the figure captions. Mean values were compared pairwise with the use of the Tukey correction (*lsmeans* function). For the *in vivo* experiment, a linear model was used, followed by ANOVA (*lm* and *Anova* functions), and mean values were compared pairwise with the use of the Tukey correction (*lsmeans* function). For correlation analysis, Spearman correlation coefficient (*r*) was calculated (*cor.test* function). For microarray experiments, raw data were normalized with the robust multiarray average method, and mean expression values were compared using multiple *t* tests in the Transcriptome Analysis Console software (Affymetrix, Santa Clara, CA, USA). Differentially expressed genes (DEG) were defined with *P* < 0.05 and a fold change of ± 1.2 or more between the 2 groups (LPS and LPS + indole). Functional analysis was performed on the list of DEG with the Ingenuity Pathway Analysis software (Qiagen). Significantly enriched biologic functions were

defined with enrichment $P < 0.05$ and at least implicating 4 DEG. For all statistical tests, $P < 0.05$ was considered to be significant. All plots were generated by GraphPad Prism 5 and 7 software (GraphPad Software, La Jolla, CA, USA). Heat maps were generated in R with the package *made4* (*heatmap* function).

RESULTS

Tryptophan-derived bacterial metabolite indole prevents LPS-induced inflammation in PCLS

As a first screening step on PCLS, we tested the effects of bacterial metabolites (1 mM) produced from AAA (phenylacetate, benzoate, *p*-cresol, and indole) and known to reach the liver *via* the portal circulation (15). None of the metabolites significantly altered the activity of ALAT and ASAT in the incubation medium, suggesting the absence of cytotoxicity (Fig. 1A). Moreover, no significant effect of the metabolites was observed at the mRNA level for the key proinflammatory genes *Ccl2*, *Il-1B*, and *Tnf* coding for the proteins chemokine ligand 2, IL-1 β , and TNF, respectively (Fig. 1B). Next, we evaluated potential interactions between the AAA-derived bacterial metabolites and LPS that can also be transported from the gut to the liver *via* the portal circulation. LPS (100 ng/ μ l) did not alter ALAT and ASAT activities in the incubation medium, while it induced a 2- to 3-fold increase in the mRNA levels of *Ccl2*, *Il-1B*, and *Tnf* (Fig. 1A, B). *p*-Cresol and indole totally prevented the LPS-induced up-regulation of *Ccl2* and *Il-1B*, whereas phenylacetate and benzoate had no

effect on proinflammatory gene expression (Fig. 1B). In contrast, *p*-cresol and indole did not significantly decrease *Tnf* mRNA levels. Because *p*-cresol has been previously shown to be toxic for intestinal epithelial cells (24) while indole has beneficial effects on gut barrier function (25, 26), we decided to further explore the hepatic effects of this potentially protective bacterial metabolite derived from tryptophan. In order to evaluate whether the effects of indole were dose dependent, PCLS exposed or not to LPS were treated with 10 to 1000 μ M indole. None of the treatments was cytotoxic (Fig. 2A). Indole dose dependently decreased the LPS-induced up-regulation of *Ccl2* and *Il-1B*, with these effects being statistically significant from 10 to 100 μ M, respectively (Fig. 2B). Moreover, the LPS-induced secretion of CCL2 and IL-1B proteins in the culture medium was strongly inhibited by indole (100 μ M) (Fig. 2C). Of note, the LPS-induced secretion of IL-1B by PCLS was low in our short-term experiment. In contrast, indole had no effect on the LPS-induced TNF protein secretion, in agreement with the mRNA profiles. Overall, our results showed that the bacterial metabolite indole has antiinflammatory effects in the PCLS model.

Antiinflammatory effects of indole are partly dependent on KC in PCLS

KC play a key role in the response to LPS in the liver (5, 27). To investigate the potential implication of these cells in the antiinflammatory effects of indole, we depleted KC with CL (28). Then PCLS were prepared from mice with or without KC. As expected, the mRNA level of the *Adgre1*

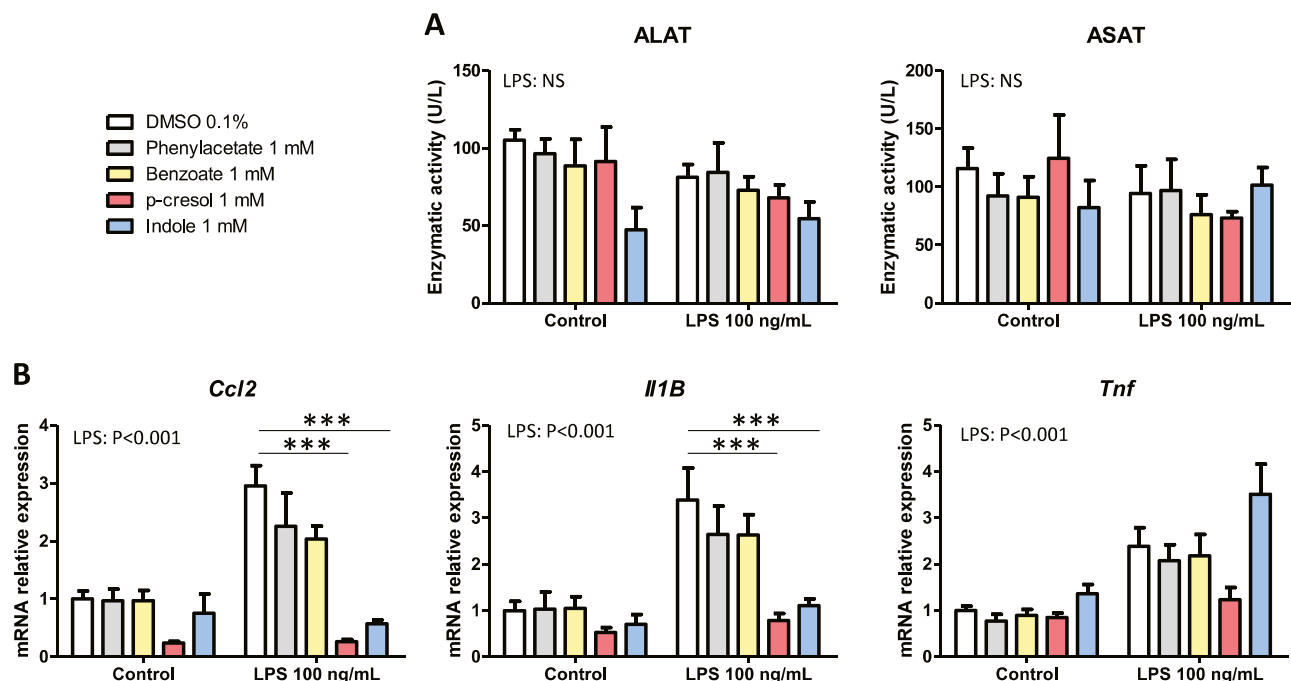


Figure 1. Effects of bacterial metabolites produced from AAA on PCLS. PCLS were treated with or without LPS (100 ng/ml) and bacterial metabolites (1 mM). A) ALAT and ASAT activities were measured in culture medium. B) mRNA levels were quantified in PCLS using real-time quantitative PCR. Data are presented as means $\pm$ SEM, $n \geq 4$ mice in each condition (experiment 1). Mixed-model ANOVA was used with LPS and metabolite treatments as fixed effects and mouse as random effect. For pairwise comparison, mean value of each metabolite-treated group was compared to mean of respective vehicle-treated group in water or LPS conditions. NS, not significant. *** $P < 0.001$ (adjusted by Tukey method).

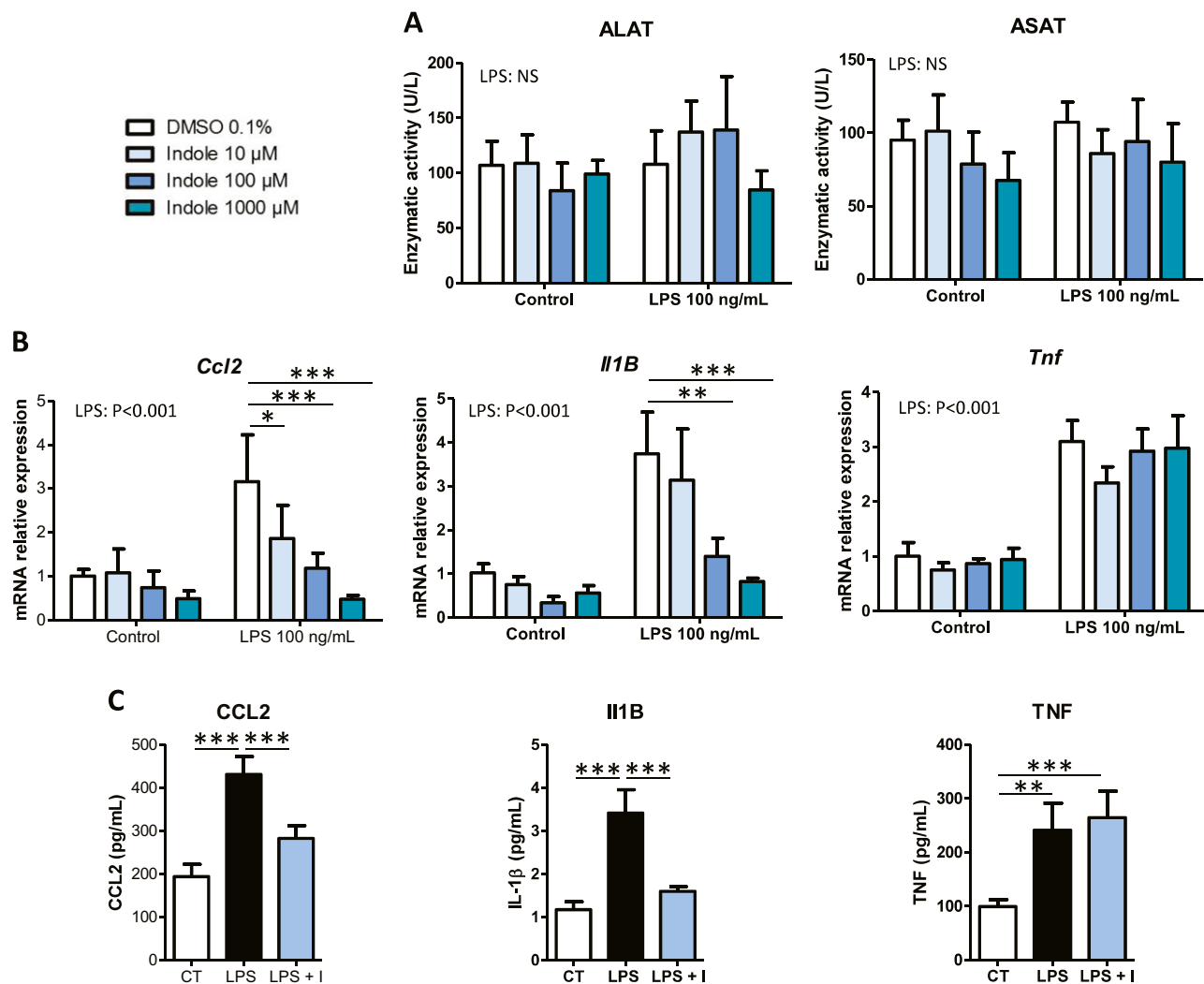


Figure 2. Dose effects of indole on PCLS. PCLS were treated with or without LPS (100 ng/ml) and indole (10–1000 μ M). **A**) ALAT and ASAT were measured in culture medium. **B**) mRNA levels were quantified in PCLS using real-time quantitative PCR. **C**) Cytokines were quantified in culture medium using Luminex. For this experiment, intermediate concentration of indole was used (100 μ M). For IL-1 β , concentration in control and LPS + indole group were extrapolated beyond standard range, but fluorescent signal was always higher than signal measured in blank. Data are presented as means $\pm$ SEM, $n \geq 5$ mice in each condition (experiment 1). Mixed-model ANOVA was used with LPS and indole (I) treatments as fixed effects and mouse as random effect. For pairwise comparison, mean value of each indole-treated group was compared to mean of respective vehicle group in water- or LPS-treated conditions. NS, not significant. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (adjusted by Tukey method).

gene (coding for the mature macrophage marker protein F4/80) was decreased in PCLS prepared from CL-treated mice compared to control mice (Fig. 3A). The basal expression level of *Ccl2* was reduced by 2-fold in PCLS prepared from CL-treated mice (Fig. 3A), showing a significant contribution of KC to the expression of this gene. However, LPS was still able to increase its expression, suggesting that KC are not solely responsible for *Ccl2* induction by LPS. Indole (100 μ M) also prevented LPS effect after CL treatment, suggesting that it may target pathways in other cell types activated by LPS. Interestingly, the expression profile of *Nos2* (coding for the key oxidative stress inducer protein NOS2) was similar in PCLS prepared from control and CL-treated mice, suggesting that KC did not contribute to the regulation of the expression of this gene by LPS and indole. The LPS-induced up-regulation of *Nox2* (coding for NADPH oxidase 2, another protein

implicated in oxidative stress) was also prevented by indole in a KC-independent manner, despite a lower basal expression level after KC depletion. To confirm these results, isolated KC were treated with LPS (100 ng/ml) and indole (100 μ M) (Fig. 3B). The expression of *Adgre1* was similar in all experimental conditions, suggesting similar purity of the KC preparation. In isolated KC, indole did not prevent the LPS-induced up-regulation of *Ccl2*, *Nos2*, and *Nox2*. Collectively, these results show that the presence of other cells than KC is required for the indole-induced down-regulation of *Ccl2*, *Nos2*, and *Nox2*.

LPS failed to significantly up-regulate *Il1b* mRNA levels in PCLS prepared from CL-treated mice, and the effects of indole were strongly attenuated (Fig. 3C), showing that KC play a pivotal role in the regulation of *Il1b* expression in our model. In agreement with this finding, *Il18* and nucleotide-binding domain and leucine-rich

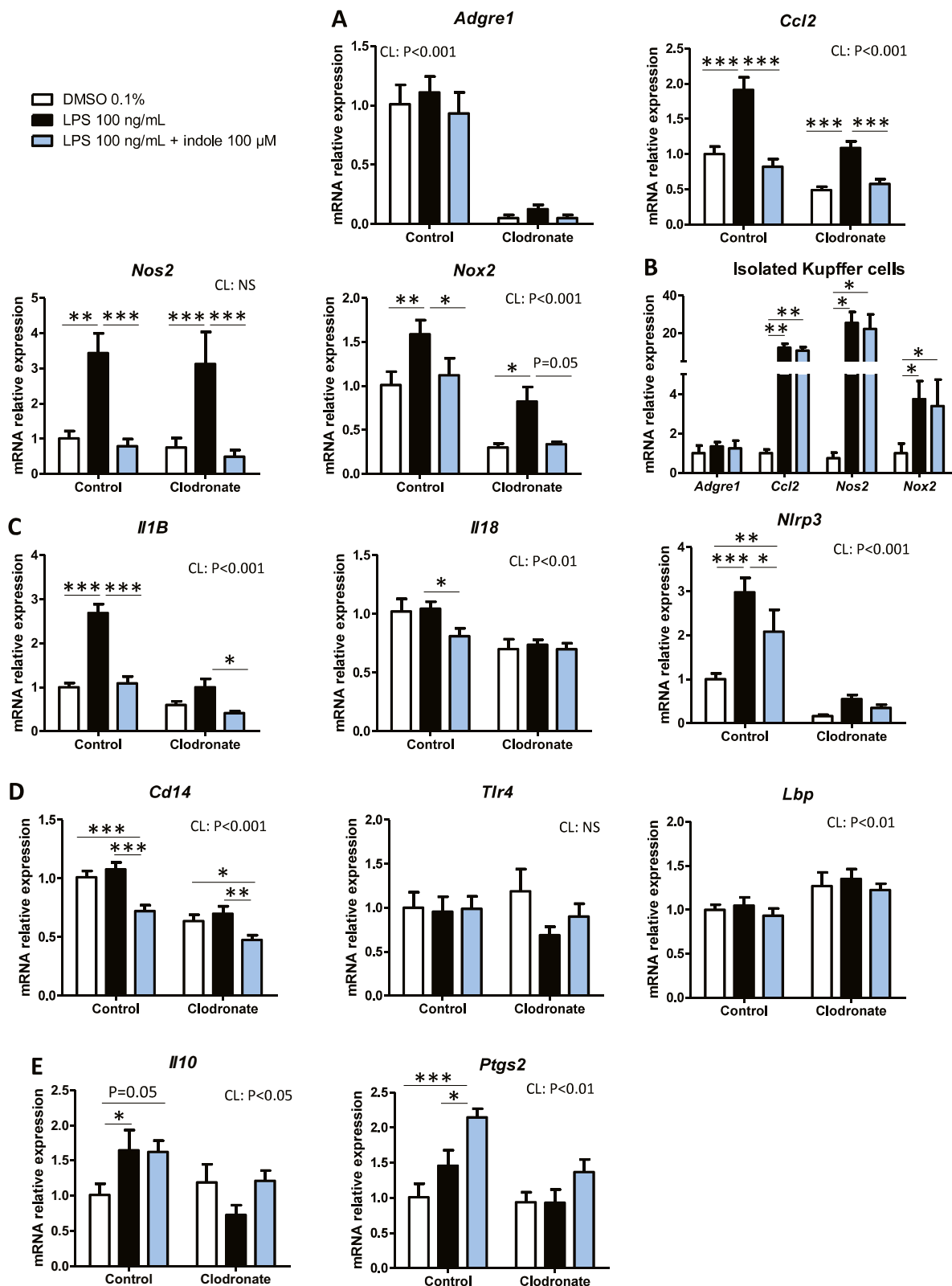


Figure 3. Effects of indole on PCLS prepared from mice without KC. PCLS were prepared after injection of CL or NaCl (control) before treatment with or without LPS (100 ng/ml) and indole (100 μ M). A, C–E) mRNA levels were quantified in PCLS using real-time quantitative PCR, $n \geq 8$ mice in each condition (experiment 2). Mixed-model ANOVA was used with CL, LPS, and indole treatments as fixed effects and mouse as random effect. Mean values were compared pairwise in control or CL conditions. B) mRNA levels were quantified by real-time quantitative PCR in isolated KC treated with or without LPS (100 ng/ml) and indole (100 μ M), $n = 4$ mice in each condition (experiment 1). Mixed-model ANOVA was used with LPS and indole treatments as fixed effects and mouse as random effect. Mean values were compared pairwise. Data are presented as means $\pm$ SEM. NS, not significant. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (adjusted by Tukey method).

repeat containing (NLR) family pyrin domain-containing 3 (*Nlrp3*) mRNA levels (coding for the protein IL-18 and the inflammasome NLR family pyrin domain-containing 3) were reduced by indole in PCLS prepared from control mice but not in PCLS prepared from CL-treated mice, suggesting that indole down-regulates the NLRP3–IL-1B–IL-18 pathway in a KC-dependent manner. Regarding the sensing of LPS at the cell surface, indole down-regulated the expression of *Cd14* independently of the presence of KC, while no effect was observed for *Tlr4* and *Lbp* coding for the Toll-like receptor 4 (TLR4) and lipopolysaccharide binding protein (Fig. 3D). Last, we found that indole did not regulate the expression of the regulatory cytokine *Il-10* but induced a 2-fold up-regulation of *Ptgs2* [coding for the prostaglandin-endoperoxide synthase 2 (Fig. 3E), implicated in the release of tolerogenic bioactive lipids (5)] in a KC-dependent manner. Our results showed that the antiinflammatory effects of indole were partly mediated by KC but that other liver cell types (e.g., hepatocytes, or stellate or endothelial cells) are required for some of the beneficial effects of indole.

Indole interacts with liver xenobiotic metabolism in PCLS

Indole, as a xenobiotic, can be metabolized in the liver by the cytochrome P450, family 2, subfamily E, member 1 (*Cyp2e1*), as well as through sulfate-transfer implicating sulfotransferase family 1A, member 1 (*Sult1a1*) (29, 30). We tested whether the antiinflammatory effects of indole were associated with an alteration of xenobiotic metabolism. Indole (100 μ M) had no effect on *Cyp2e1* and *Sult1a1* mRNA levels in PCLS (Fig. 4A). In contrast, indole induced a strong up-regulation of cytochrome P450, family 1, subfamily A, polypeptide 1 (*Cyp1a1*) and cytochrome P450, family 1, subfamily B, member 1 (*Cyp1b1*) (Fig. 4A), which are key target genes of the aryl hydrocarbon receptor (AhR), a pivotal transcription factor in xenobiotic metabolism (31). As a next step, we wanted to explore whether indole metabolism by the host into I3S could alter its biologic activity. I3S (100 μ M) failed to prevent the LPS-induced up-regulation of *Il-1B* and *Nos2*, even if it induced a strong up-regulation of *Cyp1a1* and *Cyp1b1* in PCLS (Fig. 4B). These data suggest that indole itself—and not its metabolite, I3S—was antiinflammatory and that up-regulation of *Cyp1a1* and *Cyp1b1* by indolic compounds may occur independently of their effects on inflammation. In order to confirm this hypothesis, we repeated the experiments with a simultaneous incubation with the AhR inhibitor CH-223191 (10 μ M). However, these experiments were inconclusive because CH-223191 down-regulated *Cyp1a1* expression in the control condition but failed to prevent its induction by indole in the PCLS model.

Indole down-regulates proinflammatory gene expression in PCLS prepared from genetically obese mice

In order to test whether the effects of indole could be observed in a model of NAFLD associated with chronic

liver inflammation, PCLS were prepared from *ob/+* (control) or *ob/ob* (leptin deficient) mice. As expected, PCLS prepared from *ob/ob* mice overexpressed the proinflammatory genes *Il-1B*, *Nos2*, *Ccl2*, *Tnf*, and *Cd14* (Fig. 5). Interestingly, treating PCLS with indole (100 μ M) reduced the expression of all these genes, except *Tnf*, a finding in good agreement with the results obtained in the acute LPS exposure model. Interestingly, *ob/ob* mice overexpressed the AhR target genes *Cyp1a1* and *Cyp1b1* in basal conditions, and their expression was strongly induced by indole. In summary, our results show that the antiinflammatory effects of indole, as well as its ability to induce the expression of AhR target genes, are observed also in a mouse model characterized by metabolic endotoxemia and hepatic inflammation.

Oral administration of indole reduces LPS-induced liver inflammation *in vivo*

In order to test if our findings on PCLS were relevant *in vivo*, indole was administered by gavage to mice 30 min before intraperitoneal injection of LPS. As expected, LPS strongly induced proinflammatory gene expression (Fig. 6A). Indole oral administration reduced the LPS-induced up-regulation of the cytokines *Il-1B*, *Il-6*, and *Il-15*. Indole also down-regulated the gene expression of *Nos2* and *Nox2*, suggesting a decrease in the induction of oxidative stress. However, LPS did not significantly increase the hepatic levels of ROS and TBARS, maybe because of the short time period between LPS injection and the animal's death (Fig. 6B). Of note, some differences were observed compared to the PCLS model because the amplitude of the effects of indole was lower *in vivo* and indole did not significantly regulate *in vivo* the expression of *Ccl2*, *Il-18*, and *Ptgs2* (Fig. 6A). These results might be related to the very important induction of proinflammatory gene expression by LPS *in vivo*, and the concentration of indole reaching the liver might be lower than the concentration tested *ex vivo*. Because the transcription of most of the genes down-regulated by indole are controlled by the NF- κ B pathway (6), we quantified by Western blot NF- κ B p65 and I κ B α . Indole markedly reduced the protein level of the total and phosphorylated forms of NF- κ B p65 and I κ B α , resulting in a similar ratio between the 2 forms (Fig. 6C). In agreement with the results obtained in PCLS, our results obtained *in vivo* show that orally administered indole has antiinflammatory effects in the liver and that these effects are associated with a down-regulation of the level of key proteins of the NF- κ B pathway.

Indole regulates hepatic cholesterol metabolism in mice treated with LPS

In order to further characterize the effects of indole, we performed transcriptome profiling in the liver of the mice treated with LPS after oral administration of indole or vehicle. We identified 217 genes that

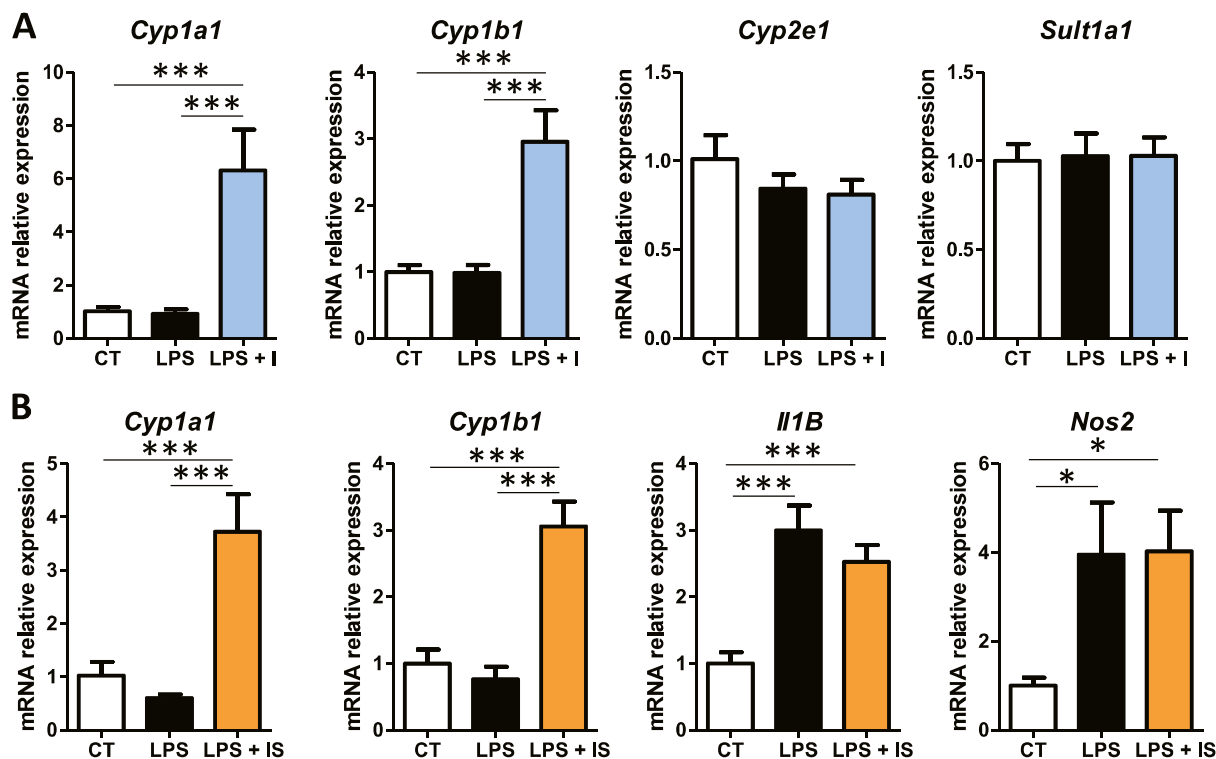


Figure 4. Interactions between indole and xenobiotic metabolism in PCLS. PCLS were treated with or without LPS (100 ng/ml) and indole or I3S (100 μ M). mRNA levels were quantified by real-time quantitative PCR in PCLS treated with LPS and indole (A) or I3S (B) (experiment 1). Data are presented as means $\pm$ SEM, $n \geq 4$ mice in each condition. CT, control; I, indole; IS, I3S. Mixed-model ANOVA was used with LPS and metabolite treatments as fixed effects and mouse as random effect. Mean values were compared pairwise. *** $P < 0.001$, * $P < 0.05$ (adjusted by Tukey method).

were differentially expressed between the LPS- and LPS + indole-treated mice (Supplemental Table 2). Functional analysis revealed that there was an

overrepresentation of genes implicated in lipid metabolism, and more specifically in cholesterol metabolism (Fig. 7A). In total, 52 DEG (24% of the total number of

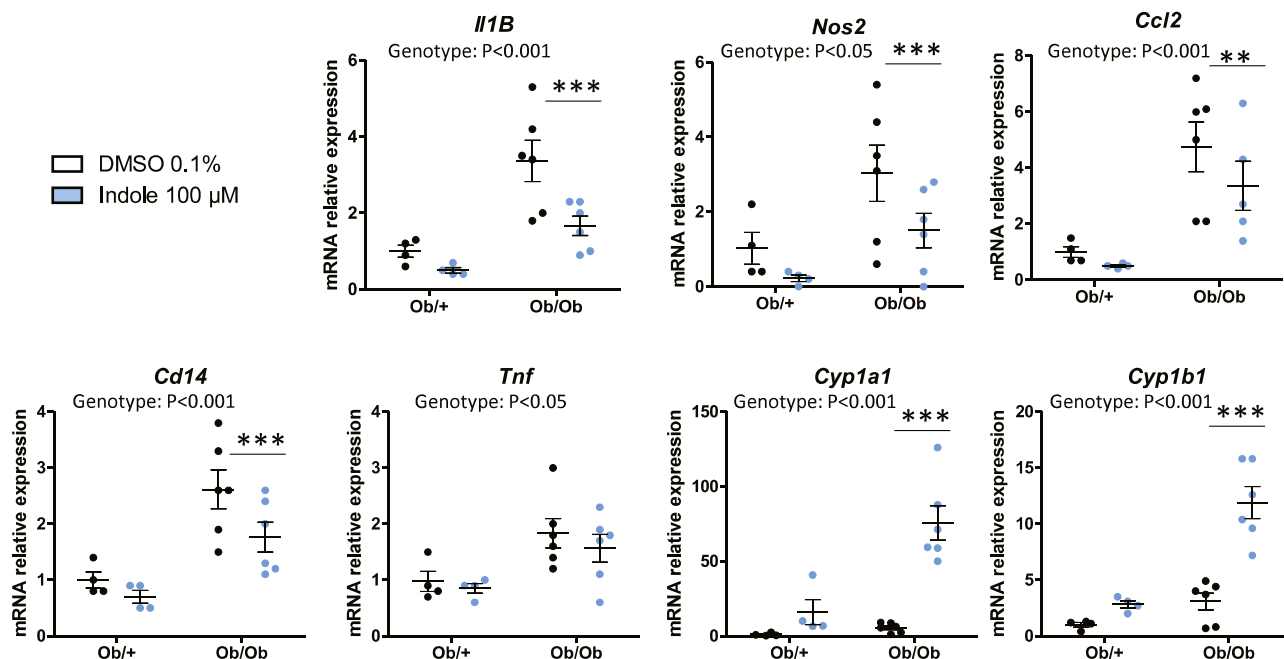


Figure 5. Effects of indole on PCLS prepared from genetically obese (*ob/ob*) mice. PCLS were treated with or without indole (100 μ M). mRNA levels were quantified in PCLS using real-time quantitative PCR. Data are presented as means $\pm$ SEM, $n \geq 4$ mice in each group (experiment 3). Mixed-model ANOVA was used with genotype and indole treatment as fixed effects and mouse as random effect. Mean values were compared pairwise in *ob/+* or *ob/ob* groups. *** $P < 0.001$, ** $P < 0.01$ (adjusted by Tukey method).

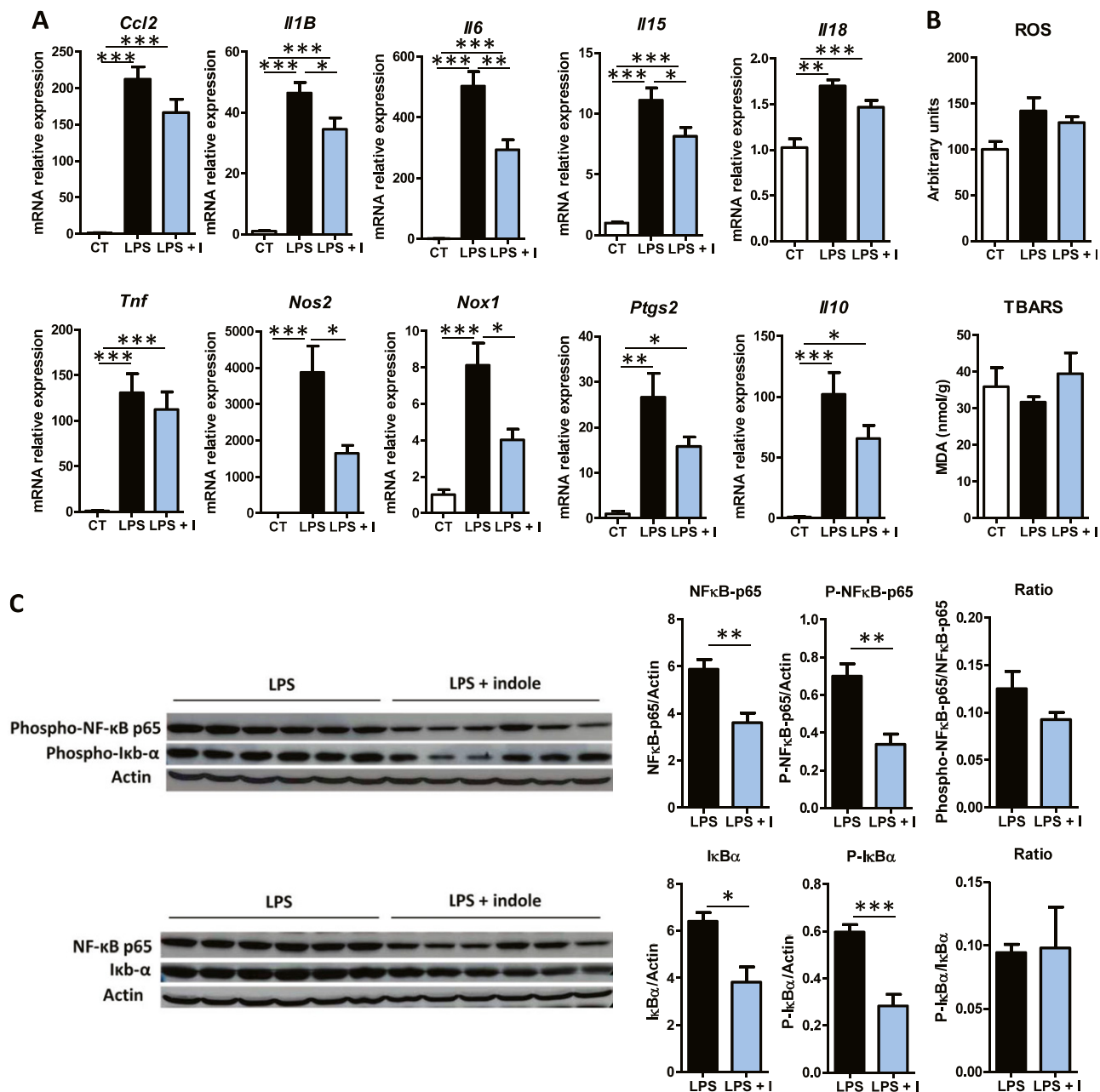


Figure 6. Effects of indole on liver inflammation *in vivo*. Mice orally received water or indole (3 μ mol/20 g of body weight) before intraperitoneal injection of vehicle or LPS (10 mg/kg of body weight). **A**) mRNA levels were quantified in liver using real-time quantitative PCR. Results are expressed relative to mRNA level measured in control group (set at 1). **B**) ROS and TBARS were quantified in liver. **C**) Left: Western blot of β -actin, and total and phosphorylated forms of NF- κ B p65 and I κ B α in liver lysate. right: band intensities were quantified relative to band intensity of β -actin, used as loading control. Data are presented as means $\pm$ SEM, $n = 6$ mice in LPS and LPS + indole (LPS + I) groups, $n = 4$ in control group (CT) (experiment 4). ANOVA was used to test treatment effect, and mean values were compared pairwise. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ (adjusted by Tukey method).

DEG) were implicated in lipid metabolism (Supplemental Table 3). Interestingly, most of these genes (92%) were up-regulated in mice treated with LPS + indole compared to LPS alone (Fig. 7B). In order to confirm the physiologic relevance of these findings, we measured hepatic cholesterol levels. LPS tended to decrease hepatic cholesterol levels, while indole prevented this effect (Fig. 7C). Because several DEG are targets of the liver X receptor (LXR) (32), we sought to evaluate whether indole regulated the hepatic concentration of oxysterols that are key ligands for LXR.

Interestingly, we found that among the panel of 10 oxysterols analyzed (Supplemental Table 4), the concentration of 4 β -hydroxycholesterol was higher in the liver of mice treated with indole and LPS compared to mice treated with LPS alone (Fig. 7D). Finally, we observed that 4 β -hydroxycholesterol concentration was negatively correlated with the expression of genes related to inflammation, as exemplified for *Nos2* mRNA levels (Fig. 7E), suggesting a potential link between the antiinflammatory effects of indole and its effects on cholesterol metabolism.

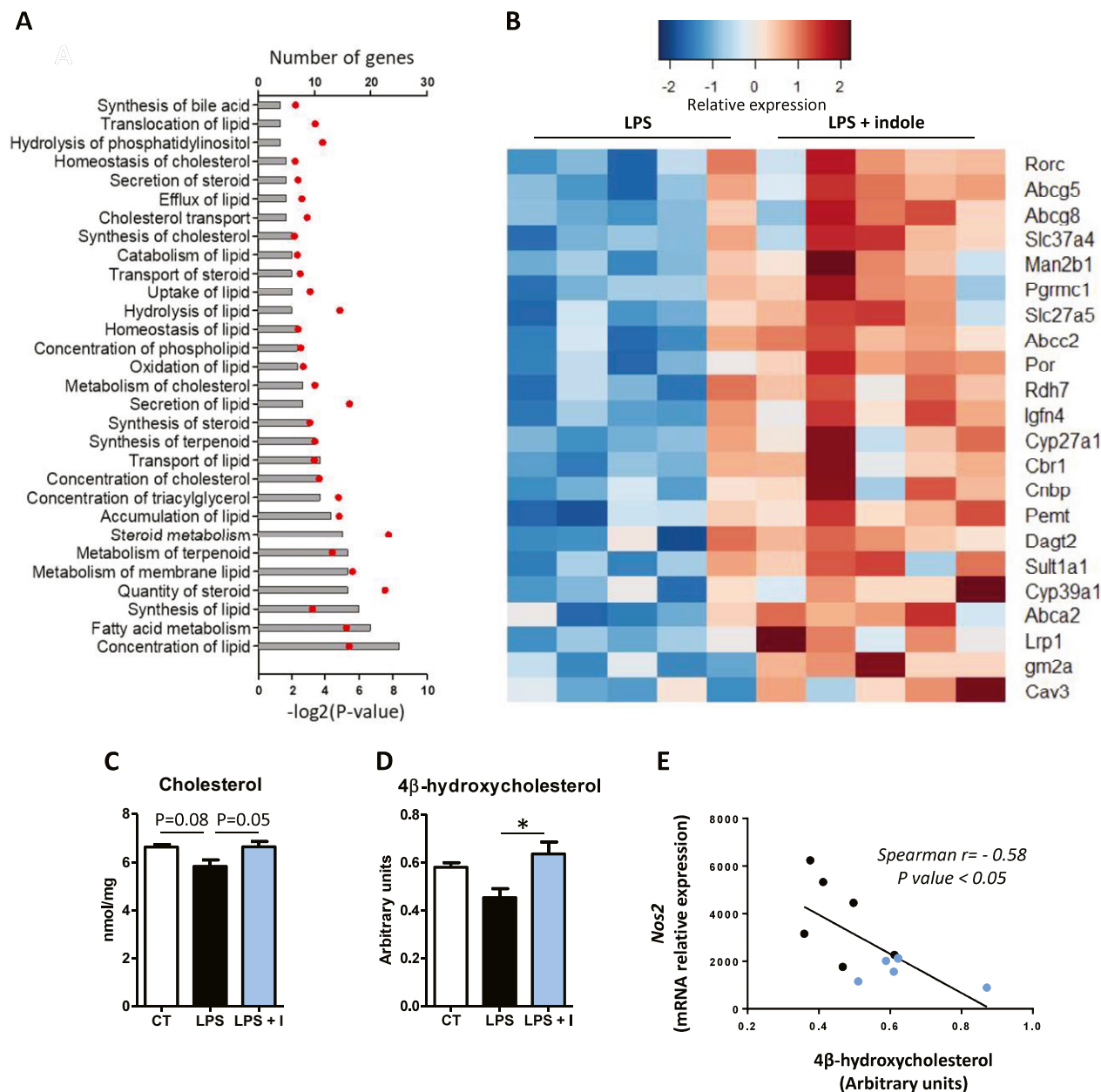


Figure 7. Effects of indole on liver sterols metabolism *in vivo*. Mice orally received water or indole (3 μmol per 20 g of body weight) before intraperitoneal injection of vehicle or LPS (10 mg/kg of body weight). Liver transcriptome was compared between LPS and LPS + indole (LPS + I) groups using microarrays ($n = 5$ mice in each group) (experiment 4). **A**) Significantly enriched functions implicated in biologic category “Lipid metabolism” identified by Ingenuity Pathway Analysis software. Bars show number of DEG implicated in each function. Red dots represent $-\log_2(P\text{-value})$ of enrichment. **B**) Heat map representing mRNA levels ($\log_2$) of selected DEG participating to functions enriched in biologic category, “Lipid metabolism.” **C, D**) Cholesterol (**C**) and 4 β -hydroxycholesterol (**D**) were quantified in liver. Data are presented as means $\pm$ SEM, $n = 6$ mice in LPS and LPS + I groups, $n = 4$ in control group. ANOVA was used to test treatment effect, and mean values were compared pairwise. $*P < 0.05$ (adjusted by Tukey method). **E**) Spearman correlation between 4 β -hydroxycholesterol hepatic concentration and *Nos2* mRNA levels in liver of mice treated with LPS (black dots, $n = 6$) or LPS + I (blue dots, $n = 6$).

DISCUSSION

Indole is produced from L-tryptophan by the bacteria-specific enzyme tryptophanase (encoded by *TnaA* in more than 80 gram-positive and gram-negative species) and can be detected in human and mouse feces at low millimolar concentration (33, 34). This metabolite and related bacterial compounds (indole-3-aldehyde, indole-3-lactate, and indole-3-propionate) were previously shown to reduce

intestinal inflammation and to prevent gut barrier dysfunction (19, 25, 26, 35–38). In the present study, we show for the first time that indole alleviates inflammation in the liver, away from the gut. In the PCLS *ex vivo* model, indole dose-dependently reduced acute hepatic inflammation induced by LPS both at mRNA and protein levels. Similarly, indole decreased proinflammatory gene expression in PCLS prepared from *ob/ob* mice that are characterized by metabolic endotoxemia and liver inflammation

(2, 3). Last, *in vivo* oral administration of indole partly prevented LPS-induced liver inflammation and associated alterations of cholesterol metabolism.

Indole is able to cross the intestinal epithelium and reach the liver, probably *via* its capacity to freely diffuse through lipid membranes (39). On the basis of the results obtained in the present study, several mechanisms underlying the antiinflammatory effects of indole in the liver can be proposed. The first step of endotoxin-induced inflammation is LPS recognition at cell surface, a process involving 3 key proteins: CD14, lipopolysaccharide binding protein (LBP), and TLR4 (40). In PCLS, indole reduced the mRNA levels of *Cd14*, potentially leading to a reduction of LPS signaling that could partly contribute to its antiinflammatory effects. After LPS binding to TLR4 at the cell membrane, the NF- κ B pathway is activated and up-regulates proinflammatory gene expression (6). Interestingly, indole down-regulated the expression of several NF- κ B-responsive genes (*e.g.*, *Nos2*, *Il-1B*, *Ccl2*). Moreover, we found that indole reduced *in vivo* the expression of 2 key proteins of the NF- κ B pathway in the liver, potentially contributing to the immunoregulatory effects of this bacterial metabolite. In our model, indole regulated the protein level of NF- κ B p65 and I κ B α rather than their phosphorylation. Our results are in agreement with a previous study demonstrating that indole modulated the NF- κ B pathway in intestinal epithelial cells *in vitro* (25). Then, downstream of NF- κ B signaling, LPS induces activation of the NLRP3 inflammasome pathway that initiates the release of IL-1B and IL-18 (8). Interestingly, indole down-regulated the expression of *Nlrp3*, *Il-1B*, and *Il-18* in PCLS, suggesting an inhibition of this pathway. These effects were not observed (or strongly attenuated) in PCLS prepared from CL-treated mice, in agreement with previous results showing that NLRP3 activation is specific to KC in the liver (41). Last, indole up-regulated the *Ptgs2* gene expression in PCLS, potentially leading to an increased production of prostaglandin E₂ that has immunoregulatory properties in the liver and is protective against liver injury (5, 42, 43). These effects were lost in PCLS prepared from CL-treated mice, consistent with the major role of KC in the expression of PTGS2 in the liver (44).

Tryptophan-derived bacterial metabolites (indole, tyramine, indole-3-acetate, and skatole) have been shown to be (weak) AhR agonists (34, 45–47). Because this receptor tunes inflammatory responses (31), its activation by indole could contribute to its antiinflammatory effects. In PCLS, indole strongly up-regulated *Cyp1a1* and *Cyp1b1* gene expression, confirming that this bacterial metabolite is an AhR agonist in the liver. Interestingly, these effects were amplified in PCLS prepared from *ob/ob* mice, in agreement with the previously reported higher expression of *Cyp1a1* in *ob/ob* mice (48). I3S was previously shown to be also an AhR agonist (49), so we explored whether this major metabolite of indole could have similar antiinflammatory effects than its precursor. However, I3S was not able to reduce LPS-induced up-regulation of proinflammatory genes in PCLS, suggesting that the metabolism of indole by the liver might reduce its beneficial effects. Divergent effects of indole and its derivative, I3S, were already observed regarding their ability to induce the

expression of tight junction proteins in intestinal epithelial cells *in vitro* (26). In contrast, similar to indole, I3S strongly induced the expression of the AhR target genes *Cyp1a1* and *Cyp1b1*, confirming a previous observation that I3S is also an AhR agonist (49). Because both indole and I3S activated AhR while only indole reduced proinflammatory gene expression, we concluded that the antiinflammatory effects of indole are likely to involve other mechanisms than AhR signaling.

Transcriptome profiling revealed that oral administration of indole up-regulated the expression of genes implicated in cholesterol transport in the liver of mice treated with LPS. Endotoxins are known to impair cholesterol homeostasis through a decreased expression of LXR target genes implicated in cholesterol efflux, such as ATP-binding cassette transporters (50, 51). Conversely, LXR activation attenuates inflammatory responses through antagonism of NF- κ B signaling (50). Interestingly, indole prevented the LPS-induced decrease in the concentration of hepatic cholesterol and 4 β -hydroxycholesterol that is an LXR agonist (52). Moreover, the negative correlation between *Nos2* mRNA levels and 4 β -hydroxycholesterol concentration suggest potential links between inflammation and cholesterol metabolism in our model. Further experiments will be required to determine whether the regulation of cholesterol metabolism by indole is a cause or a consequence of its antiinflammatory effects in the liver, but LXR could be the link between both events.

Our data provide strong evidence that indole has antiinflammatory effects in the liver. However, more experiments are needed to prove the physiologic relevance of this finding, notably in the context of chronic liver diseases. For instance, long-term administration of indole to *ob/ob* mice might reveal beneficial effects of this bacterial metabolite that could be relevant for NAFLD or nonalcoholic steatohepatitis. Another important perspective of this work would be to determine if indole produced *in vivo* by the gut microbiota is able to regulate hepatic inflammation. Indeed, a limitation of our study is that the beneficial effects of indole were observed at a relatively high concentration (100 μ M). The estimation of liver exposition to indole *in vivo* is difficult because this bacterial metabolite is continuously produced in the gut and absorbed through the mucosa before being metabolized into I3S in the liver (29, 30). An interesting experiment would be to up-regulate the production of indole by the microbiota and evaluate the consequences for hepatic inflammation. Increasing tryptophan availability for the microbiota would be the most straightforward strategy to promote indole production *in vivo*. Interestingly, tryptophan supplementation in mice was shown to modulate the progression of NAFLD, although conflicting results were obtained and the potential link with the production of indole was not explored (53, 54). In a preliminary experiment, we found that free dietary tryptophan supplementation (0.5% w/v in drinking water) in mice failed to increase indole production, as evaluated by excretion of I3S in the urine, a commonly used proxy for intestinal production of indole (55, 56). Because free amino acids are highly absorbed in the small intestine, they might not reach the distal part of the gut, where the bacterial density is higher (17). In

contrast, some dietary proteins escape the small intestine (<10%) (57) and might be an important source of exogenous tryptophan for the microbiota (17). Indeed, in humans, high-protein diets increase indole production by the microbiota, as shown by an increase in I3S urinary excretion (58). However, increasing dietary protein intake lacks specificity because it also increases the production of many other amino acid-derived bacterial metabolites. Alternatively, increased exposure of the liver to indole could be obtained through administration of indole-producing bacteria (probiotic approach) (34) or through direct administration of indole (postbiotic approach) (59).

In summary, our results show that the tryptophan-derived bacterial metabolite indole has antiinflammatory effects in the liver. We propose that targeting indole might be a promising strategy for the management of liver inflammation. Our work emphasizes the interest of studying microbiota-derived metabolites to discover new regulators of hepatic functions. **[FJ]**

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AUTHOR CONTRIBUTIONS

M. Beaumont, A. M. Neyrinck, and N. M. Delzenne conceived the experiments; M. Beaumont, A. M. Neyrinck, M. Olivares, J. Rodriguez, A. de Rocca Serra, and M. Roumain conducted the experiments; G. G. Muccioli supervised oxysterols measurements; J.-B. Demoulin supervised microarray experiments; P. D. Cani provided *ob/ob* mice; M. Beaumont, A. M. Neyrinck, and N. M. Delzenne wrote the report; M. Olivares, J. Rodriguez, A. d. R. Serra, M. Roumain, G. G. Muccioli, L. B. Bindels, P. D. Cani, P. Evenepoel, and J.-B. Demoulin provided intellectual input and contributed to the writing of the report; and N. M. Delzenne planned and supervised all experiments.

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