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Phosphatidylglycerols are induced by gut dysbiosis and inflammation, and favorably modulate adipose tissue remodeling in obesity

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ABSTRACT: Lipidomic techniques can improve our understanding of complex lipid interactions that regulate metabolic diseases. Here, a serum phospholipidomics analysis identified associations between phosphatidylglycerols (PGs) and gut microbiota dysbiosis. Compared with the other phospholipids, serum PGs were the most elevated in patients with low microbiota gene richness, which were normalized after a dietary intervention that restored gut microbial diversity. Serum PG levels were positively correlated with metagenomic functional capacities for bacterial LPS synthesis and host markers of low-grade inflammation; transcriptome databases identified PG synthase, the first committed enzyme in PG synthesis, as a potential mediator. Experiments in mice and cultured human-derived macrophages demonstrated that LPS induces PG release. Acute PG treatment in mice altered adipose tissue gene expression toward remodeling and inhibited *ex vivo* lipolysis in adipose tissue, suggesting that PGs favor lipid storage. Indeed, several PG species were associated with the severity of obesity in mice and humans. Finally, despite enrichment in PGs in bacterial membranes, experiments employing gnotobiotic mice colonized with recombinant PG overproducing *Lactococcus lactis* showed limited direct contribution of microbial PGs to the host. In summary, PGs are inflammation-responsive lipids indirectly regulated by the gut microbiota *via* endotoxins and regulate adipose tissue homeostasis in obesity.—Kayser, B. D., Lhomme, M., Prifti, E., Da Cunha, C., Marquet, F., Chain, F., Naas, I., Pelloux, V., Dao, M.-C., Kontush, A., Rizkalla, S. W., Aron-Wisniewsky, J., Bermúdez-Humarán, L. G., Oakley, F., Langella, P., Clément, K., Dugail, I. Phosphatidylglycerols are induced by gut dysbiosis and inflammation, and favorably modulate adipose tissue remodeling in obesity. *FASEB J.* 33, 4741–4754 (2019). www.fasebj.org

KEY WORDS: phosphatidylglycerol synthesis · adipocyte lipolysis · lipidomic profiling · serum phospholipidome

Altered lipid homeostasis is central to chronic metabolic disorders, including obesity, type 2 diabetes, liver, and cardiovascular diseases. These diseases are also commonly

characterized by low-grade inflammation, a well-established component of their pathophysiology. Going beyond standard clinical measurements of triglycerides and

ABBREVIATIONS: BMI, body mass index; CRP, C-reactive protein; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, Δ^9 -*cis*; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, Δ^9 -*cis*; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol); EpiAt, epididymal adipose tissue; HGC, high gene count; KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, KEGG Orthologs; LGC, low gene count; LL-pgsA, recombinant strain of *Lactococcus lactis* overexpressing PgsA; LL-WT, *L. lactis* MG1363 containing pGK-empty plasmid; NC, normal chow; OB, obese; OW, overweight; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGS1, phosphatidylglycerol phosphate synthase; PIA, N⁶-phenylisopropyl adenosine; SO, severe obesity

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cholesterol, a better understanding of the lipidome may uncover novel pathways of these diseases and identify predictors of their progression (1). Phospholipids are one of the most diverse human lipid classes (2), yet their roles outside of membrane lipids are only partially understood.

Phospholipids are major structural components of cells and lipoproteins, and alterations in specific phospholipid classes are implicated in the dysregulation of cellular responses such as insulin resistance (3). Going beyond membranes, serum phospholipids are affected by metabolic disorders including overweight (OW) or obesity (4), diabetes (5), and nonalcoholic fatty liver disease (6). These amphiphilic lipids can contribute to disease by altering the structure-function relationship of lipoproteins (7) or as endocrine signaling molecules (8). Along these lines, our team has identified altered circulating phospholipids with nonalcoholic steatohepatitis in severe obesity (SO) (9) as well as improvements in these profiles following bariatric surgery, which results in metabolic and inflammatory improvements (10).

The gut microbiota has emerged as an important factor at the interface of nutrition and host metabolism and inflammation. This symbiotic relationship involves direct and indirect signals from gut microbes that can shape the immune system, and a growing list of these bacteria-derived molecules includes trimethylamine or trimethylamine *N*-oxide (11), hippurate (12), and LPS (13). Increased exposure to LPS in obesity has been termed metabolic endotoxemia and may be an important contributor to chronic low-grade inflammation. More globally, thanks to next generation sequencing, loss of microbial gene richness in the gut is now recognized as a condition associated with chronic inflammation and degraded metabolic health (14–16).

Based on our previous findings that circulating phospholipids are altered in the hepatic portal system of non-alcoholic steatohepatitis patients with morbid obesity (9), a condition highly associated with gut dysbiosis (17), the aim of the current study was to directly investigate the links between gut microbiota status, low-grade inflammation, and serum phospholipids in patients with obesity.

MATERIALS AND METHODS

Study population

Patients with obesity were followed at Pitié-Salpêtrière Hospital (Nutrition Department, Centre de Recherche en Nutrition Humaine Ile de France). First, a group of patients with moderate obesity (NCT01314690; <https://clinicaltrials.gov/>) consisted of 49 participants (8 males, 41 females) who were OW ($n=11$) or obese (OB; $n=38$). The intervention consisted of a 6-wk period on a low-calorie diet enriched with soluble fiber, high protein intake, and low glycemic index carbohydrates as well as a 6 wk weight stabilization phase. Further details of this study have been previously published (14, 18). Baseline measurements from a group of patients with SO, consisting of 63 women, were included in some analyses and have also been previously described (NCT01454232; <https://clinicaltrials.gov/>) (10).

Study approval

Clinical investigations were authorized by the Committees for the Protection of Persons at Hôtel Dieu Hospital, and all

participants gave written and informed consent. For gnotobiotic mice, all procedures previously described were carried out according to European Community Rules of Animal Care and with authorization (3441-2016010614307552) from the French Ministry of Research. For LPS-treated mice, experiments were approved by Newcastle University's Ethical Review Board. Animals were maintained as specific pathogen free according to the Federation for Laboratory Animal Science Associations Guidelines. Work was carried out under project and personal licenses approved by the UK Home Office.

Targeted lipidomics

Targeted lipidomics analysis of phospholipids was performed on lipid extracts from serum by HPLC tandem mass spectrometry using both negative- and positive-ion modes and selective multiple reaction monitoring, which have been previously detailed (7, 10). Phosphatidylglycerols (PGs) were analyzed in positive-ion mode to distinguish from isobaric bis(monoacylglycerol) phosphate (19). Human samples and high-fat diet (HFD)-fed and germ-free mice were analyzed with the above method, whereas all other samples were analyzed using a modified protocol that increased the number of detectable PG species and is described in the Supplemental Data. External standards for the different phospholipid classes were used for calibration curves (Avanti Polar Lipids, Alabaster, AL, USA). The number of PG species quantified in a given experiment varied as a function of sample type (serum, culture medium, or bacterial cell lysate). Human peripheral blood mononuclear cells were isolated from buffy coats of healthy donors over a Lymphoprep gradient (Stemcell), and monocytes were purified by magnetic cell sorting using anti-CD14-coated beads (Miltenyi Biotech, Bergisch Gladbach, Germany).

Fecal metagenomic analysis

Total fecal DNA was extracted and sequenced on a high-throughput ABI SOLiD (Thermo Fisher Scientific, Waltham, MA, USA) sequencer. Reads were cleaned for quality and origin, mapped to the 3.9 million gene reference catalog (20), and normalized by nucleotide length. Observed gene richness was calculated after downsizing the data to 7 million reads. Metagenomic species were constructed from total-sum normalized gene abundance data using the catalog provided by Nielsen *et al.* (20). Computations were performed using the *momr* R package (15, 21). Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/>) Orthologs (KO) annotation was provided by the reference catalog, and each gene was summed into its respective KO. Enrichment of KEGG modules with higher PG levels were tested using the reporter features algorithm [*piano*] (22), which was given Spearman's correlation coefficients and their adjusted *P* values between each KO and total serum PG.

Analysis of public databases

Data for the Gene-Tissue Expression database (23) were downloaded from the Harmonizome project (Tissue Sample Gene Expression Profiles; <http://amp.pharm.mssm.edu/Harmonizome/download>) and log transformed (24). The microarray data from the studies of Wynn *et al.* (25) (with samples restricted to survivors) and Calvano *et al.* (26) (excluding the 2 h time point) were downloaded from the Gene Expression Omnibus (27) with accession numbers GSE26378 and GSE3284, respectively. See Supplemental Data for details.

Mouse experiments

C57BL/6J mice who were 8 to 12 wk old were used in all studies [dietary studies, antibiotic treatment, and lipid

micelles 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, Δ^9 -*cis* (DOPG) injections] and were provided by Janvier Labs (St. Berthevin, France) unless otherwise stated. Mice were housed in the UMS28 facility at la Pitié-Salpêtrière Faculty of Medicine under specific pathogen-free conditions at a constant temperature (23°C) with *ad libitum* access to food and water. All protocols received agreement from the local ethical committees.

OB mouse models

Mice were made OB using 60% HFD (D12492i; Research Diets, New Brunswick, NJ, USA). *ob/ob* mice who were 6 to 8 wk old on a C57Bl6 background were obtained from Janvier Labs.

LPS infusion

Mice obtained from Harlan Laboratories (Indianapolis, IN, USA) were treated with a single intraperitoneal injection of saline or Pure LPS (300 µg; InvivoGen, San Diego, CA, USA). One hour before and 6 h after LPS injection, 1 group also received an intraperitoneal injection of IKK-2 Inhibitor VI (5 mg/kg; MilliporeSigma, Burlington, MA, USA).

Exogenous administration of DOPG in mice

Purified DOPG and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, Δ^9 -*cis* (DOPE) (Avanti Polar Lipids) in chloroform were evaporated and reconstituted into 20% intralipid (I141; MilliporeSigma) to obtain 15 µg/25 µl for DOPG and 300 µg/25 µl for DOPE. Then, intralipid concentration was adjusted to 5% with PBS. Lean mice received 100 µl of 5% intralipid for vehicle or DOPG/DOPE liposomes as intraperitoneal injection. The *ob/ob* mice were intraperitoneally injected with DOPG-only liposomes, which were prepared similarly, except that DOPE was omitted. Composition of 5% intralipid solution is 50 µg/µl triacylglycerol (from soybean oil), 3 µg/µl phosphatidylcholine (PC; from egg phospholipids), and 5.5 µg/µl glycerol as indicated by the manufacturer.

Microbiota depletion with antibiotics

For antibiotic treatment, a cocktail consisting of ampicillin (7.5 mg/ml), neomycin (10 mg/ml), metronidazole (10 mg/ml), vancomycin (5 mg/ml), and amphotericin-B (0.1 mg/ml) were given by oral gavage (0.01 ml/g weight) once per day for 10 d.

Germ-free mice

Serum samples used to compare PG levels in conventional and germ-free female Swiss Webster mice were a generous gift from Robert Caesar (Department of Molecular and Clinical Medicine, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden) and Fredrik Bäckhed (Wallenberg Laboratory, Institute of Medicine, University of Gothenburg) and collected from mice maintained as previously described (28).

Gnotobiotic mice

Lactococcus lactis MG1363 containing pGK-empty plasmid (LL-WT) or pGroESL-psgA (LL-PgsA) plasmids were generated using previously described Materials and Methods (29) detailed in the Supplemental Data. Animals were housed in Anaxem-Micalis germ-free rodent facility (INRA, Jouy-en-Josas, France) and were orally administered with *L. lactis* MG1363 containing

LL-psgA or LL-WT (1×10^9 CFU in 200 µl of sterile PBS per gavage) for 4 d.

Lipolysis

Adipose tissue samples were obtained from elective liposuction in 8 healthy non-OB subjects [44.3 ± 4.6 yr, body mass index (BMI) 23.0 ± 1.1 kg/m²]. After overnight wash-out in complete DMEM supplemented with 10% fetal calf serum, adipose tissue fragments were incubated in fresh medium for 24 h with indicated concentrations of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DPPG; Avanti Polar Lipids) using ethanol as a vehicle. Glycerol release into the medium was recorded during the last 2 h after adding fresh phenol red-free medium containing 2% bovine serum albumin and DPPG. Glycerol concentration in culture medium was measured using a Colorimetric Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) and normalized to cell protein content. The schematic representations of regulation of adipose tissue lipolysis by activating or inhibiting GPCRs and their ligands were as follows: PIA (MilliporeSigma), an adenosine analog acting through G_i-coupled adenosine receptor; adenosine deaminase (ADA; MP Biomedical, Santa Ana, CA, USA), which degrades extracellular adenosine; isoproterenol (ISO; MilliporeSigma), a nonspecific β -adrenergic agonist acting through G_s; and AM6445, an antagonist of G_i-coupled CB1 receptor (gift from Dr. P. Degrace, Université de Bourgogne, Dijon, France).

Gene expression and mRNA quantification

mRNAs were extracted from frozen tissue using commercially available kits (Qiagen, Venlo, The Netherlands) and reverse transcribed, and cDNA were quantified by real-time RT-PCR using qPCR MasterMix Plus for SYBG Green (Eurogentec, Liège, Belgium). Primer efficiency was assessed using standard curves. Expression data were evaluated using the $\Delta\Delta C_t$ method. Glyceraldehyde-3-phosphate dehydrogenase and 18S mRNA were used for normalization.

Statistical analysis

Statistical analyses were performed in the R Language and Environment with packages listed in brackets. Lipidomics data were log₂ transformed for all statistical tests to mitigate skewness, although these data are sometimes presented on the raw scale to enhance interpretation. Spearman correlation was used for bivariate associations (base R). Two-sample comparisons were tested using Welch's *t* test (base R). Both 1- and 2-way ANOVA were used for multiple group comparisons [car]. Longitudinal data were analyzed using mixed effects models with random intercepts for subject (lme4, car, multcomp). Following a significant interaction, simple effects were tested using Welch's *t* tests. Following a significant main effect, *post hoc* comparisons were made using generalized linear hypothesis tests with the Bonferroni-Holm adjustment.

RESULTS

Gut dysbiosis is associated with increased serum PGs

Targeted lipidomics can provide deep profiling of specific lipid groups. As such, our method successfully quantified low-abundance classes such as PGs, phosphatidylserines, and phosphatidic acids in human and mouse serum, in

addition to highly abundant PCs and phosphatidylethanolamines (PEs) (Fig. 1A, B). Importantly, the relative abundance of each phospholipid class was relatively similar between humans and mice (Fig. 1A, B). For example, PG(34:1), PG(36:2), and PG(36:1) together comprised 75% of the total PG and shared the same rank order across species, validating the use of clinical and animal models to study these phospholipids.

We have examined the relationship between the serum phospholipidome and the gut microbiome in OW/OB subjects (14). Almost half of the phospholipid species in serum, distributed across several classes, inversely correlated with gut microbiome richness, indicating intricate links (ρ between -0.3 and -0.6 ; Fig. 1C). A high correlation between PG and PE, both highly abundant phospholipids in microbes, was observed (Supplemental Fig. S1). Dichotomizing patients into high gene count (HGC) and low gene count (LGC) at a 480,000 gene threshold has previously revealed increased metabolic risk and low-grade inflammation in LGC individuals (14, 15). PGs were the most increased phospholipid class and elevated by $\sim 50\%$ in LGC subjects (Fig. 1D, E). At the class level, other phospholipids were also elevated in LGC group, including PE. In agreement with the high correlation between PG and PE, many PE species were also elevated in the LGC group (Supplemental Fig. S2A). Conversely, a fiber-enriched hypocaloric diet, which improves metagenomic richness (14), resulted in normalization of serum PGs in LGC individuals to that of HGC (Fig. 1F). PE concentrations showed similar evolution, albeit that PE levels also declined in HGC individuals (whose richness slightly decreased with weight loss (14) (Supplemental Fig. S2B). Relative to baseline, diet-associated changes in serum PG concentrations were more marked in the LGC group, with a greater effect achieved during active weight loss than weight stabilization (Fig. 1G). Thus, both cross-sectional and longitudinal studies identified a strong link between gut dysbiosis and altered PG and PE phospholipids.

Serum PGs are associated with metabolic endotoxemia potential and low-grade inflammation

First focusing on metagenomic abundance of KO reflecting bacterial PG metabolism, PG synthesis genes were not correlated with serum PG levels (adjusted $P > 0.05$; Fig. 2A), indicating equal potential for PG synthesis among bacterial communities irrespective of overall gene richness. However, an untargeted analysis of metagenomic functional capacity revealed several KEGG modules that were significantly correlated with serum PG levels (all adjusted $P < 0.01$; Fig. 2B). Of particular note were the positive associations with 2 modules involved in LPS biosynthesis. In this analysis, PE levels associated non-significantly with the PE synthesis module (Supplemental Fig. S2C, D).

This observation prompted us to examine the associations between PG and PE phospholipid levels and low-grade inflammation in an extended patient sample that included the OW/OB subjects and a patient cohort with SO (Supplemental Table S1). Serum PGs were positively

correlated with serum IL-6 (Fig. 2C) and C-reactive protein (CRP; Fig. 2D), and inversely associated with the anti-inflammatory adipokine adiponectin (Fig. 2E). Importantly, the correlations between PG and IL-6 and adiponectin remained after adjustment for percent body fat, percent android fat, age, and fasting triglycerides using multiple linear regression (Fig. 2F). These data suggest that PGs may be inflammation-responsive lipids that are elevated in gut dysbiosis. Regarding serum PEs, we found no association with CRP ($\rho = -0.02$), negative correlation with IL-6 ($\rho = -0.20$, $P < 0.05$), and positive correlation with adiponectin ($\rho = 0.32$, $P < 0.001$), which is inconsistent with links to gut dysbiosis and related inflammation.

PGs are induced by endotoxin and inflammation

The Gene-Tissue Expression project was queried to home in on the source of elevated PGs. Phosphatidylglycerol phosphate synthase (PGS1), the first committed enzyme in PG synthesis, is expressed across numerous tissues but was most highly expressed in whole blood (Fig. 2G), even greater than that of lung, where PGs is an important component of surfactant (30–32). The Gene Expression Omnibus database was also searched for experiments in which PGS1 expression was increased. In a data set of children admitted to the hospital with sepsis (25), whole blood PGS1 expression was markedly increased (4-fold increase, adjusted $P < 0.001$; Fig. 2H, top). Another study (26) experimentally corroborated inflammation-related PGS1 regulation, in which PGS1 expression was increased 3.5-fold after intravenous LPS infusion (adjusted $P < 0.001$; Fig. 2H, bottom). Interestingly, PGS1 was regulated independently of the other genes in the PG-synthesis pathway, with the exception of *LPGAT1* (adjusted $P < 0.001$; Fig. 2H, bottom). Changes in expression of genes involved in PE synthesis were not consistent across the 2 data sets (Supplemental Fig. S2E). Next, gene coexpression networks for PGS1 were used to uncover its molecular regulation. This analysis revealed marked agreement between the 2 data sets, and PGS1 expression was directly related to pathogen-associated molecular pattern recognition [*e.g.*, Toll-like and Nod-like receptor signaling (Fig. 2I)]. Given the lack of association between PE and markers of inflammation, a search was not performed for data sets reporting altered expression of PE synthesis genes.

Rodent models identify the host as the primary source of circulating PGs

PGs are considerably enriched in bacterial membranes relative to eukaryotic cells (33, 34), leaving open the possibility that the gut microbiota can contribute directly to circulating PGs in addition to the above documented inflammation-dependent PG induction. Therefore, we engineered a “super PG producing” recombinant strain of *L. lactis* that overexpresses the bacterial homolog of PGS1,

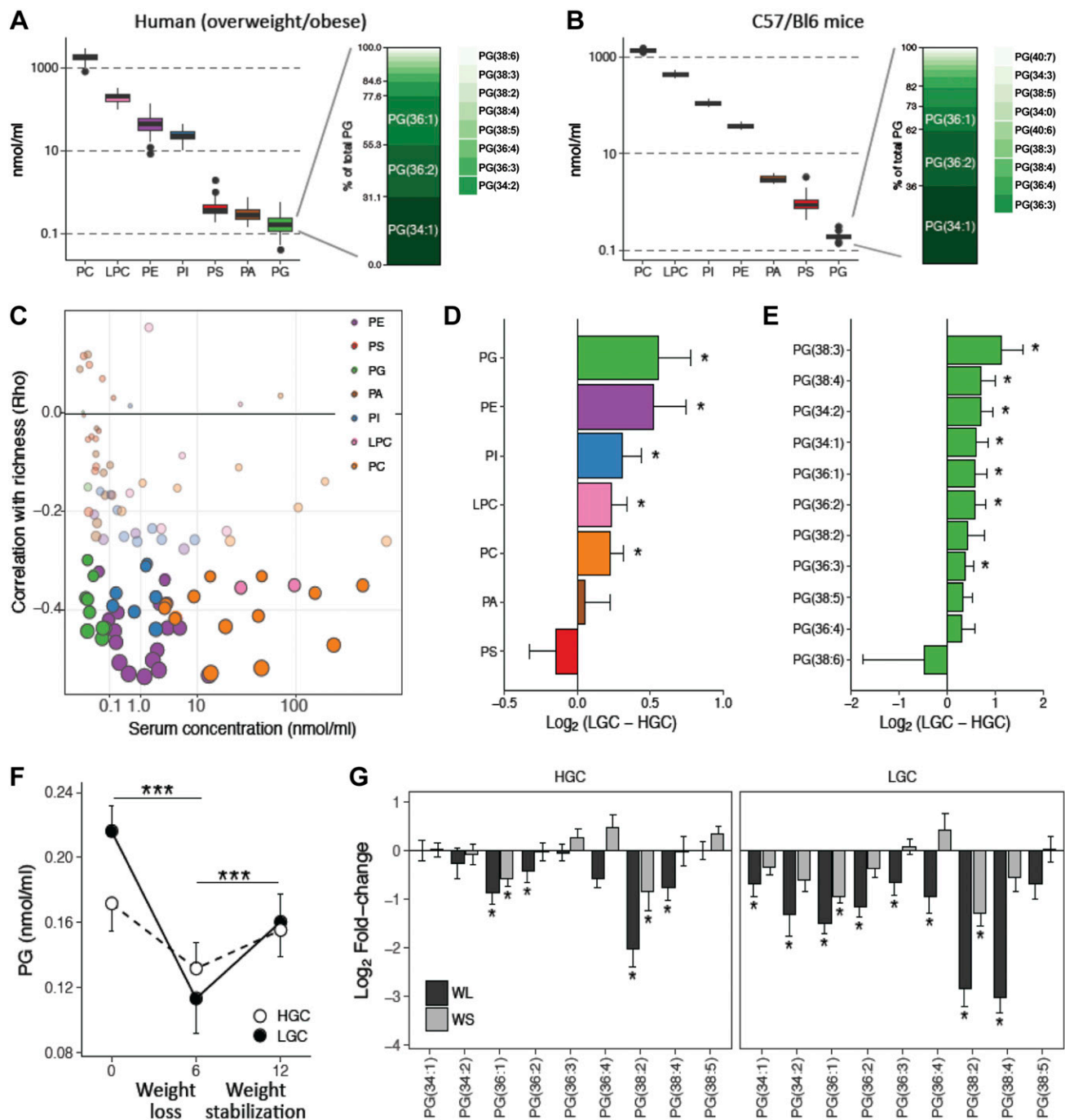


Figure 1. Relationship between serum PGs and human gut microbiota profiles in obesity. *A, B*) Serum concentrations of phospholipids in 49 humans with OW/OB (*A*) and NC ($n = 10$) (*B*) mice, respectively. Differences in green shading of the PG profile are used to differentiate species. Numbers in parenthesis indicate total carbon number and number of double bonds in the fatty acid chains. *C*) Correlation between individual phospholipid species and metagenomic richness in 45 individuals with OW/OB. Color code for phospholipid species as in *D*, size is proportional to correlation coefficient, and darkend points are adjusted $P < 0.05$. *D*) Fold differences in serum phospholipids between LGC ($n = 18$) and HGC ($n = 27$) subjects (with a threshold $< 640,000$ unique genes). *E*) Serum concentrations of PG species. Significant differences between groups are indicated as $P < 0.05$. *F, G*) Change in serum PG with dietary intervention in patients stratified by gene richness. $*P < 0.05$, $***P < 0.001$ vs. baseline after weight loss (WL) or weight stabilization (WS) periods (6 wk each). For baseline, WL, and WS, group sizes are 18, 14, and 15 for LGC, and 27, 21, and 23 for HGC, respectively. Bar height is mean, and error bars \pm SEM.

PgsA (LL-*pgsA*), which were successfully enriched with PGs compared with the wild-type *L. lactis* (LL-WT; Fig. 3A and Supplemental Fig. S3). Compared with gnotobiotic mice colonized with the LL-WT strain, female mice receiving the LL-*pgsA* strain had a statistically significant

18% increase in total serum PG (Fig. 3B). Across similar gnotobiotic status and comparable colonization efficiencies (data not shown), male mice did not respond to LL-*pgsA* gavage by increasing PG serum concentrations, but females did. The largest increase in females occurred

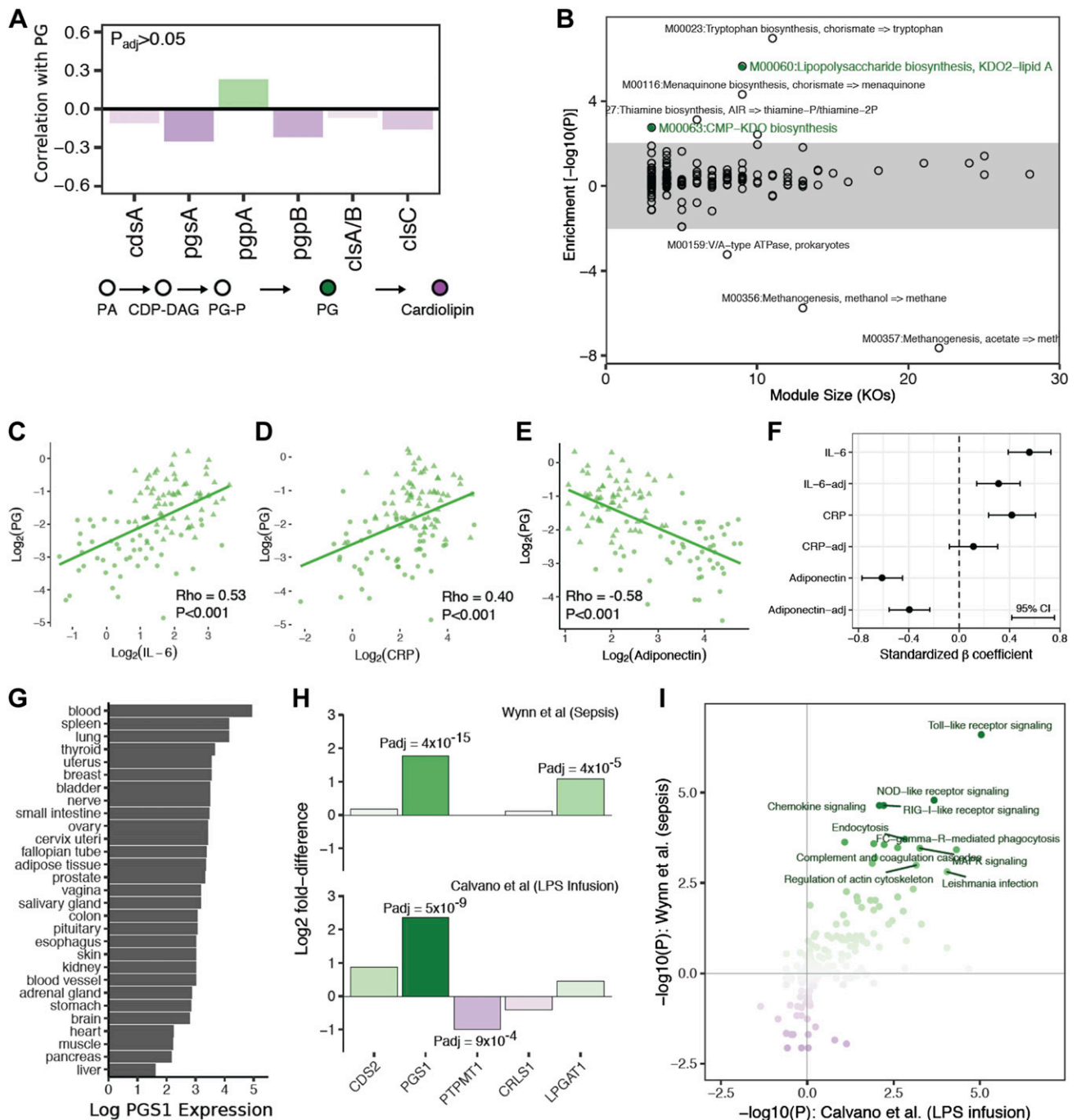


Figure 2. Relationships between PG and metagenomics identify host response to endotoxin. **A**) Correlation coefficients between gut metagenomic abundance of individual KO of the PG/cardiolipin synthesis pathway and serum PG levels in 45 individuals with OW/OB. Gene names and catalytic steps corresponding to KO groups in the x axis are indicated. **B**) Reporter KEGG modules (*i.e.*, enriched gene sets) for correlations between metagenomic KO abundance and serum PG levels in the same subjects. Green modules are involved in LPS biosynthesis. **C–E**) Correlation between serum IL-6 (**C**), CRP (**D**), or adiponectin (**E**) and PG. Circles are the group with OW or OB ($n = 49$), and triangles are the group with morbid obesity ($n = 59$). **F**) Standardized regression coefficients for unadjusted and adjusted (adj; percent fat mass, percent android fat, serum triglycerides, and age as covariates) linear regression models. **G**) Log-transformed PGS1 expression from the Gene-Tissue Expression database. **H**) Effect of sepsis (top; $n = 21$ control and 81 septic shock) or intravenous LPS (bottom; $n = 6$ at 0 and 6 h post-LPS) on PG synthesis-related gene expression from 2 studies extracted from the Gene Expression Omnibus database. **I**) Reporter KEGG pathways for the correlations between PGS1 and all other genes in each data set. The top 10 most significant pathways are labeled. P_{adj} is Benjamini-Hochberg adjusted P value.

with saturated species, namely PG(32:0), PG(34:0), and PG(36:0) (Fig. 3C); however, these are low abundance species, and therefore the effect on total PG was modest.

The direct contribution of the gut microbiota to serum PG levels was also tested following a microbiota-depleting course of antibiotics that recapitulates the germ-free

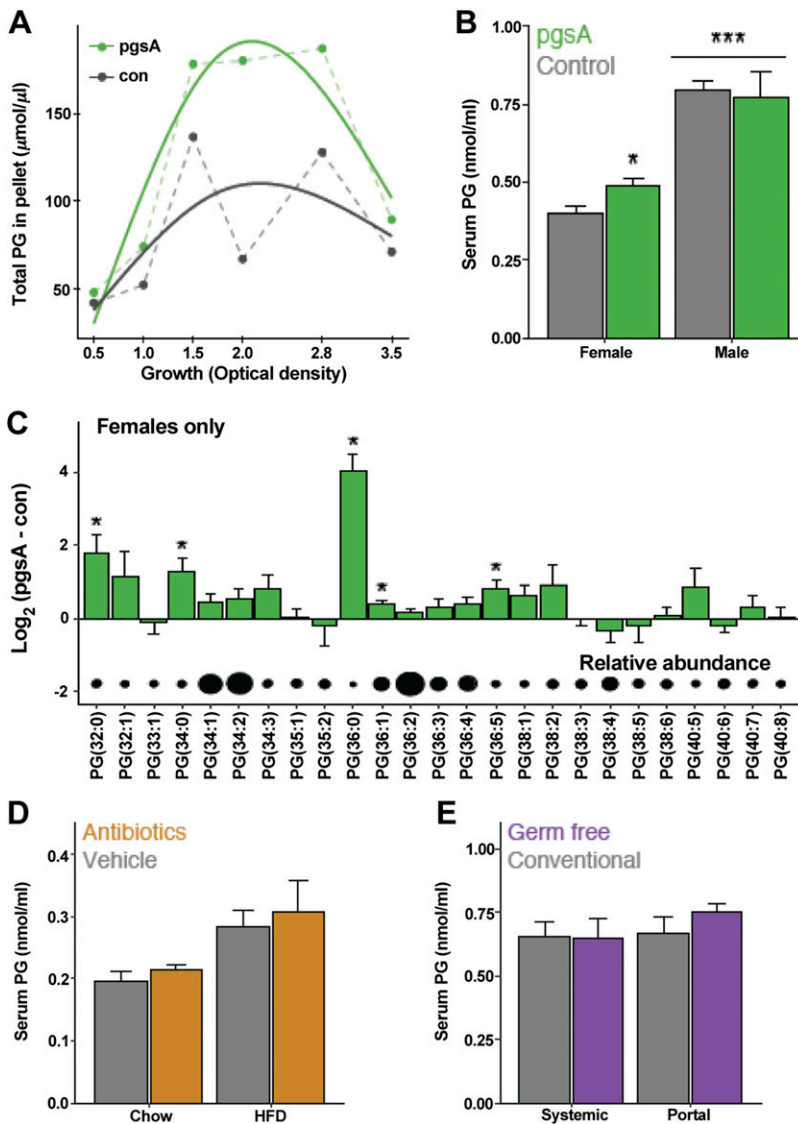


Figure 3. Gut bacteria relationship to host serum PG concentrations. **A)** PG content of bacterial pellets collected during growth of recombinant *Lactococcus* LL-pgsA or LL-WT (control) with 1 culture per optical density. Optical density 0.5–1.5 are the exponential growth phase, and 2.0–3.5 are the stationary growth phase. **B)** Total serum PG in gnotobiotic mice treated for 4 d with recombinant LL-pgsA overexpressing pgsA or empty vector control ($n = 3/\text{group}$). The horizontal bar indicates significant difference between males and females. **C)** Relative change in serum PG species between pgsA overexpressing and control strain treated females. Circle size is proportional to the relative abundance of the PG species in serum; asterisks indicate a significant difference between mice receiving pgsA vs. control *Lactococcus* ($n = 3/\text{group}$). **D, E)** Total serum PG in antibiotic or vehicle treated (**D**; $n = 10/\text{group}$) and conventional or germ-free (**E**; $n = 5/\text{group}$) mice, respectively. $*P < 0.05$, $***P < 0.001$. Bar height is mean, and error bars \pm the SEM.

phenotype without altering body weight (Supplemental Fig. S4A–C). In such microbiota-depleted mice, neither total PG nor individual PG species were altered with normal chow (NC) or HFD (Fig. 3D and Supplemental Fig. S4D), and PE slightly declined (Supplemental Fig. S4F, G). Serum PG and PE levels were both unaltered by germ-free housing of mice (Fig. 3E and Supplemental Fig. S4H). Thus, though gut microbes can increase host PG levels directly under certain conditions, at least in female mice, PG production by the host appears to be the primary contributor to circulating PGs.

Direct link from inflammation to PG synthesis

To directly demonstrate regulation of PG synthesis by inflammation, mice were treated with intraperitoneal LPS with or without an IKK2-inhibitor. LPS injection doubled total serum PG, which was fully blunted by the inhibitor (Fig. 4A, B). To validate this effect in endotoxin-sensitive human cells, we treated primary human monocyte-derived macrophages, either in the naïve state (M0) or after M2 polarization (M2), with a broad range of LPS doses. After 24 h, both M0 and M2 demonstrated a curvilinear

dose response to the release of PG into the culture medium, with a more responsive effect in M2 cells (Fig. 4C, D).

The expression of PGS1 mRNA across a number of metabolic tissues was quantified in HFD-fed OB mice, which developed inflammation and endotoxemia. In control mice, PGS1 expression in adipose tissue depots and intestine was greater than in liver (Fig. 4E). PGS1 mRNA was increased by HFD in several adipose tissue depots, with an increase of 80% in epididymal adipose tissue (EpiAT) and 46% in subcutaneous fat pads (Fig. 4F). PGS1 gene expression was strongly correlated with both *Cd114* and *Tlr4* gene expression in EpiAT across controls and HFD mice (Fig. 4G, H). Furthermore, over half of the top 15 serum phospholipids positively correlated with EpiAT Tlr4 mRNA (Fig. 4I). Collectively, these experiments identify PG as inflammation-responsive phospholipids.

Exogenous PGs promote gene expression related to adipose tissue remodeling

We next investigated whether PG could exert metabolic effects by applying an acute treatment with exogenous PG

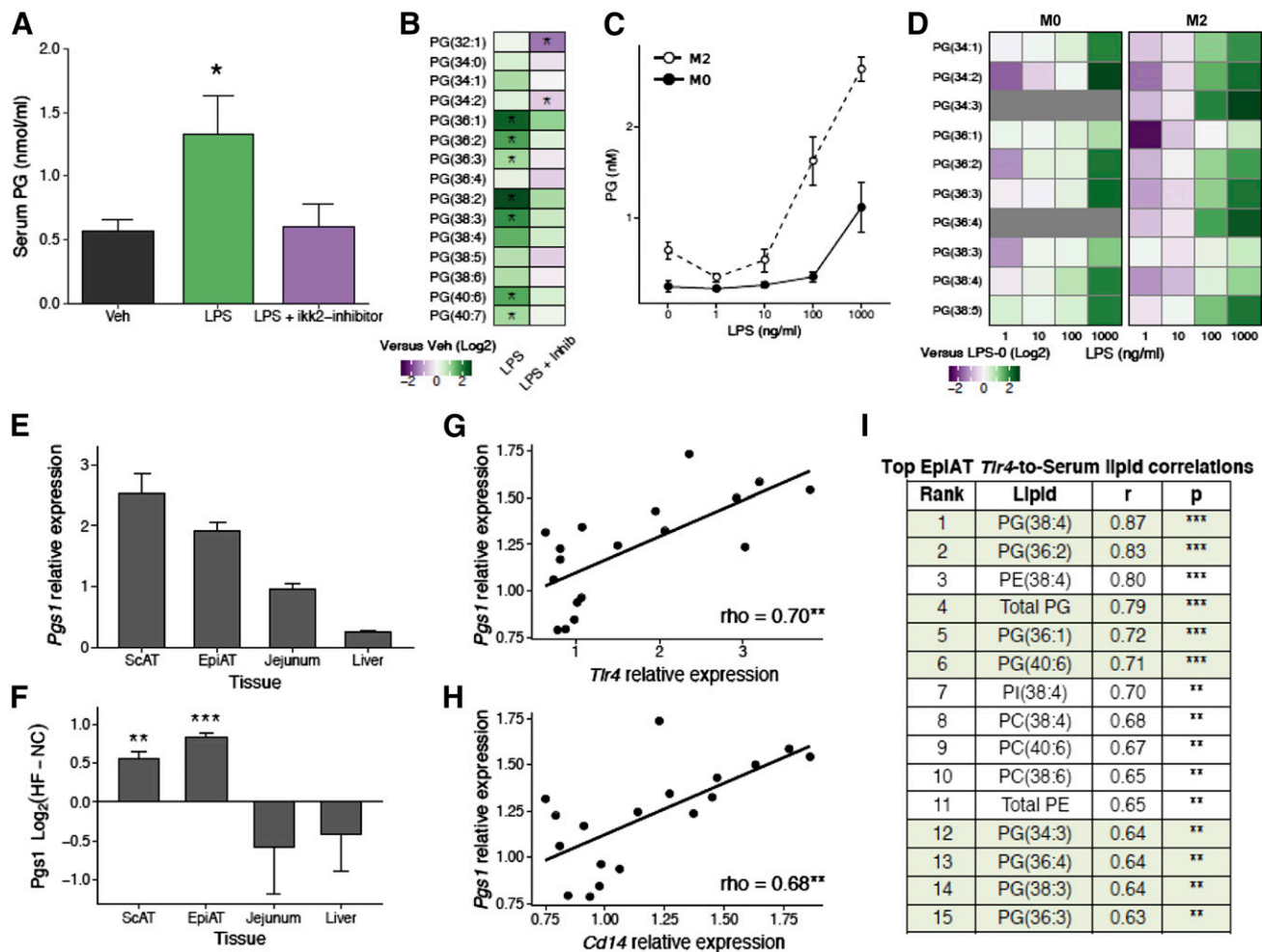


Figure 4. LPS-induced PG response in mice and cell models. *A, B*) Serum PG 24 h after single intraperitoneal injection of 300 μ g LPS with or without 5 mg/kg IKK-2 inhibitor VI ($n = 4$ –5/group). *C, D*) Release of PG into the serum-supplemented culture medium in naive (M0) or IL-4-polarized macrophages (M2) from primary human monocytes. To generate M0 monocyte-derived macrophages, CD14⁺ cells were cultured with 10 ng/ml M-CSF. After 7 d, M0 cells were cultured with 10 ng/ml IL-4 for 24 h to generate M2 monocyte-derived macrophages. M0 and M2 were stimulated with LPS for 24 h before collecting supernatants for lipidomics. All conditions were analyzed in triplicate. *E, F*) PGS1 expression (relative to glyceraldehyde-3-phosphate dehydrogenase) across different tissues in NC-fed mice ($n = 8$). ScAT, Subcutaneous adipose tissue. *E, F*) HFD ($n = 10$) (*E*) vs. NC-fed ($n = 8$) mice (*F*). *G, H*) Correlation between PGS1 and *Tlr4* (*G*) and *Cd14* (*H*) expression (relative to *18S*) in EpiAT. *I*) The top correlations between serum lipids and EpiAT *Tlr4* expression (relative to *18S*) across NC and HFD mice. Green highlight indicates PG species. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Bar height is mean, and error bars \pm the SEM.

and PE, both highly correlated in human serum (Supplemental Fig. S1). Lean chow-fed mice received a daily dose of DOPG and DOPE (0.5 and 10 μ g/g, respectively) in a 5% intralipid vehicle, injected intraperitoneally for 5 consecutive days. PG treatment increased 187 and decreased 127 adipose tissue genes compared with vehicle controls (false discovery rate < 5%; Supplemental Table S2). Pathway analysis indicated significant alterations in genes related to the regulation of TLR by endogenous ligands and tissue remodeling (Fig. 5A). Extracellular matrix-related genes included *Col4a5*, *Col4a6*, *Col6a6*, and *Fbln2*, all related to structural components, and M2-macrophage markers such as *Mgl2* (Cd103b), *Clec10a* (Cd301), and *Cd163* (Fig. 5B and Supplemental Table S2). The absence of changes in body weight, food intake, or fat mass (data not shown) suggested direct PG effects. PG potential on adipose tissue remodeling was also observed in an OB setting,

as treatment of *ob/ob* mice with DOPG alone or vehicle confirmed the PG-induced increases in M2-related genes (Fig. 5C). No significant effects on M1-related genes were observed (data not shown). As a whole, these data indicate that PG may favor adipose tissue remodeling and maintenance of homeostasis in obesity.

PGs inhibit adipose tissue lipolysis

We next explored if PG might affect adipose tissue lipid metabolism, and we therefore examined whether PGs would modulate lipolysis. Intracellular cAMP, regulated by GPCR, is critical to adipocyte lipolysis (Fig. 6A). Human adipose tissue explants were highly responsive to lipolytic modifiers (Fig. 6B). We observed that treatment of adipose tissue fragments with DPPG up to 1 μ M dose-dependently decreased glycerol release in basal conditions

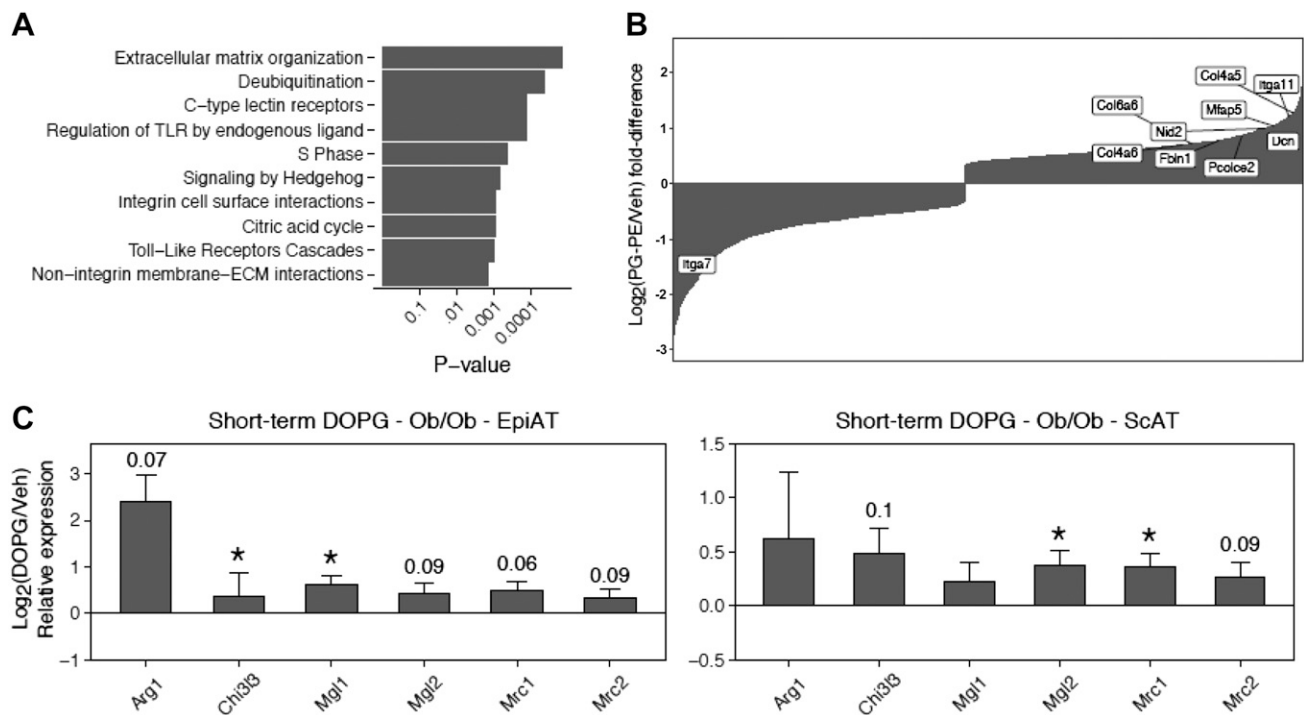


Figure 5. DOPG treatment in mouse models. *A*) Gene set enrichment of EpiAT genes regulated by 5 d daily DOPG/DOPE treatment in NC-fed mice ($n = 5/\text{group}$). *B*) Fold difference in genes across the microarray, with genes from significant sets highlighted. *C*) Relative expression of genes involved in adipose tissue remodeling following 7 d daily treatment (short term) with DOPG in *ob/ob* mice ($n = 5/\text{group}$). Gene expression is relative to *18S* expression. * $P < 0.05$. Bar height is mean, and error bars \pm SEM.

($P < 0.05$; Fig. 6C). Importantly, DPPG was still able to reduce glycerol release when isoproterenol was combined with N6-phenylisopropyl adenosine (PIA), an adenosine receptor agonist (Fig. 6D), but had no effect in the presence of the β -adrenergic agonist isoproterenol, alone (Fig. 6E), indicative of an inhibitory G protein (G_i) mediated effect of DPPG rather than inhibition of a stimulatory G protein (G_s) dependent formation of cAMP or activation of cAMP catabolism by phosphodiesterases. Indeed, a dose-dependent response to PIA in the presence or absence of increasing DPPG concentrations (Fig. 6F) indicated a left-shifted dose-response curve. Glycerol release was almost totally inhibited with 40 nM PIA in the absence of DPPG, whereas 20 nM PIA was sufficient in the presence of 0.2 μM DPPG. We found no evidence that PG could inhibit lipolysis indirectly *via* endogenous antilipolytic molecules. Glycerol release remained suppressed by DPPG despite depletion of extracellular adenosine antilipolytic by adenosine deaminase (Fig. 6G) or addition of AM6445, a CB1 receptor antagonist (Fig. 6H). Similarly, DPPG did not stimulate extracellular release of lactate, another antilipolytic metabolite (Fig. 6I). Together, these data suggest that exogenous PGs may favor the maintenance of adiposity by modulating adipocyte lipid mobilization.

PG levels associated with adiposity in mice and humans

Total serum PG was $\sim 50\%$ higher in HFD-induced OB mice compared with NC controls, a difference which

exceeded the other elevated phospholipid classes, PI, PC, and PE (Fig. 7A). In HFD mice, where PG species homogeneously increased (Fig. 7B), PE species were evenly increased or decreased depending on chain length and unsaturation (Supplemental Fig. S5). PG was also the most affected phospholipid class with increasing obesity grade in humans, as patients with SO (mean percent body fat, 49.9 ± 3.6) had ~ 3 -fold higher levels of serum PG compared with OW/OB (mean percent body fat, 39.4 ± 6.6) (Fig. 7C), with all individual PG species affected (Fig. 7D). Importantly, PG elevation with body fat occurred in generally normolipidemic patients, as fasting triglycerides and cholesterol were not increased in SO compared with OW/OB (Supplemental Table S1). Furthermore, a positive correlation across the range of BMI was observed for PG concentrations ($\rho = 0.63$, $P < 0.001$) but not PC (Fig. 7E), indicating that the PG/corpulence relationship was not simply because of globally elevated phospholipids. Notably, PE declined with obesity severity as well as LPC (Fig. 7C) and negatively associated with BMI (Fig. 7E). Thus, in mice as well as humans, elevated PGs are a signature of increased adiposity, which corroborates PG potential to preserve adipose tissue expandability in the presence of catabolic inflammation.

DISCUSSION

This report sheds new lights on the metabolic role of PGs, a poorly understood phospholipid class. Our work follows recent lipidomic studies that reported altered circulating

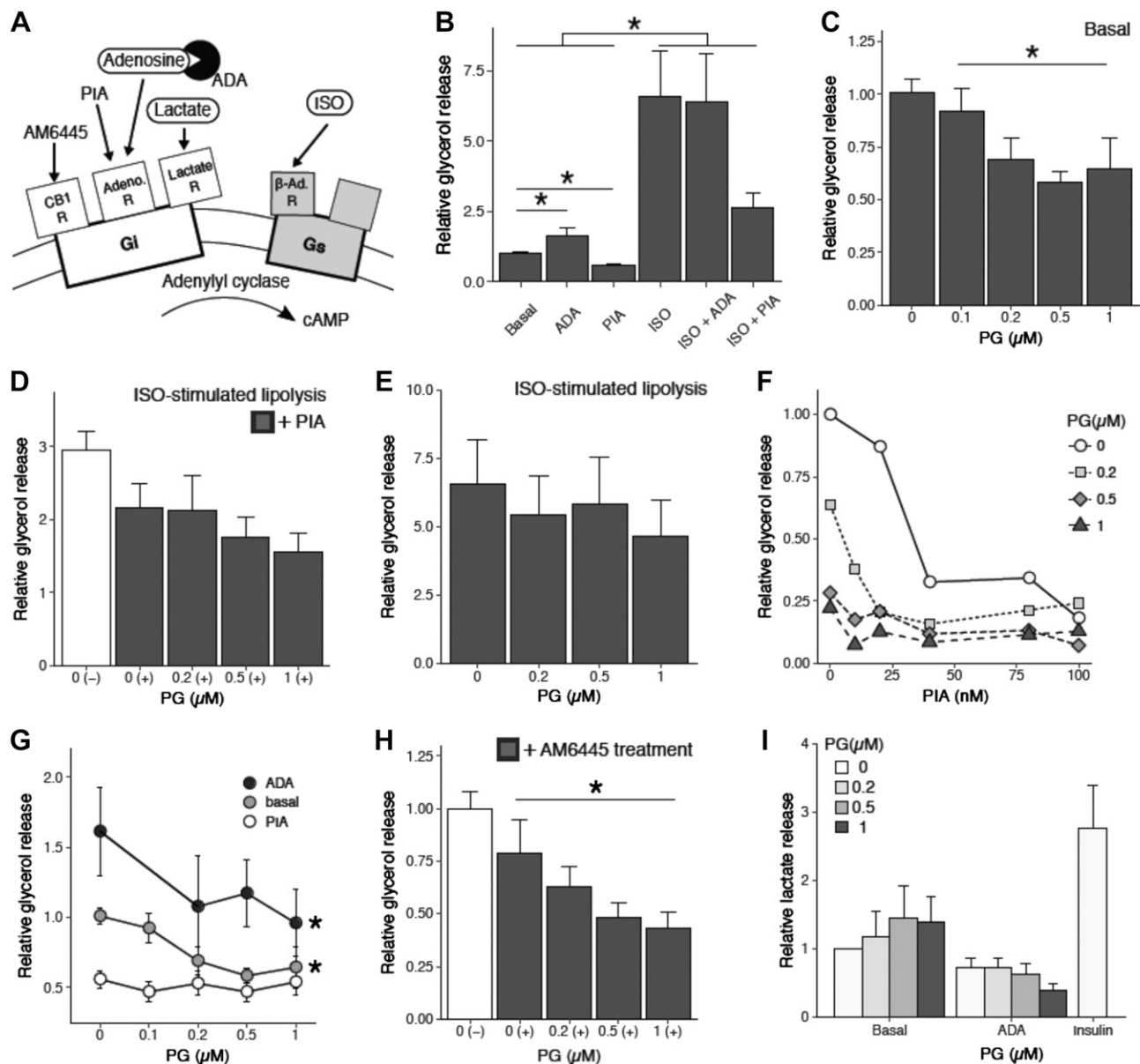


Figure 6. PG inhibits *in vitro* adipose tissue lipolysis. A) Schematic representation of regulation of adipose tissue lipolysis by activating of inhibiting GPCRs and their ligands: PIA (MilliporeSigma), ADA (MP Biomedical) ISO, and AM6445. B–H) Relative glycerol release as a lipolytic index of adipose tissue samples treated for 4 h with the indicated effectors and Dipalmitoyl-PG (PG). Relative glycerol release is calculated from values corrected for tissue protein content. Bars are the mean \pm SEM from 4 to 8 independent experiments with subcutaneous adipose tissue samples of non-OB donors. B) Pairwise comparisons by Student's *t* test between effectors. C–H) Significance of PG effects in dose response curves are tested by Kruskal-Wallis or Friedman test. F) Dose response to PIA in the presence of PG (a representative experiment is shown). I) Lactate release into the culture medium. Values are means of triplicate dishes. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

PG concentrations in different metabolic diseases, including diabetes and nonalcoholic steatohepatitis (5, 9, 10). However, this is the first study to demonstrate a relationship between gut dysbiosis and low-grade inflammation in the regulation of serum PG levels in obesity. Moreover, we demonstrated that PGs are induced by endotoxin and act in a negative feedback loop in inflammation to modulate adipose tissue response in obesity.

Low-grade inflammation is a general comorbidity in metabolic diseases, likely initiated by diverse alterations linked to fatty acid excess, tissue accumulation of reactive oxygen species, gut microbiota imbalance, and presumably

other factors such as genetic susceptibility. Intricately linked within each other, it is impossible to identify a single cause to metabolic inflammation, but recent studies have depicted a prominent contribution of gut microbiota dysbiosis. Low metagenomic richness is associated with altered metabolic and inflammatory conditions (15, 35), but the molecular mechanisms behind these links are unclear. To this end, we report profound alterations in the circulating phospholipidome of patients with low metagenomic richness, most notably PG, PE, and PI. Though the current report focused on PGs, an otherwise poorly understood phospholipid, the relationship between the microbiota and other

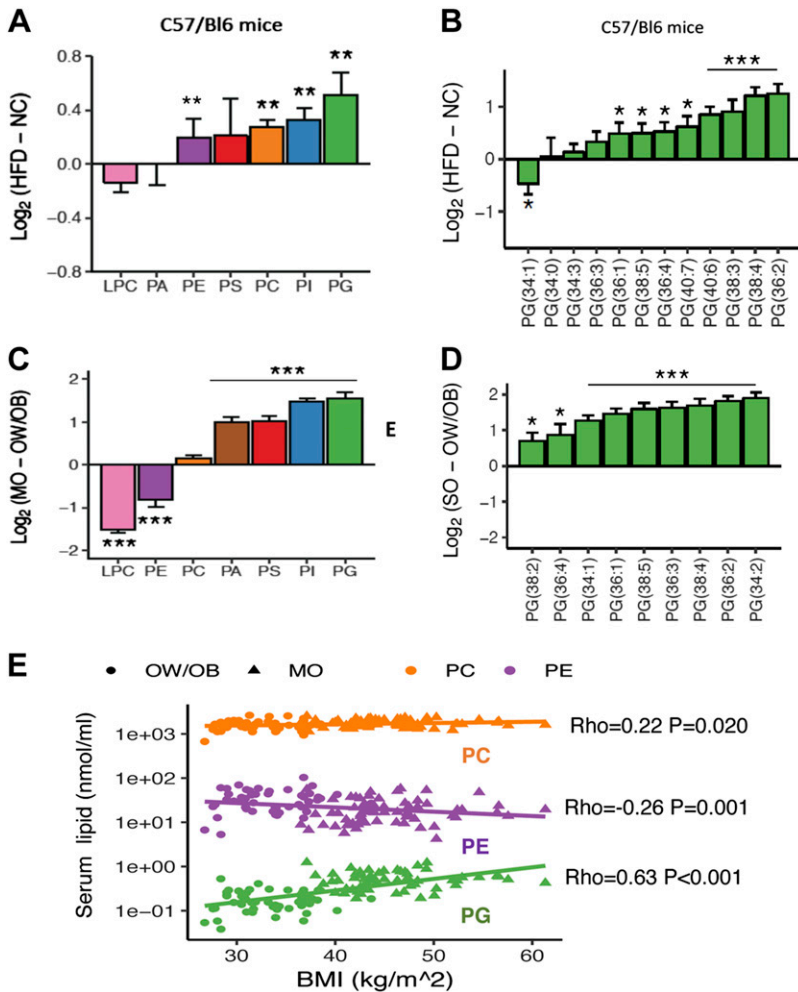


Figure 7. Serum PG concentrations correlate with increasing obesity grade in mice and humans. *A, B*) Differences in serum concentrations of total phospholipid classes (*A*) or individual PG species (*B*) between mice fed HFD *vs.* NC (10 mice/group). *C, D*) Comparison of phospholipid concentrations (*C*) or individual PG species (*D*) in 49 patients with OW/OB *vs.* 63 with SO or morbid obesity (MO), respectively. *E*) Correlation between serum PG and PC across patient body mass index (BMI; circles are OW/OB, *n* = 49). Triangles are those with MO. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the corresponding control within the same phospholipid class. Bar height is mean, and error bars \pm SEM. *E*) Correlations between total serum phospholipid classes and BMI across subjects with OW/OB and SO.

phospholipid classes offers important future work. Serum phospholipids correlate with increasing BMI in large epidemiologic cohorts (4), and our findings suggest that the gut microbiota may be an important contributor to these changes. However, other inflammatory mediators, including other phospholipids, cannot be definitively excluded from this regulatory pathway. Furthermore, our study did not include normal weight individuals. Whether changes in gut microbiota in healthy controls would affect PG levels and long-term metabolic risk requires an independent investigation.

We found that PGs were positively associated with microbiomes enriched with endotoxin-synthesis genes and associated with markers of inflammation. Furthermore, elevation of PG levels by LPS was directly demonstrated in mice and human cells. Though PG synthesis is ubiquitous, it might be particularly important in endotoxin-responsive leukocytes. That PGS1 expression is increased in adipose tissue of OB mice, which become infiltrated by an abundance of macrophages and other leukocytes (36), corroborates the important role of inflammation in the control of circulating PG levels. Notably, previous studies in mice have demonstrated that the gut microbiota regulates adipose tissue macrophage activation and recruitment *via* TLR signaling (28, 37). In line with those studies, increased serum PGs in mice following

HFD-induced obesity are found with increased PGS1 expression in adipose tissue. The primary mechanism for inflammation-dependent increased PG levels appears to be due to increased synthesis (as indicated by increased PGS1 expression) rather than degradation of cardiolipin, a potential store of PG, where even under severe cardiolipin degradation PG levels are unaltered (38). Given that numerous inflammatory pathways are up-regulated in obesity, an important question for future studies is to determine if TLR is a required inflammatory pathway that triggers PG synthesis (39). Though PE levels were initially related to gut dysbiosis, PE abundance at the class level was not consistently associated with markers of inflammation in our study.

We also provided evidence that elevated PG levels may participate in the defense of adipose tissue function in the context of low-grade inflammation. Short-term treatment in mice demonstrated that PGs can induce transcriptional regulation of M2-like macrophage polarization genes in adipose tissue. PGs were also found to reduce adipose tissue lipolysis *ex vivo*, thereby potentially moderating fatty acid leak from OB adipose tissue. The strong association between PGs and adiposity in mice and humans is consistent with PG being a signal for the preservation of adipose tissue stores. Consistent with the characterized anti-inflammatory effect of PG in the lung (31, 32), the

anti-lipolytic and proremodeling actions of PG indicate that these phospholipids may favor an overall protective effect on adipose tissue remodeling. In the future, it will be important to determine whether PGs cooperate with the broader class of bioactive lipids involved in the resolution of inflammation (40), such as those derived from ω -3 polyunsaturated fatty acids that can stimulate phagocytic M2-like macrophages (41). The possibility that PGs act alongside proresolving lipids would explain the counterintuitive observation that a gut dysbiosis-related metabolite would exert beneficial metabolic actions in obesity similar to other beneficial mediators, such as resolvin D1 and IL-10, which are also elevated yet dysfunctional in obesity (42). An important next step will be to ablate the induction of PG synthesis in response to microbiota-derived inflammation *in vivo*.

Given that PGs are enriched in the membrane of bacterial cells (33), we initially hypothesized that microbial PGs would contribute to serum levels in the host. However, our findings on host PGS1 regulation as well as those with gnotobiotic mice colonized with recombinant PG-overproducing bacteria that resulted in a sex-specific and moderate modulation of host PG levels, presumably through direct transfer of fully saturated but an otherwise rare subset of PG species (32:0, 34:0, and 36:0), indicate that this is likely a minor contributing event. Moreover, we found that serum PGs were unaltered in microbiota-depleted mice, demonstrating that host cells are the primary source of circulating PGs. Interpretation of these results, however, is limited by the fact that inoculation by a single species, *L. lactis* in particular, does not recapitulate the complex ecosystem of the gut microbiota. Furthermore, the sex-dependent response is unexpected.

In summary, we propose a novel PG-dependent regulatory loop in the complex relationship linking gut microbiota to host metabolism and inflammation. We demonstrate excessive PG production and release related to low-grade inflammation and gut dysbiosis, which may in turn favor continued adipose tissue expansion in the face of catabolic inflammatory signals. These findings highlight PG as a novel inflammation-related lipid actor in metabolism and emphasize the need for a deeper understanding of its role in metabolic diseases. FJ

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AUTHOR CONTRIBUTIONS

B. D. Kayser generated the hypotheses, conducted experiments, analyzed the data, and wrote the manuscript together with K. Clément and I. Dugail; M. Lhomme conducted lipidomic analyses; E. Prifti performed bioinformatics for metagenomic data; C. Da Cunha, F. Marquet, F. Chain, and I. Naas designed and conducted the experiments; V. Pelloux designed and conducted microarray and gene expression experiments; M.-C. Dao and J. Aron-Wisniewsky performed data analysis; A. Kontush (lipidomics), S. W. Rizkalla and J. Aron-Wisniewsky (clinical investigations), L. G. Bermúdez-Humarán and P. Langella (recombinant bacteria and gnotobiotic mice), and F. Oakley (endotoxin treatments) designed and supervised the experiments; K. Clément and I. Dugail generated the hypotheses, analyzed data, and supervised the experiments and clinical investigations; K. Clément and I. Dugail are the guarantors of this work; and all authors provided critical review of the manuscript.

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