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The endogenous bioactive lipid prostaglandin D_2-glycerol ester reduces murine colitis via DP1 and PPARγ receptors

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ABSTRACT: Cyclooxygenase-2 (COX-2) has long been implicated in the pathogenesis of inflammatory bowel diseases (IBDs). COX-2 is mostly known for the production of prostaglandins (PGs) from arachidonic acid. However, it also metabolizes the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide into the less well-studied bioactive lipids PG-glycerol esters (PG-Gs) and PG-ethanolamides (PG-EAs or prostamides). We previously showed that PGD_2-G, a product of 2-AG oxygenation by COX-2, has anti-inflammatory effects. Therefore, we used the dextran sulfate sodium (DSS)-induced model of colitis in mice to explore the role of PGD_2-G in murine models of IBD. Colon inflammation was assessed using macroscopic and histologic scores, myeloperoxidase activity, and expression of inflammatory mediators by real-time quantitative PCR and ELISA. We also compared the effects of PGD_2-G with those of PGD_2 and PGD_2-EA. Finally, we used receptor antagonists to gain mechanistic insight into the receptors responsible for the observed effects. PGD_2-G reduced DSS-induced colitis, but PGD_2 and PGD_2-EA did not have the same effect. Furthermore, we showed that PGD_2-G is an agonist of the PGD_2 receptor 1 (DP1) and that some of the effects of PGD_2-G were blocked by antagonism of peroxisome proliferator-activated receptor γ and DP1. Therefore, PGD_2-G could be one of the products from the COX-2/prostaglandin D synthase axis to exert beneficial effects in colitis.—Alhouayek, M., Buisseret, B., Paquot, A., Guillemot-Legris, O., Muccioli, G. G. The endogenous bioactive lipid prostaglandin D_2-glycerol ester reduces murine colitis *via* DP1 and PPARγ receptors. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: Crohn's disease · eicosanoids · prostanoid · 15d-PGJ2-G

Inflammatory bowel diseases (IBDs) are chronic, incurable diseases of the gastrointestinal tract. They are characterized by immune cell infiltration, epithelial cell destruction, and mucosal ulceration of the intestine and/or colon. Although not completely elucidated, the pathogenesis of IBD is believed to be multifactorial, involving genetic susceptibility and environmental factors leading to an aberrant immune and inflammatory response. Nonsteroidal anti-inflammatory drugs, which inhibit the cyclooxygenase (COX-1 and COX-2) enzymes, and selective COX-2 inhibitors (termed COXIBs) are among the environmental factors thought to increase the risk of relapse of IBD (1). However, experimental and clinical data are inconsistent in this regard, thus giving COX-2 a controversial role in IBD (2). The COX enzymes, followed by specific prostaglandin (PG) synthases, constitute the first step for PG production from arachidonic acid. Therefore, the success of nonsteroidal anti-inflammatory drugs gave PGs a bad reputation as proinflammatory lipids.

PGs have been shown to play divergent roles depending on the PG and the setting. For instance, PGD_2 derived from COX-2 and hematopoietic PGD synthase (HPGDS) has been shown to be a mediator of allergic inflammation and asthma (3). Nonetheless, other studies showed anti-inflammatory effects of COX-2, HPGDS, and PGD_2 and its metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (4, 5). In the more specific context of IBD, HPGDS and PGD_2 seem to play an anti-inflammatory role (6, 7), although this might be dependent on the receptor activated by PGD_2 because

ABBREVIATIONS: 2-AG, 2-arachidonoylglycerol; 15d-PGJ_2-G, 15-deoxy-$\Delta^{12,14}$-PGJ_2-G; ABHD6, α/β hydrolase domain 6; ACN, acetonitrile; COX-2, cyclooxygenase-2; DP, prostaglandin D; DSS, dextran sulfate sodium; HPGDS, hematopoietic prostaglandin D synthase; HTAB, hexadecyltrimethylammonium bromide; IBD, inflammatory bowel disease; MAGL, monoacylglycerol lipase; MMP, matrix metalloproteinase; mPGES1, microsomal prostaglandin E synthase 1; MPO, myeloperoxidase; MRM, multiple reaction monitoring; PG, prostaglandin; PG-EA, prostaglandin-ethanolamide; PG-G, prostaglandin-glycerol ester; PPAR, peroxisome proliferator-activated receptor; qPCR, quantitative PCR; TNBS, trinitrobenzene sulfonic acid

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the PGD_2 receptor (DP)1 plays a protective role in experimental colitis, whereas DP2 has a deleterious role (8). On the other hand, the increase in microsomal prostaglandin E synthase 1 (mPGES1) expression that accompanies the increase in COX-2 expression in murine models of IBD pointed to PGE_2 as the culprit in the detrimental effects of COX-2 in colon inflammation. Indeed, the concordant induction of COX-2 and mPGES1 was shown to lead to a preferred production of PGE_2 over PGD_2 (9).

Other metabolites of COX-2 that could be implicated in its effects on colon inflammation are the glycerol ester (PG-G) and ethanolamide (PG-EA) derivatives of the PGs. These PG-Gs and PG-EAs are formed after the oxygenation of the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide, respectively, by COX-2 (10). These PG-Gs and PG-EAs were characterized about a decade ago (11), although not much was known about their effects until more recently. However, they are not just metabolic intermediates in PG synthesis or mere degradation products of the endocannabinoids but are gradually being unraveled as new bioactive lipids with biologic effects different from the corresponding endocannabinoid or PG (10, 12). The endocannabinoids, along with their biosynthetic and hydrolytic enzymes and their receptors, have pleiotropic effects in the organism, including in inflammatory settings (13, 14). The endocannabinoid system is altered in colitis, and endocannabinoids have been shown to exert anti-inflammatory effects in the setting of colon inflammation (15–17). The effects of the endocannabinoids are often mediated by their receptors, the cannabinoid receptors CB_1 and CB_2. However, we and others have shown that some of the effects of endocannabinoids are independent of cannabinoid receptor activation and blocked by COX-2 inhibition (18, 19). Indeed, we showed that increasing 2-AG levels reduces LPS-induced activation of macrophages and that this effect was blocked by HPGDS inhibition and substrate-selective inhibition of COX-2 (19). Moreover, we also showed that PGD_2-G, a product of 2-AG metabolism by COX-2 and PGDS, has anti-inflammatory properties, whereas PGE_2-G and $PGF_{2\alpha}$-G, derived from COX-2 followed by mPGES1 or PGF synthase, respectively, increase LPS-induced macrophage activation (19).

Because the COX-2/HPGDS axis is thought to be protective in colitis and because we showed that PGD_2-G decreases macrophage activation, we investigated the effect of PGD_2-G in colitis. We also compared the effects of PGD_2-G with those of PGD_2 and PGD_2-EA because they are also products of the COX-2/HPGDS axis, and not much is known about the biologic effects of PGD_2-EA.

MATERIALS AND METHODS

Chemicals and reagents

PGD_2-G, PGD_2, PGD_2-EA, MK0524 and GW9662 were purchased from Sanbio (Uden, The Netherlands). The lipid standards used for quantification were also from Sanbio. Hexadecyltrimethylammonium bromide (HTAB) and *o*-dianisidine were from Sigma-Aldrich (Bornem, Belgium). Dextran sulfate sodium (DSS) was purchased from TdB Consultancy (Uppsala, Sweden).

Animals

Male C57BL/6 mice (8–10 wk of age) were obtained from Janvier Labs (Le Genest-Saint-Isle, France), housed in standard filter-top cages, and provided food and drinking water *ad libitum*. Animals were kept in a 12-h light/dark cycle (lights off at 6 PM) at controlled temperature (22°C) and humidity. All animal care and experimental procedures were conducted in accordance with the European recommendation 2010/63/EU (transformed into the Belgian Law of May 29, 2013) regarding the protection of laboratory animals. The local ethics committee approved the protocol of the study (study agreement 2010/UCL/MD/022; lab agreement LA1230314).

DSS-induced acute murine colitis model

To induce colitis, 3% DSS was added to the drinking water over a 5-d period, and this solution was replaced with normal drinking water for an additional 2 d. Mice (n = 8 per group) were euthanized on d 7 under isoflurane anesthesia (20). PGD_2-G (0.1, 0.3, or 1 mg/kg) or vehicle (saline/ethanol/tween 80 18:1:1, v/v/v) were administered i.p. once daily starting on the day of addition of DSS to the drinking water (pretreatment group, PGD_2-G I) or on d 5 (treatment group, PGD_2-G II). The d-5 time point was chosen based on previous studies indicating that, at this point, colitis is established based on weight loss and the presence of occult blood in the feces (20). Control mice received only normal drinking water and injections of vehicle. Body weight was monitored daily. PGD_2 (1 mg/kg), PGD_2-EA (1 mg/kg), the DP1 antagonist MK0524 (1 mg/kg), and the peroxisome proliferator–activated receptor (PPAR)γ antagonist GW9662 (1 mg/kg) were administered using the same administration scheme and vehicle as PGD_2-G I. The dose regimen of PGD_2 and PGD_2-EA was based on that of PGD_2-G. Doses of MK0524 and GW9662 were based on previous reports (8, 21–23).

Tissues were removed at the time of death and snap frozen in liquid nitrogen for real-time quantitative PCR (qPCR), myeloperoxidase activity, and HPLC-MS or fixed in 4% buffered formalin for histologic assessment. Before freezing, colons were examined for weight, stool consistency, gross macroscopic appearance, and length.

Macroscopic and histologic grading of colitis

Macroscopic colonic damage was assessed at the time of death based on 2 main characteristics of the pathology: colon length shortening and colon weight gain. The excised colons were measured and weighed, and we determined the colon weight/colon length ratio, which is a reliable and sensitive indicator of the severity and extent of the inflammatory response in colitis (24).

For histologic scoring, small segments of the colon were fixed in 4% buffered formalin overnight and embedded in paraffin. Two sets of 3 serially cut sections (5 μm) were cut at a distance of 100 μm, and the 6 sections were evaluated for each mouse. For this, sections were stained with hematoxylin and eosin, and histologic scores were blindly determined as previously described (25). The histologic score represents the sum of an infiltration score and a tissue damage score. Infiltration was graded semiquantitatively from 0 to 4 as follows: 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate extending in the submucosa; 3, extensive infiltration reaching the submucosa with edema and/or slight infiltration of the muscularis propria; 4, extensive infiltration of the muscularis propria. Tissue damage was graded from 0 to 3 as follows: 0, normal morphology; 1, punctuate mucosal erosions, muscularis mucosa intact; 2, mucosal erosions in large areas and/or deeper areas of ulcerations; 3,

extensive mucosal damage and extension into deeper structures of the bowel wall (*i.e.*, the muscularis propria).

mRNA extraction and real-time qPCR

Total RNA was extracted using the TriPure reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). qPCR was performed in duplicate for each sample using a SYBR Green mix (GoTaq qPCR Master Mix; Promega) with a STEP one PLUS instrument and software (Applied Biosystems, Foster City, CA, USA) as previously described (26). Products were analyzed by performing a melting curve at the end of the PCR reaction. Data are normalized to the 60S ribosomal protein L19 (RPL19) mRNA expression. For each study, we verified that our experimental conditions did not affect the expression of the reference gene. Primer sequences for qPCR are listed in **Table 1**.

Ultraperformance liquid chromatography–MS analysis of PGs and endocannabinoids

Frozen tissues were homogenized in a glass vial containing 8 ml of cold $CHCl_3$, and d_5-2-AG (200 pmol) and d_4-PGE_2 (250 pmol) were added as internal standards. Then, 4 ml of cold methanol and 2 ml of bidistilled water were added to the extraction vials before thorough mixing. After centrifugation (10 min, 700 *g* at 4°C), the organic layer was recovered and evaporated to dryness under a nitrogen stream. Samples were then prepurified by solid-phase extraction using silica as stationary phase. The highly lipophilic compounds were removed using 4 ml $CHCl_3$, and endocannabinoids and PGs were eluted using 3.6 ml of $CHCl_3$-MeOH (8:2, v/v). The fraction of interest was dried under a nitrogen stream, and the dry residue was reconstituted with acetonitrile (ACN)/water (66:33, v/v) for ultraperformance liquid chromatography–MS/MS analysis. Quantification was conducted using a mass spectrometer (Xevo TQ-S; Waters, Milford, MA, USA) coupled to an Acquity UPLC class H (Waters). Analyte separation was achieved using a Kinetex EVO C18 column (1, 7 μm, 2.1 × 100 mm) (Phenomenex, Torrance, CA, USA) connected to a SecurityGuard C18 guard column (Phenomenex) and maintained at 40°C. Mobile phases A and B consisted of H_2O/ACN/acetic acid 94.9:5:0.1 (v/v/v) and ACN/acetic acid 99.9:0.1 (v/v), respectively. The injection volume was 5 μl. The gradient (0.3 ml/min) was designed as follows: transition from 80% A to 62.5% A linearly over 7 min, followed by transition to 95% B linearly to 11 min, followed by 6 min at 95% B and subsequent re-equilibration at 80% A. An electrospray ionization source in positive mode was used. Precursor ions, product ions, multiple reaction monitoring (MRM) transitions, optimal cone voltage, and collision energies were established for each analyte. The MRM mode was used to profile 2 transitions per compound, 1 quantitative (Q) and 1 qualitative (q). The MRM transitions used were: 379,2→287,3 (Q) and 379,2→269,3 (q) for 2-AG; 317,2→90,9 (Q) and 317,2→130,9 (q) for PGD_2 and PGE_2; 317,2→299,2 (Q) and 317,2→90,9 (q) for 15-deoxy-$\Delta^{12,14}$-PGJ_2; 391,3→299,3 (Q) and 391,3→317,2 (q) for PGD_2-G; 373,1→299,2 (Q) and 373,1→317,2 (q) for 15-deoxy-$\Delta^{12,14}$-PGJ_2-G; 384,3→287,3 (Q) and 384,3→269,3 (q) for d_5-2-AG; 321,2→275,0 (Q) and 321,2→134,9 (q) for d_4-PGE_2. Electrospray ionization conditions were set as follows: capillary voltage at 3000 V; desolvatation temperature at 550°C; desolvatation and cone gas flow at 1100 and 170 L/H, respectively; and nebulizer gas flow at 7.0 bar. The autosampler was set at 5°C during sample analysis. For data acquisition and processing, the MassLynx software (Waters) was used. The area under the curve for the peaks of interest was normalized using the area under the curve of the relevant internal standard and then to the tissue weight.

Binding and functional tests

DP1 and DP2 binding experiments were conducted at Eurofins SA (Gosselies, Belgium) using [^{3}H]-PGD_2 as radioligand and membrane from cells recombinantly expressing DP1 and DP2, respectively. Binding of PGD_2 was performed in the same setting as for PGD_2-G. DP1 and PPARγ functional assays were performed at Eurofins-Cerep SA (Celles l'Evescault, France) according to standardized operational protocols. The DP1 assay consisted of measuring cAMP levels upon ligand incubation with

TABLE 1. *Primer sequences*

Gene	Primers, (5′–3′)	
	Forward	Reverse
ABHD6	CTGTCCATAGTGGGGCAAGT	TCAGATGGGTAGTAAGCGGC
COX-2	TGACCCCCAAGGCTCAAATAT	TGAACCCAGGTCCTCGCTTA
DAGLβ	ACCTGATAGTCCTCCTTGTTCTCC	CACTGGATACCTTTTGCCACC
DP1	CACTTTGTGCTGCTGGCTCT	AGGCTTGGAGGTCTTCTGAGT
DP2	CTGAGGCTGACAACTGCTCT	ATGGCAAGGTGGAAGAAAGGA
IL-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
IL-6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
iNOS	AGGTACTCAGCGTGCTCCAC	GCACCGAAGATATCTTCATG
$LYPLA_2$	GTGATGCCCTCCTGGTTTG	ATGTTCTCTGCGGCCTTCTT
MAGL	ATGGTCCTGATTTCACCTCTGGT	TCAACCTCCGACTTGTTCCGAGACA
MIP-1α	AGATTCCACGCCAATTCATC	CTCAAGCCCCTGCTCTACAC
MMP-2	ACACCTACACCAAGAACTTCCG	GGCCAGTACCAGTGTCAGTATC
MMP-9	TCACACGACATCTTCCAGTACCA	TGGAAACTCACACGCCAGAAGA
15-PGDH	GTGGGACCTATCTTGGTTTGG	CATGAGCCCTGCTAATGAAGAC
HPGDS	TGGGAAGACAGCGTTGGAG	AGGCGAGGTGCTTGATGTG
mPGES1	ATGAGGCTGCGGAAGAAGG	GCCGAGGAAGAGGAAAGGATAG
RPL19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
TNF-α	AGCCCCCAGTCTGTATCCTT	GGTCACTGTCCCAGCATCTT

15-PGDH, 15-hydroxyprostaglandin dehydrogenase; DAGLβ, diacylglycerol lipase β; $LYPLA_2$, lysophospholipase A_2; MIP-1α, macrophage inflammatory protein-1α; RPL19, 60S ribosomal protein L19.

Chinese hamster ovary cells expressing the DP1 receptor. The PPARγ functionality was assessed by measuring a coactivator recruitment upon ligand incubation. The assays were performed by operators blinded to the drug assessed. The data were compared in the same experiment to the effect of known agonists and antagonists of these receptors (*i.e.*, BW245c and MK0524 for DP1 and rosiglitazone and GW9662 for PPARγ).

Myeloperoxidase activity

The tissue-associated myeloperoxidase (MPO) assay was performed to quantify the degree of neutrophil infiltration as previously described (25). Briefly, tissue was placed in HTAB buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6) on ice and gently homogenized. The homogenate was centrifuged at 2000 *g* for 10 min and centrifuged at 18,000 *g* for 20 min at 4°C. Seven microliters of supernatant, in duplicate, were then added to 96-well plates together with 200 μl of a solution of 0.167 mg/ml *o*-dianisidine and 500 ppm hydrogen peroxide in 50 mM potassium phosphate buffer at pH 6. Total protein concentration was determined using the DC protein assay (Bio-Rad, Nazareth, Belgium). MPO activity in the supernatant was measured at 460 nm and normalized for protein concentration. Results are expressed as percentage of vehicle.

Cytokine quantification by ELISA

Levels of TNF-α, IL-1β, and IL-6 in tissues were determined by a sandwich-type ELISA technique using the Ready-Set-Go! Kit (eBioscience, Vienna, Austria) following the manufacturer's instructions. Proteins were isolated from the same tissue samples used for real-time qPCR using TriPure reagent (Roche) after RNA extraction according to the manufacturer's instructions. Total protein concentration was determined using the DC protein assay (Bio-Rad) before the ELISA assays were run.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA, USA). We used the 2-tailed Student's *t* test for unpaired values to compare 2 groups and Mann-Whitney test when relevant.

One-way ANOVA with Dunnett's posttest or Kruskal-Wallis test with Dunn's posttest were used when comparing more than 2 groups.

RESULTS

DSS-induced colitis affects prostanoid production

We started our study by assessing the impact of DSS-induced colitis on the expression of COX-2, the PG synthases, and the 2-AG metabolic enzymes. DSS-induced colitis increased the mRNA expression of COX-2 and mPGES1 and decreased the expression of 15-hydroxyprostaglandin dehydrogenase (15PGDH), the enzyme involved in PG degradation (**Fig. 1**). This was accompanied by an increase in PGE_2 levels (**Fig. 2*A***). On the other hand, mRNA expression of HPGDS was decreased in mice with DSS-induced colitis compared with control mice (Fig. 1), as were the levels of the PGD_2 metabolite 15d-PGJ_2, which were below detection limit in the colitis group (Fig. 2*A*). We could not measure PGD_2-G levels in the colon because the ultraperformance liquid chromatography–MS method lacks the sensitivity needed to measure PG-Gs *in vivo* in the small colon samples we used to measure PG levels. Therefore, we performed a new experiment and

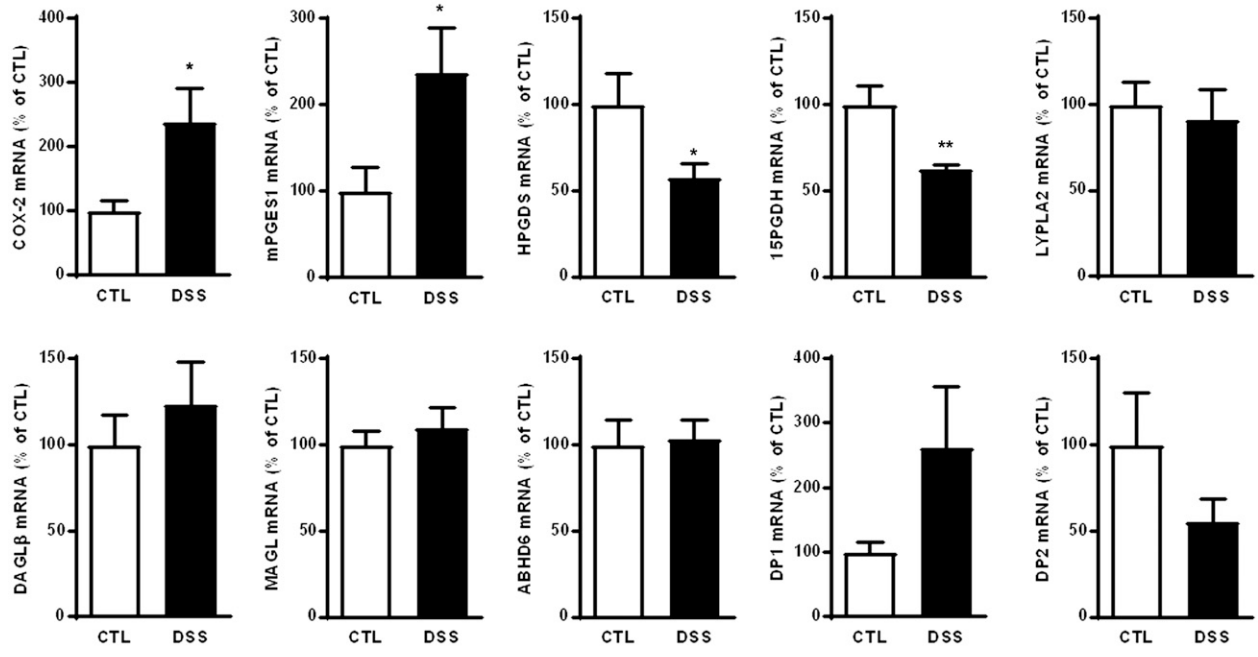

Figure 1. DSS-induced colitis alters the expression of enzymes involved in prostanoid synthesis in the colon. Colitis was induced by the addition of 3% DSS in the drinking water for 5 d, followed by 2 d of normal drinking water. Control mice (CTL) had access to drinking water throughout the study. Mice were euthanized on d 7. mRNA expression was assessed by real-time qPCR. Data were normalized to 60S ribosomal protein L19 mRNA expression and expressed relative to the control group set at 100%. Results are expressed as means ± SEM ($n = 8$ mice/group). *$P < 0.05$, **$P < 0.005$ *vs.* the control group.

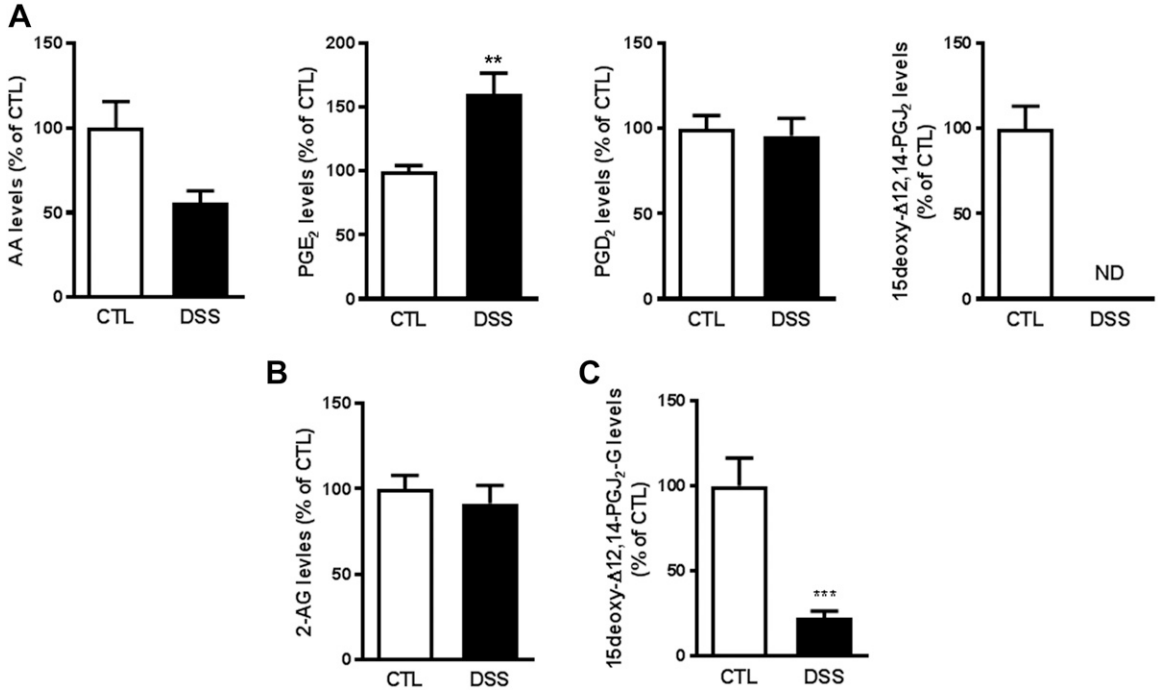

Figure 2. DSS-induced colitis alters prostanoid levels in the colon. Colitis was induced by the addition of 3% DSS in the drinking water for 5 d, followed by 2 d of normal drinking water. Control mice (CTL) had access to drinking water throughout the study. Mice were euthanized on d 7. Levels of PG (*A*), 2-AG (*B*), and 15d-PGJ_2-G (*C*) were measured by ultraperformance liquid chromatography MS. Data are expressed relative to the control group set at 100% and are shown as means ± SEM ($n = 8$ mice/group). $^{**}P < 0.005$, $^{***}P < 0.001$ *vs.* the control group. ND, below detection limit.

attempted to measure PG-Gs using the whole colon of mice (~200 mg). PGD_2-G was below the quantification limit, but we could quantify its metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2-G (15d-PGJ_2-G), which was decreased in DSS-induced colitis compared with controls (Fig. 2*C*).

2-AG levels were not affected by colon inflammation (Fig. 2*B*); nor was the mRNA expression of the enzymes responsible for its biosynthesis [diacylglycerol lipase β, hydrolysis, monoacylglycerol lipase (MAGL), and α/β-hydrolase domain 6 (ABHD6)] (Fig. 1). The metabolic pathways of 2-AG and PG-Gs are intertwined because PG-Gs are produced from COX-2 oxygenation of 2-AG and can be metabolized by its hydrolytic enzymes MAGL and ABHD6 (27). Recently, lysophospholipase A_2 was identified as a PG-G–specific hydrolase (28). However, similarly to MAGL and ABHD6, mRNA expression of lysophospholipase A_2 was not affected by colon inflammation (Fig. 1).

PGD_2-G reduces DSS-induced colitis

To assess the effect of PGD_2-G on colon inflammation, we administered this bioactive lipid (0.1, 0.3, or 1 mg/kg) to mice with DSS-induced colitis starting on d 1 of DSS addition to the drinking water. The colon weight/length ratio is a reliable indicator to assess colitis in mice (24). This ratio is increased in mice with colitis compared with control mice (**Fig. 3*A***) due to an increase in colon weight after edema and inflammatory cell infiltrates and a decrease in colon length due to smooth muscle contractions. PGD_2-G administration decreased this colon weight/length ratio at 1 and 0.3 mg/kg compared with untreated mice with colitis (Fig. 3*A*). We also measured protein expression of the proinflammatory cytokine IL-6 and mRNA expression of other proinflammatory cytokines and chemokines involved in colon inflammation, such as TNF-α, IL-1β, IL-6, and macrophage inflammatory protein-1α. These inflammatory markers were increased by colitis. mRNA expression of COX-2 was also increased after colon inflammation. PGD_2-G administration decreased these proinflammatory markers (Fig. 3*B*, *C*), although only the 1 mg/kg dose of PGD_2-G decreased IL-6 production and TNF-α expression.

Treatment with PGD_2-G after colitis is induced reduces colitis

To further assess the effect of PGD_2-G on colon inflammation, we administered this bioactive lipid (1 mg/kg) to mice with DSS-induced colitis, starting either on d 1 of DSS addition to the drinking water (PGD_2-G I) or starting on d 5 (PGD_2-G II). The latter time point was chosen to see whether PGD_2-G could reduce colon inflammation after colitis was already induced. Both regimens of PGD_2-G administration decreased this colon weight/length ratio compared with untreated mice with colitis (**Fig. 4*A***). Colitis-induced alterations of the colon can also be measured by establishing a histologic score, which was increased in mice with DSS-induced colitis and decreased by PGD_2-G administration (Fig. 4*C*, *D*). Neutrophil infiltration in colitis can be assessed by measuring MPO activity in the colon. Indeed, this enzyme is most

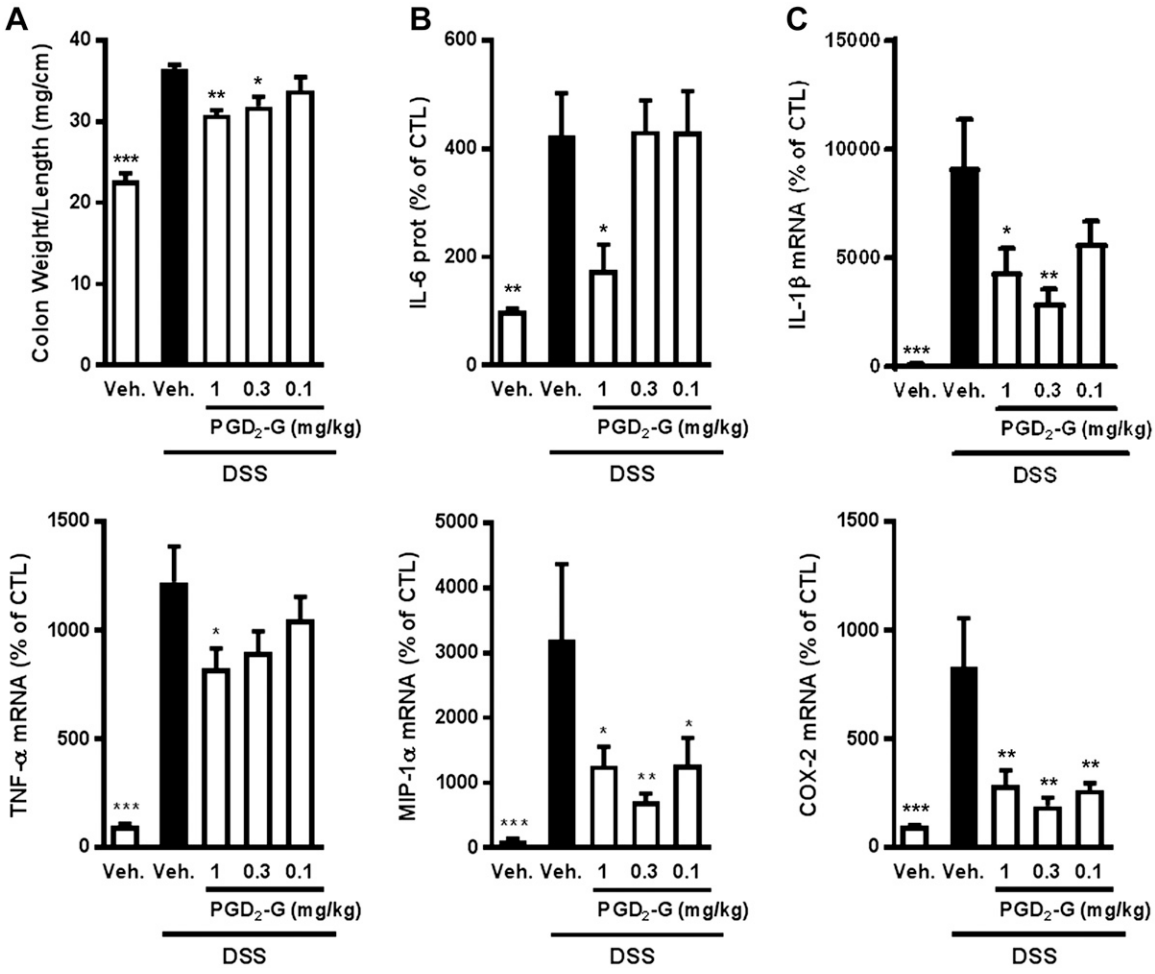

Figure 3. PGD_2-G reduces mucosal injury and inflammatory markers in DSS-induced colitis. Colitis was induced by the addition of 3% DSS in drinking water for 5 d, followed by 2 d of normal drinking water. Mice were euthanized on d 7. PGD_2-G (0.1, 0.3, or 1 mg/kg, i.p.) was administered on the first day of addition of DSS to the drinking water. The control (CTL) group and the untreated DSS group received intraperitoneal injections of vehicle. *A*) Colon weight/length ratio. *B*) IL-6 production was measured by an ELISA assay and expressed as percentage of the CTL group. *C*) mRNA expression was assessed by real-time qPCR. Data were normalized to 60S ribosomal protein L19 mRNA expression and are expressed relative to the control group set at 100%. Results are shown as means ± SEM ($n = 8$ mice/group). $*P < 0.05$, $**P < 0.005$, $***P < 0.001$ *vs.* the DSS-untreated group.

abundantly expressed in neutrophils, and an increase in MPO activity reflects an increase in neutrophil infiltration. As expected, MPO activity was increased in the colon of mice with DSS-induced colitis and decreased after PGD_2-G administration (Fig. 4*B*).

We measured mRNA expression of TNF-α, IL-1β, IL-6, and macrophage inflammatory protein-1α. These inflammatory markers were decreased by PGD_2-G administration (**Fig. 5**). mRNA expression of the enzymes iNOS and COX-2 was also increased after colon inflammation and decreased in mice receiving PGD_2-G (Fig. 5). We also measured mRNA expression of matrix metalloproteinase (MMP)-2 and MMP-9. MMPs were shown to be upregulated in colitis and in human IBD (29, 30). Although their role in the pathogenesis of IBD is not fully elucidated, MMPs are considered potential biomarkers of the disease, and their inhibition remains an area of interest (31). DSS-induced colitis increased mRNA expression of MMP-2 and MMP-9 in the colon. This increase was counteracted by administration of PGD_2-G (Fig. 5).

PGD_2-G decreased colon inflammation even when administered at d 5 after DSS addition to the drinking water (*i.e.*, when colitis is established), although this effect was not statistically significant for all the parameters measured.

PGD_2 and PGD_2-EA do not have the same effects as PGD_2-G on colon inflammation

To see if the other products of the COX-2/PGDS axis could also have an anti-inflammatory effect in colitis, we administered PGD_2 and PGD_2-EA at a similar dose as PGD_2-G (*i.e.*, 1 mg/kg) to mice with DSS-induced colitis. Although PGD_2 and PGD_2-EA decreased the colon weight/length ratio, they did not affect MPO activity, protein production, or mRNA expression of proinflammatory markers (**Fig. 6*A–C***). The fact that PGD_2 does not reproduce the effects of PGD_2-G on colon inflammation rules out the possibility that the effects of PGD_2-G are due to its hydrolysis into PGD_2.

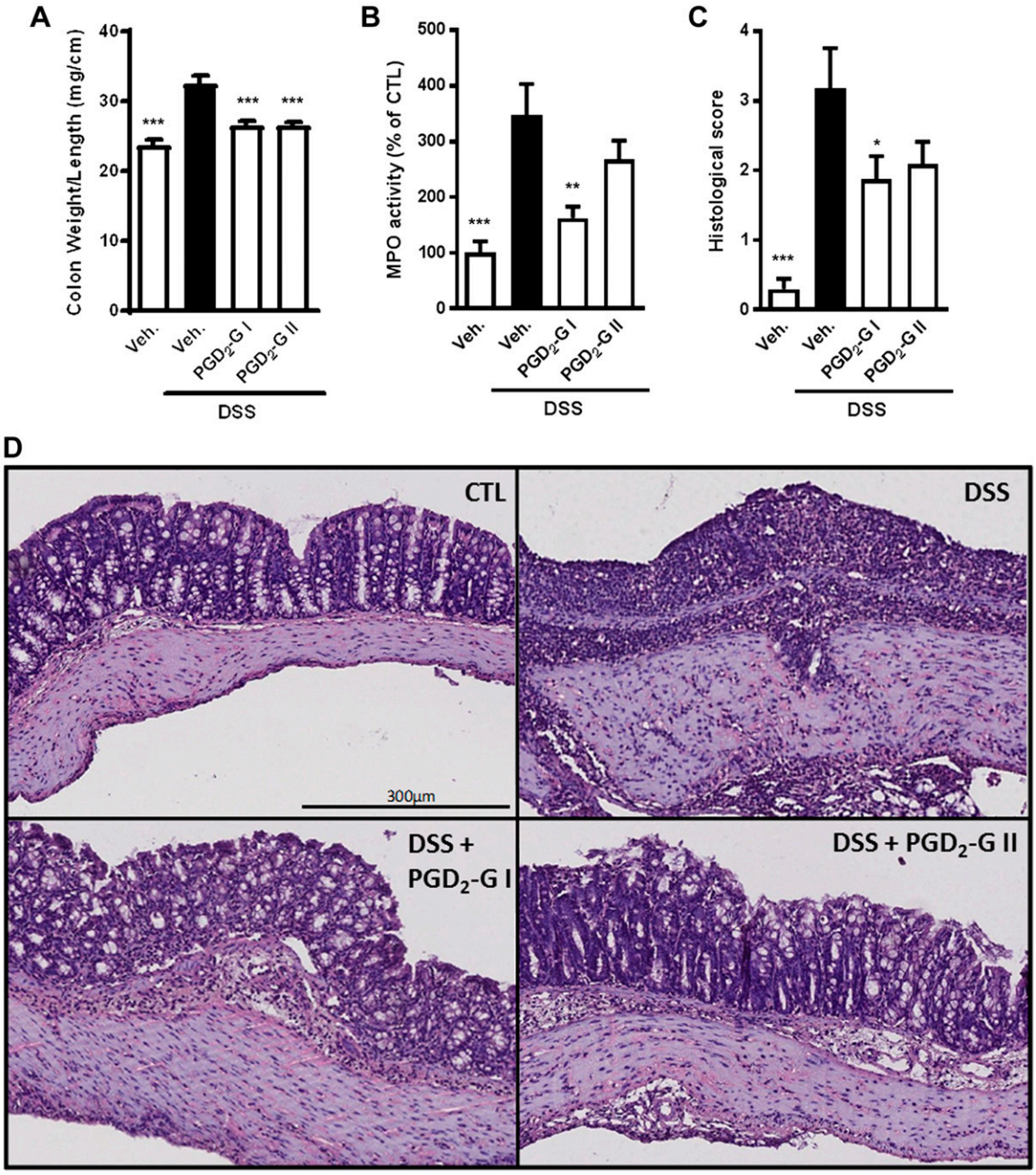

Figure 4. PGD_2-G reduces mucosal injury in DSS-induced colitis. Colitis was induced by the addition of 3% DSS in the drinking water for 5 d, followed by 2 d of normal drinking water. Mice were euthanized on d 7. PGD_2-G (1 mg/kg, i.p.) was administered once daily starting on the first day of addition of DSS to the drinking water (PGD_2-G I) or on d 5 (PGD_2-G II). The control (CTL) group and the untreated DSS group received daily intraperitoneal injections of vehicle. *A*) Colon weight/length ratio. *B*) MPO activity. Data are expressed relative to the control group set at 100%. *C*) Histologic score, blindly established after hematoxylin and eosin staining of the colon. *D*) Representative images of hematoxylin and eosin staining of the colon. Results are shown as means ± SEM ($n = 8$ mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ *vs.* the DSS-untreated group.

PGD_2-G is a ligand of DP1 and DP2

To date, there are no receptors described for PGD_2-G. Therefore, we assessed whether PGD_2-G could bind the PGD_2 receptors DP1 and DP2. Although this was not previously described for PGD_2-G, it was shown that PGE_2-EA binds to the 4 receptors of PGE_2 (*i.e.*, the PGE_1 to PGE_4 receptors) (32). We found that PGD_2-G binds to the DP1 receptor with a similar affinity as PGD_2 (IC_{50} values of 39 and 36 nM, respectively). At the DP2 receptor, the affinity of PGD_2-G is 30 times lower than the affinity of PGD_2 (IC_{50} values of 412 and 12 nM, respectively). We also wanted to determine if PGD_2-G was able to activate the DP1 receptor, thus acting as an endogenous agonist. We found that PGD_2-G behaved as an agonist, showing an EC_{50} value of 22 nM. 15d-PGJ_2-G, a chemical metabolite of PGD_2-G, is an agonist of PPARγ (33). Therefore, we also assessed whether PGD_2-G could activate PPARγ but found that it did not interact with this receptor.

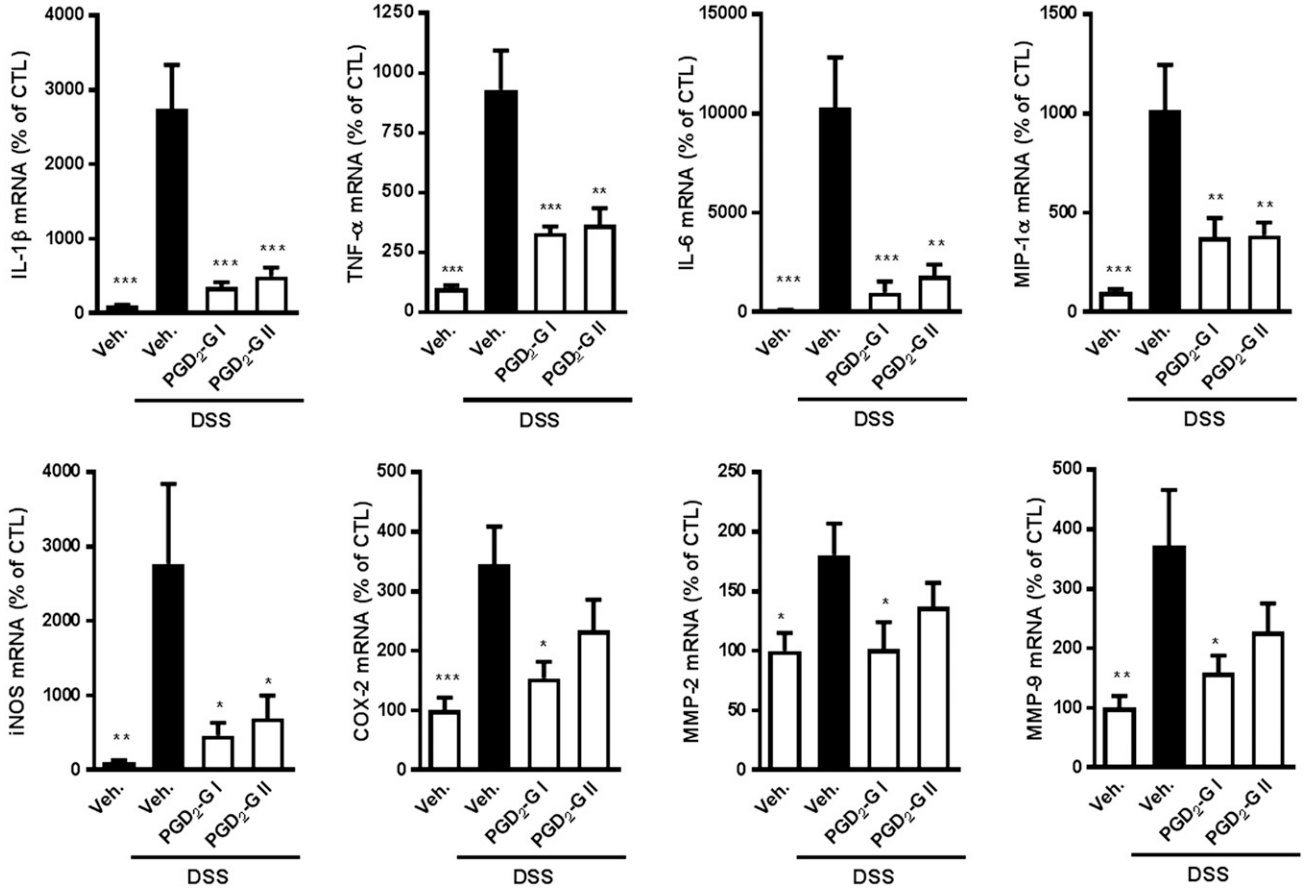

Figure 5. PGD_2-G reduces inflammatory markers in DSS-induced colitis. Colitis was induced by the addition of 3% DSS in the drinking water for 5 d, followed by 2 d of normal drinking water. Mice were euthanized on d 7. PGD_2-G (1 mg/kg, i.p.) was administered once daily starting on the first day of addition of DSS to the drinking water (PGD_2-G I) or on d 5 (PGD_2-G II). The control (CTL) group and the untreated DSS group received daily intraperitoneal injections of vehicle. mRNA expression was assessed by real-time qPCR. Data were normalized to 60S ribosomal protein L19 mRNA expression and expressed relative to the control group set at 100%. Results are expressed as means ± SEM ($n = 8$ mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ *vs.* the DSS-untreated group.

The effects of PGD_2-G are blocked by antagonism of DP1 and PPARγ

Because we showed that PGD_2-G binds to the DP1 receptor and because we know that 15d-PGJ_2-G, a chemical metabolite of PGD_2-G, is an agonist of PPARγ (33), we wanted to assess if one or both of these receptors mediates the effects of PGD_2-G on colon inflammation. Furthermore, DP1 and PPARγ have been put forth as playing a beneficial role in colitis, whereas DP2 activation was shown to be deleterious (8, 34). Therefore, we used the DP1 antagonist MK0524 (35) and the PPARγ antagonist GW9662 (36) to determine whether these pharmacological agents would prevent the effects of PGD_2-G. When assessed alone for their effects on DSS-induced inflammation, both antagonists decreased the DSS-induced increase in the colon weight/length ratio, and MK0524 decreased DSS-induced mRNA expression and protein production of IL-6 (Supplemental Fig. 1). However, the 2 antagonists had no effect on the other parameters measured, such as MPO activity and mRNA expression of COX-2, IL-1β, and TNF-α, although GW9662 slightly decreased TNF-α protein production (Supplemental Fig. 1).

We then administered them together with PGD_2-G. The DP1 antagonist MK0524 blocked some of the effects of PGD_2-G on colon inflammation, such as the colon weight/length ratio, MPO activity, mRNA expression of IL-1β, and protein production of TNF-α (**Fig. 7**). The PPARγ antagonist GW9662 also blocked some of the beneficial effects of PGD_2-G, including MPO activity and TNF-α and IL-6 expression (Fig. 7). These results clearly suggest that both the DP1 receptor and the PPARγ receptor are involved in the effects of PGD_2-G on colon inflammation.

DISCUSSION

The aim of this study was to assess the effect of PGD_2-G, a bioactive lipid derived from the oxygenation of the endocannabinoid 2-AG by COX-2 and PGDS, in a murine model of colitis, and to compare it with the other products of the COX-2/PGDS axis (*i.e.*, PGD_2 and PGD_2-EA). To our knowledge, this is the first report of DP1 activation by PGD_2-G and of anti-inflammatory effects of PGD_2-G in colitis.

We also assessed the effect of colitis on the prostanoid system. We showed that COX-2 and mPGES1 expression

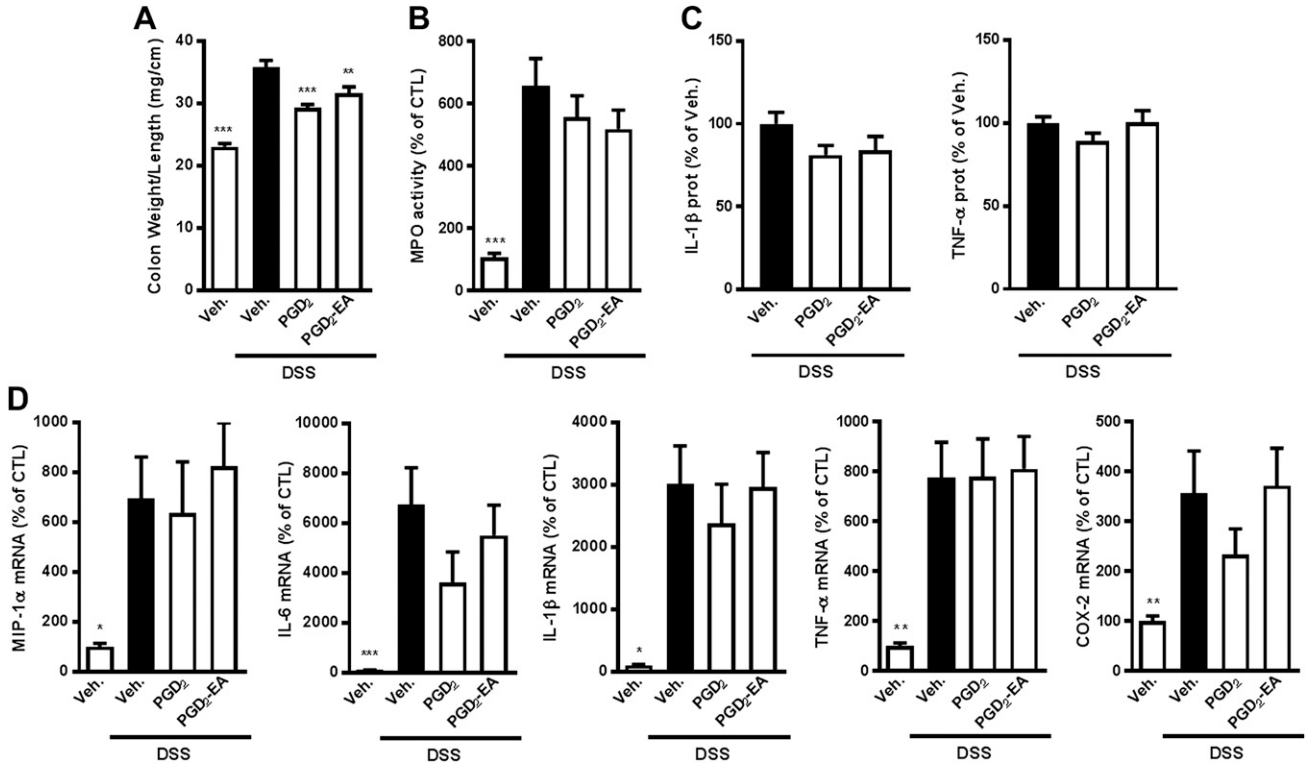

Figure 6. PGD_2 and PGD_2-EA do not reproduce the effects of PGD_2-G on DSS-induced colitis. Colitis was induced by the addition of 3% DSS in the drinking water for 5 d, followed by 2 d of normal drinking water. Mice were euthanized on d 7. PGD_2 (1 mg/kg, i.p.) and PGD_2-EA (1 mg/kg, i.p.) were administered once daily starting on the first day of addition of DSS to the drinking water. The control (CTL) group and the untreated DSS group received daily intraperitoneal injections of vehicle. *A*) Colon weight/length ratio. *B*) MPO activity. *C*) TNF-α and IL-1β production were measured by ELISA and expressed as percentage of the CTL group. *D*) mRNA expression measured by real-time qPCR and normalized to 60S ribosomal protein L19 mRNA expression. For (*B*, *C*), data are expressed relative to the control group set at 100%. *A*–*C*), Results are expressed as means ± SEM ($n = 8$ mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ *vs.* the DSS-untreated group.

were increased by colon inflammation, as was PGE_2 production, whereas HPGDS and 15PGDH expression were decreased. This is in line with studies with trinitrobenzene sulfonic acid (TNBS) or DSS-induced colitis in the rat showing increased COX-2 expression and PGE_2 production (37–39). However, another study reported decreased production of both PGE_2 and PGD_2 in DSS-induced colitis in mice (40). Moreover, in colon biopsies from patients with ulcerative colitis, 15PGDH is decreased in active disease, whereas PGE_2 is elevated (6).

Although the role of COX-2 in colon inflammation is not completely clear, some studies point to a beneficial role of the COX-2/PGDS axis in colitis. For instance, knockout of HPGDS worsened colitis and accelerated tumor formation in mice with DSS-induced colitis exposed to the procarcinogen azoxymethane (7). Here, we show that PGD_2-G reduced DSS-induced colon inflammation. Moreover, we report that PGD_2-G activates the DP1 receptor. This receptor was reported to be involved in colitis. Indeed, BW245C, a DP1 agonist, was shown to decrease colitis as well as the incidence of colitis-associated cancer in the DSS-azoxymethane model of colitis-associated cancer (7), whereas a DP1 antagonist worsened DSS-induced colitis (8). Moreover, 1 study showed that niacin administration to mice with DSS- and TNBS-induced colitis leads to an increase of PGD_2 production and subsequently to a decrease in colitis severity in a DP1-dependent manner (41). These studies point to a beneficial effect of DP1 in colitis that PGD_2-G could be part of. Moreover, because DP2 activation was shown to be deleterious in colitis (8, 21), the fact that the affinity of PGD_2-G for DP1 is 10-fold higher than its affinity for DP2 might explain why PGD_2-G exerts beneficial effects not reproduced by PGD_2, which has the same affinity for both receptors. Indeed, although anti-inflammatory effects of PGD_2 have been reported in other models (4, 5), the effect of PGD_2 itself was not studied in colitis, although some reports would suggest potential beneficial effects. For instance, PGD_2 was shown to be up-regulated in colon biopsies from patients in long-term remission from ulcerative colitis (6) and to be elevated for several weeks after resolution of TNBS-induced colitis in rats (42). One study reported an early (1–3 h) increase in PGD_2 production in rats with TNBS-induced colitis. This increase was blocked by COX-2 inhibition, which concomitantly worsened inflammatory infiltration, suggesting a beneficial role of PGD_2 (43). Another study showed anti-inflammatory effects of the PGD_2 metabolite 15d-PGJ_2, a PPARγ ligand, on dinitrobenzene sulfonic acid–induced colitis in rats (34). However, in the present study, we could not show beneficial effects of PGD_2 or PGD_2-EA on

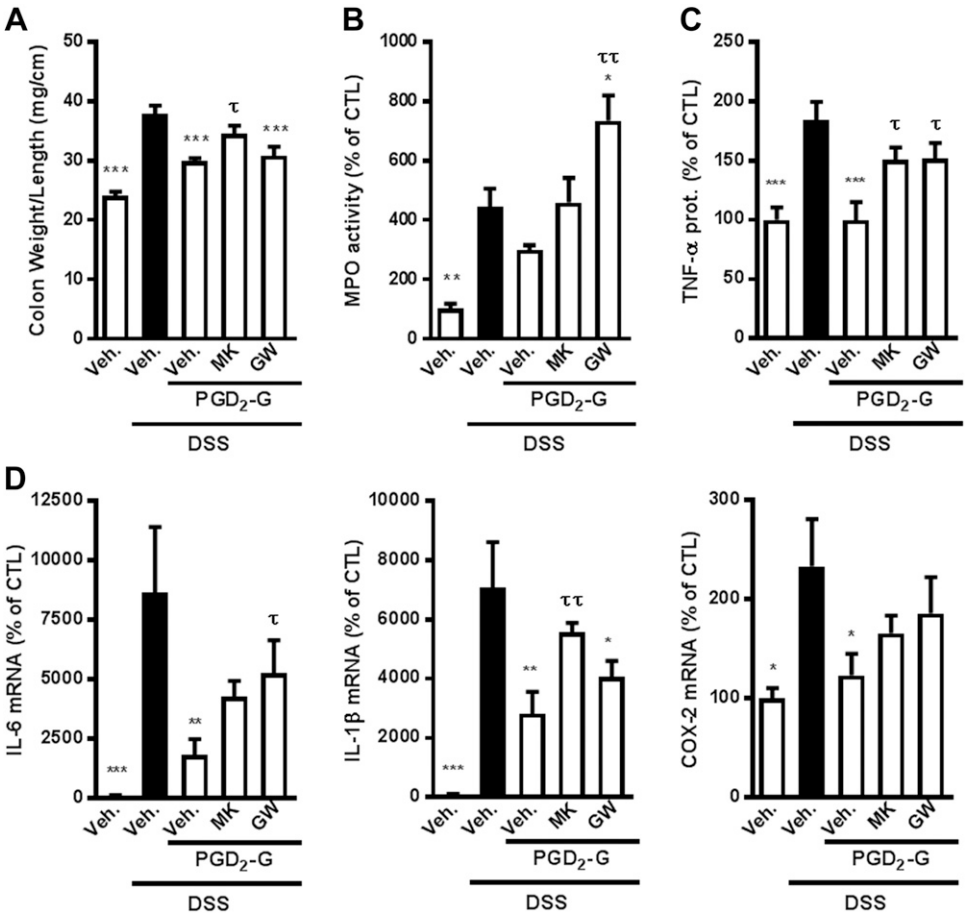

Figure 7. The effects of PGD_2-G on DSS-induced colitis are blocked by DP1 and PPARγ antagonism. Colitis was induced by the addition of 3% DSS in the drinking water for 5 d, followed by 2 d of normal drinking water. Mice were euthanized on d 7. PGD_2-G (1 mg/kg, i.p.) was administered alone or in combination with the DP1 antagonist MK0524 (MK, 1 mg/kg) or the PPARγ antagonist GW9662 (GW, 1 mg/kg), once daily starting on the first day of addition of DSS to the drinking water. The control (CTL) group and the untreated DSS group received daily intraperitoneal injections of vehicle. *A*) Colon weight/length ratio. *B*) MPO activity expressed as percentage of the CTL group. *C*) TNF-α production was measured by ELISA and expressed as percentage of the CTL group. *D*) mRNA expression was assessed by real-time qPCR. Data were normalized to 60S ribosomal protein L19 mRNA expression and expressed relative to the control group set at 100%. Results are expressed as means ± SEM ($n = 8$ mice/group). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ *vs.* the DSS-untreated group. $^{\tau}P < 0.05$, $^{\tau\tau}P < 0.005$ *vs.* the DSS + PGD_2-G group.

DSS-induced colon inflammation. Although anti-inflammatory effects have been previously described for PGD_2, not much is known concerning PGD_2-EA except for a role in reducing apoptosis in colorectal carcinoma cells (44).

Because PGD_2-G administration was efficient in reducing DSS-induced colitis, it would have been interesting to show how colitis affects its endogenous levels. Although we and others have measured PGD_2-G levels in cell models, the methods available lack the sensitivity needed to measure PG-Gs *in vivo* (19, 45). Here, despite our efforts using either a high-resolution mass spectrometer or a triple quadrupole mass spectrometer, we were not able to reliably quantify PGD_2-G in mice colon weighing up to 200 mg. A similar issue was reported by others for PG-Gs in several tissues (46–49). Moreover, supporting the endogenous nature of this bioactive lipid are our previous data showing that most effects of the endocannabinoid 2-AG on macrophage activation are blocked by COX-2 and HPGDS inhibition (*i.e.*, by preventing PGD_2-G production) (19). In the same model, we could also show that PGD_2-G was produced in LPS-activated J774 macrophages (19), whereas others have shown it to be produced by activated RAW264.7 macrophages (50). PGD_2-G can therefore be produced in inflammatory settings. Unraveling how its levels are altered upon colitis awaits the setup of more sensitive quantification methods. Nevertheless, we were able to quantify 15d-PGJ_2-G, a chemical metabolite of PGD_2-G, which was altered by the colitis. The drastic decrease in 15d-PGJ_2-G levels in colon inflammation points to a decrease in PGD_2-G levels.

In summary, we have shown here that PGD_2-G administration reduces DSS-induced colitis *via* a DP1-dependent mechanism that is in line with our data showing that PGD_2-G is a novel ligand for the DP1 receptor. PPARγ was also implicated in the effects we observed with PGD_2-G, probably through its metabolite 15d-PGJ_2-G. FJ

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AUTHOR CONTRIBUTIONS

M. Alhouayek and G. G. Muccioli designed the research; M. Alhouayek, B. Buisseret, A. Paquot, O. Guillemot-Legris, and G. G. Muccioli performed the experiments and analyzed the data; M. Alhouayek and G. G. Muccioli wrote the paper; and all authors read and approved the manuscript.

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