Lactobacillus crispatus M247-Derived H_2O_2 Acts as a Signal Transducing Molecule Activating Peroxisome Proliferator Activated Receptor-γ in the Intestinal Mucosa

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Background & Aims: **Accumulating evidence indicates that the peroxisome proliferator activated receptor (PPAR)-γ is a major player in maintaining intestinal mucosa homeostasis, but whether PPAR-γ is directly involved in probiotic-mediated effects and the molecular events involved in its activation are not known.** ***Methods:*** **We investigated the role of PPAR-γ in the immunomodulatory effects of *Lactobacillus crispatus* M247 on intestinal epithelial cells (IEC) and the role of probiotic-derived H_2O_2 on PPAR-γ activity.** ***Results:*** ***L crispatus* M247 supplementation in mice significantly increased PPAR-γ levels and transcriptional activity in the colonic mucosa. *L crispatus* M247 induced PPAR-γ nuclear translocation and enhanced transcriptional activity in epithelial (CMT-93) cells, as demonstrated by the increased luciferase activity of a PPAR-γ–responsive element, PPAR-γ–responsive gene up-regulation, and reduced activity of an nuclear factor-κB–responsive element. Pharmacologic PPAR-γ inhibition or silencing by small interfering RNA cancelled the *L crispatus* M247–mediated effects in CMT-93 cells. Because *Lactobacillus* strains producing little H_2O_2 failed to activate PPAR-γ, we investigated the role of *L crispatus* M247–derived H_2O_2 in PPAR-γ activation. *L crispatus* M247 induced a transient rise in intracellular H_2O_2 and PPAR-γ transcriptional activity was cancelled by antioxidant or H_2O_2 scavenger. Toll-like receptor (TLR)-2 was not required for PPAR-γ up-regulation mediated by *L crispatus* M247 in mice, although the protective effects of *L crispatus* M247 on dextran sodium sulfate-induced colitis were less pronounced in TLR-2$^{-/-}$ mice.** ***Conclusions:*** ***L crispatus* M247 uses H_2O_2 as a signal transducing molecule to induce PPAR-γ activation in IEC, directly modulating epithelial cell responsiveness to inflammatory stimuli.**

The >400 bacterial species populating the gastrointestinal tract are instrumental to the development of the mucosa-associated immune system, but inappropriate responses to intestinal microbiota can trigger chronic inflammatory disorders.[1] Because luminal bacteria contribute to maintaining immune homeostasis, and because the intestinal microbiota of inflammatory bowel disease patients differ significantly from the situation in healthy individuals, how manipulating the intestinal flora affects the host's health has been extensively explored.[2] The immunomodulatory effects of commensal and probiotic bacteria seem to rely on intimate contact between viable bacteria and the host's intestinal epithelial cells (IEC).[3] By recognizing conserved microbial structures, toll-like receptors (TLR) and nucleotide-binding oligomerization domain isoforms expressed in the epithelial cells activate complex intracellular signal cascades to trigger innate responses.[4] Because commensal bacteria differ in their ability to stimulate TLRs, the pattern of cytokines released varies significantly and bacteria can promote different inflammatory or anti-inflammatory responses.[5] TLR-4 stimulation by lipopolysaccharide mediates Th1-type cytokine secretion, whereas TLR-2 stimulation enhances intestinal epithelial barrier function and Th2-type cytokine release. Mucosal TLR-4 up-regulation in inflammatory bowel disease patients therefore increases their sensitivity to bacterial endotoxins, whereas TLR-2 ligands have a protective effect.[6–8]

Peroxisome proliferator-activated receptors (PPARs) -α, -δ, and -γ are members of the nuclear hormone receptor family and act as ligand-activated transcription factors, regulating gene expression in several physiologic activities.[9] PPAR-γ is an endogenous regulator of intes-

Abbreviations used in this paper: AFM, antibiotic-free medium; CFU, colony-forming units; CM, complete medium; DCF, dichlorofluorescein; DPI, diphenyliodonium; DSS, dextran sodium sulfate; GSH, reduced glutathione (L-gamma-glutamyl-L-cysteinyl glycine); H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; LPL, lipoprotein lipase; MRS, DeMan-Rogosa-Sharpe medium; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptors; PRR, pattern recognition receptor; ROS, reactive oxygen species; SEM, standard error of the mean; TLR, Toll-like receptors; WB, Western blot.

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tinal inflammation; its activation prevents inflammatory damage in colitis, whereas low PPAR-γ levels are associated with chronic inflammation and a greater susceptibility to tissue damage.[10–12] Because PPAR-γ ligands have no protective effects if they are administered after the onset of experimental colitis, PPAR-γ seems to help maintain gut homeostasis rather than having any direct anti-inflammatory role.[13] In addition to specific ligands, commensal intestinal microbes can also regulate PPAR-γ expression and activity in colonic epithelial cells.[3,14]

We recently observed that administering *Lactobacillus crispatus* M247 (a probiotic strain) modified mucosal levels of TLRs, but only reduced the severity of dextran sodium sulfate (DSS)-related colitis if administered before inflammation developed, suggesting an anti-inflammatory effect due to changes in intestinal mucosa sensitivity to bacterial products.[7,15] Because certain *Lactobacillus spp* are known to produce H_2O_2 and PPAR-γ can be activated directly or indirectly by H_2O_2, we speculated that probiotics capable of intimate contact with IEC can use H_2O_2 as a signal transducing molecule to induce PPAR-γ activation in epithelial cells. In turn, PPAR-γ would contribute to regulate pattern recognition receptors (PRRs) expression and therefore influence mucosal responsiveness to inflammatory stimuli.[16–18]

Methods

Plasmids

The PPAR-γ–responsive luciferase gene reporter (PPRE-Luc; a gift from Prof A. Galli) is described elsewhere.[19] An nuclear factor (NF)-κB–responsive luciferase gene reporter construct was generated by subcloning an NF-κB–responsive sequence (NF-κB-luc) into the pGL3-luc vector. Cytomegalovirus β-galactose (CMV-β-Gal) was purchased from Promega (Madison, WI).

Bacterial Strains

L crispatus M247, isolated from a healthy weaning baby, and its spontaneous mutant named MU5, were cultured for 18 hours in DeMan–Rogosa–Sharpe medium (MRS) broth (Difco, Angus, Ontario, Canada) at 37°C under micro-aerophilic conditions. Cells were then collected by centrifugation and resuspended in fresh MRS (10^9/bacteria mL). The MU5 strain was selected from wild-type *L crispatus* M247 with no chemical or physical mutagenic treatments. It was isolated in a partition experiment after migration into hexadecane as opposed to the wild-type strain. After culture in liquid MRS broth, M247 generates visible clamps, whereas MU5 does not. *L johnsonii, L gasseri* DSM, *L fermentum* DSM, *L plantarum,* and *L paracasei* (from ATCC) were cultured in MRS, at 37°C under micro-aerophilic conditions.[7]

Animal Studies

Male C57/Bl6 and TLR-$2^{-/-}$ ($Tlr^{2tm1Kir}$ on a C57BL/6 background) mice (8–12 weeks old) were purchased from Charles River Laboratories (Lecco; Wilmington, MA) and housed under standard conditions. The animal studies were approved by the Animal Care and Use Committee of the University of Padua. Mice were randomly allocated to experimental groups (8–12 each) given either *L crispatus* (10^8 colony-forming units [CFUs]) or vehicle (10% skim milk) intragastrically every day. After 15 days, the animals were killed and samples of colonic mucosa were frozen in liquid nitrogen and fixed in 4% paraformaldehyde or placed in cryo-embedding matrix. In different experiments, wild-type and TLR-$2^{-/-}$ mice received daily *L crispatus* (10^8 CFUs) or vehicle (10% skim milk) and, after 3 days, their drinking water was supplemented with 4% (wt/vol) DSS (TDB Consultancy, Uppsala, Sweden) to induce colitis. The animals were killed after 7 days of exposure to DSS and the severity of colitis was determined as described elsewhere.[15]

Cell Culture

The mouse colonic epithelial cell line CMT-93 (European Collection of Cell Cultures, Porton Down, UK) was maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY), 100 U/mL penicillin and 100 μg/mL streptomycin (complete medium [CM]).[7] Cells were seeded (1×10^6 cells/mL) in 6-well plates and, after reaching confluence, they were washed and incubated in antibiotic-free medium (AFM) or supplemented with 10^8 CFUs of the proper *Lactobacillus* strain (MOI ~1:50). After 1 hour, cells were washed and incubated in CM for 4–24 hours, before RNA or protein extraction. When indicated, monolayers were incubated for 30 minutes with 100 nmol/L prostaglandin F2α, 2 μmol/L L-glutathione reduced (GSH), 1000 U/mL catalase or diphenyliodonium (DPI; Sigma, St Louis, MO) before exposure to *Lactobacillus*.

Cell Viability

To exclude cytotoxic effects due to *L crispatus* and epithelial cell coculture, we conducted flow cytometry analysis on the CMT-93 cycle dynamics and evaluated apoptosis by annexin V staining after 4–24 hours of exposure to bacteria.[20]

Transfection Assays

CMT-93 (1×10^5/well) were seeded in 12-well plates and transfected 24 hours later with the specified plasmids using Lipofectamine 2000 (Promega). After 24–48 hours, cells were washed, incubated in AFM, and subjected to the suitable treatment. After another 6 hours, the cells were harvested, washed with phosphate-buffered saline (PBS) and lysed to quantify luciferase activity,[19] which was normalized using an internal CMV-β-Gal control plasmid.[21]

RNA Extraction and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

The SV total RNA isolation system (Promega) was used to extract total RNA from CMT-93 and colonic samples according to the manufacturer's protocol. Tissues were homogenized in lysis buffer using a Retsch MM300 (Qiagen, Valenica, CA). Contaminating DNA was removed by DNase I treatment (Promega). Total RNA (3 μg) was reverse transcribed to cDNA using random primers and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Real-time quantitative polymerase chain reaction (qPCR) was performed on an ABI PRISM 7700 Sequence Detection System (Perkin Elmer, Norwalk, CT) using SYBR Green as dsDNA-specific binding dye.[7] Oligonucleotide primer sequences and amplification conditions are reported in the supporting document (see Supplementary material online at www.gastrojournal.org). For each sample, the amounts of the targets and endogenous reference (glyceraldehyde 3-phosphate dehydrogenase) were extrapolated from a standard curve prepared using serially-diluted correspondent cDNAs subcloned into the pGEM-T vector (Promega).

Immunohistochemistry

Immunohistochemistry for PPAR-γ on frozen colonic sections was performed as previously described.[7] Immunocomplexes were detected using a rabbit Alexa Fluo 488 anti-goat immunoglobulin (Ig)G (Invitrogen

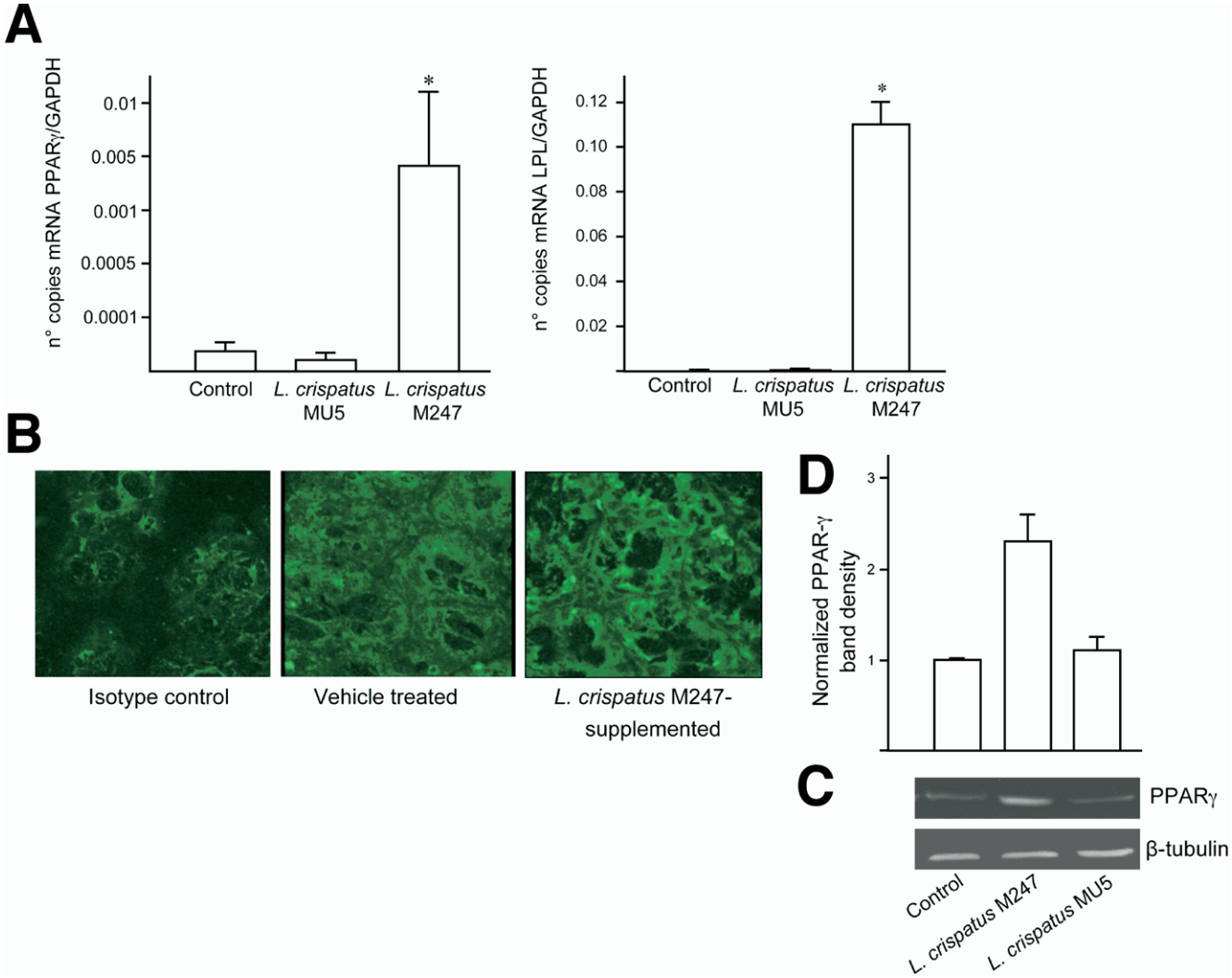

Figure 1. *L crispatus* modifies PPAR-γ levels in the colonic mucosa. C57/Bl6 mice (n = 6–9 per group) received either skim milk, 10^8 CFU *L crispatus* M247, or MU5 daily for 2 weeks. (*A*) PPAR-γ and LPL mRNA level determined by qRT-PCR. Values are expressed as mean copies of the target gene normalized to copies of glyceraldehyde 3-phosphate dehydrogenase ± SEM. *$P < .01$ versus control. (*B*) Immunofluorescence analysis of colon sections of vehicle and *L crispatus* M247-supplemented mice probed with goat anti–PPAR-γ antibody. Immunocomplexes were detected using FITC-labeled secondary antibody (×63 objective). To exclude nonspecific immunoreactivity, sections were incubated with nonimmune goat IgG. (*C*) Representative WB analysis of total mucosal protein extracts probed with anti–PPAR-γ antibody. β-Tubulin: internal standard. (*D*) Normalized mean of PPAR-γ relative band density, with the density of the band from control mice set to 1.0 (densitometric analysis of 3 experiments).

Corporation, Milan, Italy) and sections were analyzed using a Leica TCSNT/SP2 confocal microscope (original magnification, ×63). Images were stored digitally and processed with graphic software (Adobe, San Jose, CA).

Western Blot Analysis

Western blot (WB) was performed using standard procedures on total proteins extracted from CMT-93 cells or colonic mucosa of mice lysed in nondenaturing radioimmunoprecipitation buffer.[7] Bound anti–PPAR-γ or anti–ε-cadherin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were detected using an HRP-conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR) and displayed using the enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology). Membranes were photographed using a VersaDoc imaging system (Bio-Rad, Hercules, CA).

Transfection of Stealth RNA BLOCK-iT

Specific (PPAR-γ Stealth Select 3 RNAI, number MSS207863, MSS207864, MSS207865) and negative Stealth RNAs were purchased from Invitrogen and 20 μmol/L stock solutions were prepared in diethyl pyrocarbonate water. None of the BLOCK-iT Fluorescent Oligo and negative Stealth RNA have any significant homology to mouse genes (Invitrogen-held data). Stealth RNA was transfected when CMT-93 cells reached 50% confluence. Lipofectamine 2000 (1 μg/mL) and PPAR-γ–Stealth RNAi (80 nmol/L) were used in a total transfection volume of 2 mL/well. After 8 hours, 2 mL of culture medium containing 10% fetal calf serum were added to each well. Negative controls, including nontransfected CMT-93 cells and cells incubated with either Lipofectamine 2000 alone or negative Stealth RNAi, were established in parallel. CMT-93 cells were cultured for an additional 2 days before performing the experiments. RNAi MSS207863 and MSS207864 reduced PPAR-γ levels by >90% after 48 hours, whereas PPAR-γ Stealth MSS207865 was ineffective.

H_2O_2 Assay

Bacterial strains were cultured in MRS then cells were collected by centrifugation, washed twice in ice-cold PBS (pH 6.5) and resuspended in PBS supplemented with 5 mmol/L glucose. Bacterial cultures were incubated for 1–8 hours at 37°C to mimic the duration and thermal conditions of bacteria in transit through the gut. Bacteria were then removed by centrifugation and the amount of H_2O_2 released was quantified by absorbance reading at 400 nm, after colorimetric dosage with *O*-dianisine (Sigma) and peroxidase (Horseradish Type VI-A; Sigma).[22,23] Decimal counts in sterile saline were taken at each time point to correlate bacterial CFUs with H_2O_2 release.

Reactive Oxygen Species Measurement in CMT-93

Reactive oxygen species (ROS) were measured as described elsewhere.[24] CMT-93 cells were seeded in 6-well plates, then 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCFDA), 10 μmol/L (Molecular Probes) was added and the cells were incubated in the dark (37°C, 30 minutes), then exposed to *L crispatus* or 20 μmol/L phorbol 12-myristate 13-acetate (PMA; positive control). Cells were finally washed, fixed in 4% paraformaldehyde, mounted, analyzed, and photographed using a Leica TCSNT/SP2 confocal microscope. To quantify dichlorofluorescein (DCF) fluorescence intensity, cells were lysed and fluorescence in the supernatant was measured with an excitation wavelength of 485 nm and emission of 530 nm (Hitachi F2000 Fluorescence Spectrophotometer, Kyoto, Japan).

Statistical Analysis

Data are expressed as mean values ± standard error of the mean (SEM). Statistical analyses were performed using the nonparametric Kruskal–Wallis 1-way ANOVA. Statistical significance was considered when $P < .05$.

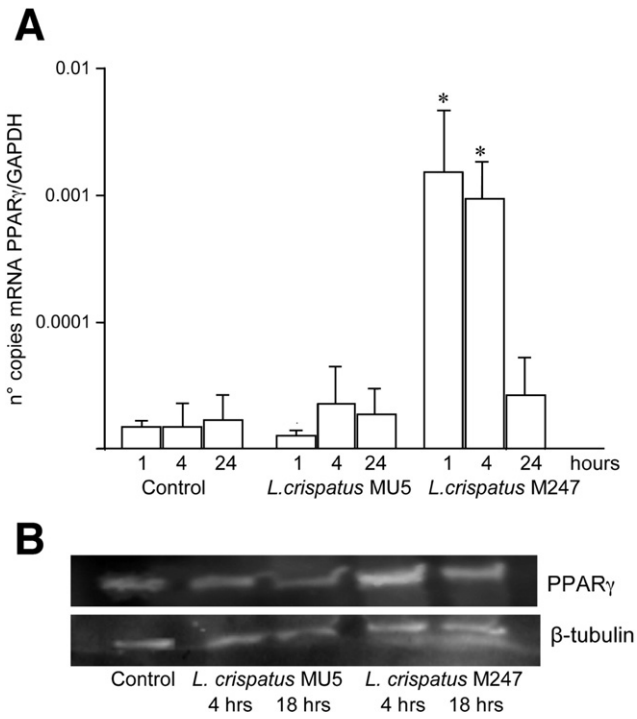

Figure 2. *L crispatus* M247 modulates PPAR-γ level in CMT-93 cells. CMT-93 monolayers were incubated for 1 hour with AFM alone or containing 10^8 CFU/ml *L crispatus* M247 or MU5, then cells were washed and cultured for 0–24 hours in CM. (*A*) PPAR-γ mRNA level determined by qRT-PCR ($n = 9$). Values are expressed as means ± SEM. *$P < .01$ versus control and MU5-treated cells. (*B*) WB analysis of total cellular protein extracts probed with anti–PPAR-γ antibody. β-Tubulin: internal standard.

Results

L crispatus *M247 Modulates Cytokines and PPAR-γ Expression In Vivo and In Vitro*

Dietary supplementation with *L crispatus* M247, but not with the MU5 mutant devoid of any anti-inflammatory activity, induced a significant increase in steady-state PPAR-γ mRNA and protein in the colonic mucosa (Figure 1).[22] Indeed, transcriptional activities consistent with PPAR-γ activation were evident after *L crispatus* M247 supplementation, as shown by the increase in lipoprotein lipase (LPL) mRNA (a PPAR-γ target gene) in the colonic mucosa (Figure 1).[25] In an in vitro coculture assay, *L crispatus* M247 increased PPAR-γ mRNA and protein level in CMT-93 cells in a time-dependent fashion (Figure 2).

L crispatus *M247 Modulates PPAR-γ Expression and Transcriptional Activity*

Because *L crispatus* M247 modulated PPAR-γ expression in colonic epithelial cells, we went on to determine the effect of *L crispatus* M247 on PPAR-γ transcriptional activity. After coculture with *L crispatus* M247, the transcriptional activity of a luciferase construct under the transcriptional control of a PPAR-γ–responsive element was significantly enhanced in the CMT-93 cells (Figure 3). In addition, ε-cadherin mRNA (a gene under PPAR-γ transcriptional control[26]) increased after coculture of CMT-93 cells with *L crispatus* M247. Finally, the interleukin(IL)-1β–induced increase in luciferase activity in CMT-93 cells transfected with a luciferase construct carrying an NF-κB–responsive element was significantly reduced in the cells exposed to *L crispatus* M247 (Figure 3). Overall, the transcriptional profile and the decrease in NF-κB activity were consistent with PPAR-γ activation in the cells exposed to *L crispatus* M247. Avoiding *L crispatus* M247 adhesion to CMT-93 cells by using a 2 coculture chamber system, PPAR-γ nuclear shuttling, ε-cadherin up-regulation, and the effects on NF-κB activity were abolished.

PPAR-γ Signaling Is Involved in L crispatus *M247 Immunomodulatory Effects*

To substantiate the role of PPAR-γ activity in the immunomodulatory effects of *L crispatus* M247, we evaluated PPAR-γ transcriptional activity in CMT-93 cells pretreated with a specific PPAR-γ inhibitor, prostaglandin F2α, or in cells transfected with Stealth RNA. As shown in Figure 4, prostaglandin F2α and siRNA cancelled the *L crispatus* M247–mediated ε-cadherin mRNA and protein up-regulation. Negligible effects were documented in CMT-93 treated with control Stealth RNA constructs.

L crispatus *M247 Produces H_2O_2 and Increases Intracellular Levels of Free Radicals*

Because several *Lactobacillus spp* are H_2O_2 producers and recent evidence indicates that ROS can affect PPAR-γ expression and activity, we tested whether *L crispatus* produces H_2O_2 and whether *L crispatus*-derived H_2O_2 can affect intracellular ROS.[17,18] As shown in Figure 5*A*, *L crispatus* M247 showed a sustained H_2O_2 pro-

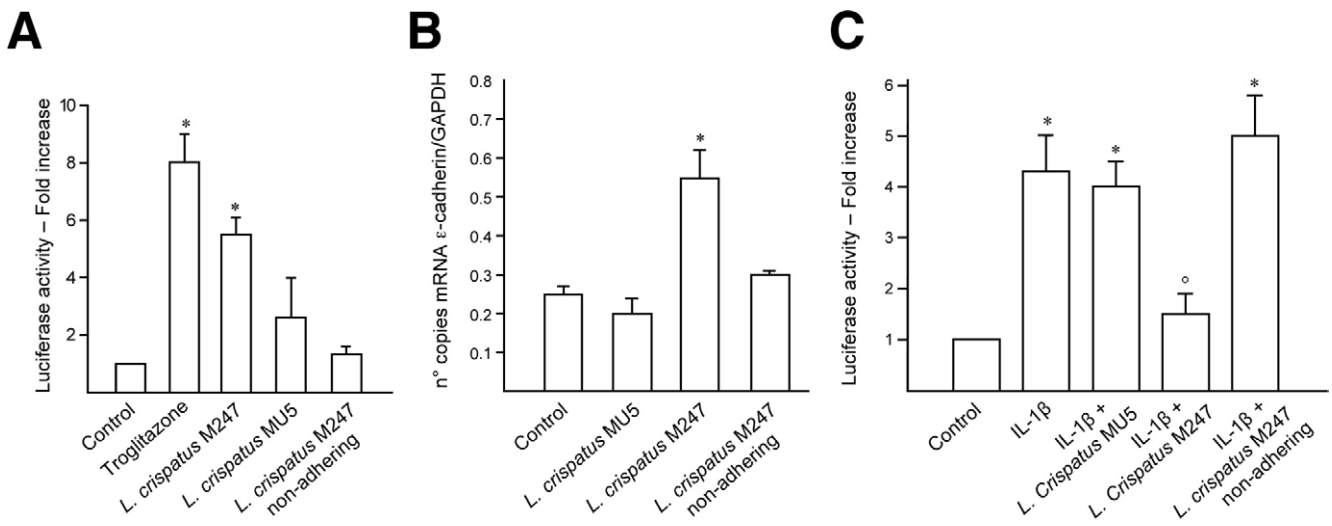

Figure 3. *L crispatus* M247 modulates PPAR-γ activity. (*A*) CMT-93 cells were cotransfected with the reporter PPRE-Luc and the expression vector CMV-βGal. After 24 hours, cells were treated with vehicle, PPAR-γ ligand (troglitazone 10 μmol/L), *L crispatus* M247, *L crispatus* MU5, or *L crispatus* M247 placed in the upper compartment of a 2-chamber culture system. After another 6 hours, luciferase and β-Gal activity were quantified. Data are expressed as mean values ± SEM ($n = 8$). *$P < .01$ versus control. (*B*) CMT-93 monolayers were washed and incubated for 1 hour in AFM alone or supplemented with *L crispatus* MU5, *L crispatus* M247, or placing the *L crispatus* M247 in the upper compartment of a 2 coculture chamber system. Then cells were washed and, after 4-hour incubation, ε-cadherin mRNA was quantified by qRT-PCR ($n = 9$ conditions). Values are expressed as means ± SEM. *$P < .01$ versus control. (*C*) CMT-93 cells were cotransfected with NF-κB-luc and CMV-βGal. After 48 hours, cells were treated with vehicle, IL-1β (10 ng/mL), IL-1β plus *L crispatus* MU5, IL-1β plus *L crispatus* M247, or IL-1β plus *L crispatus* M247 placed in the upper compartment of a 2-chamber culture system. After 6 hours of incubation, luciferase and β-Gal activity were quantified. Data expressed as mean values ± SEM ($n = 9$). *$P < .01$ versus control; °$P < .01$ versus IL-1β.

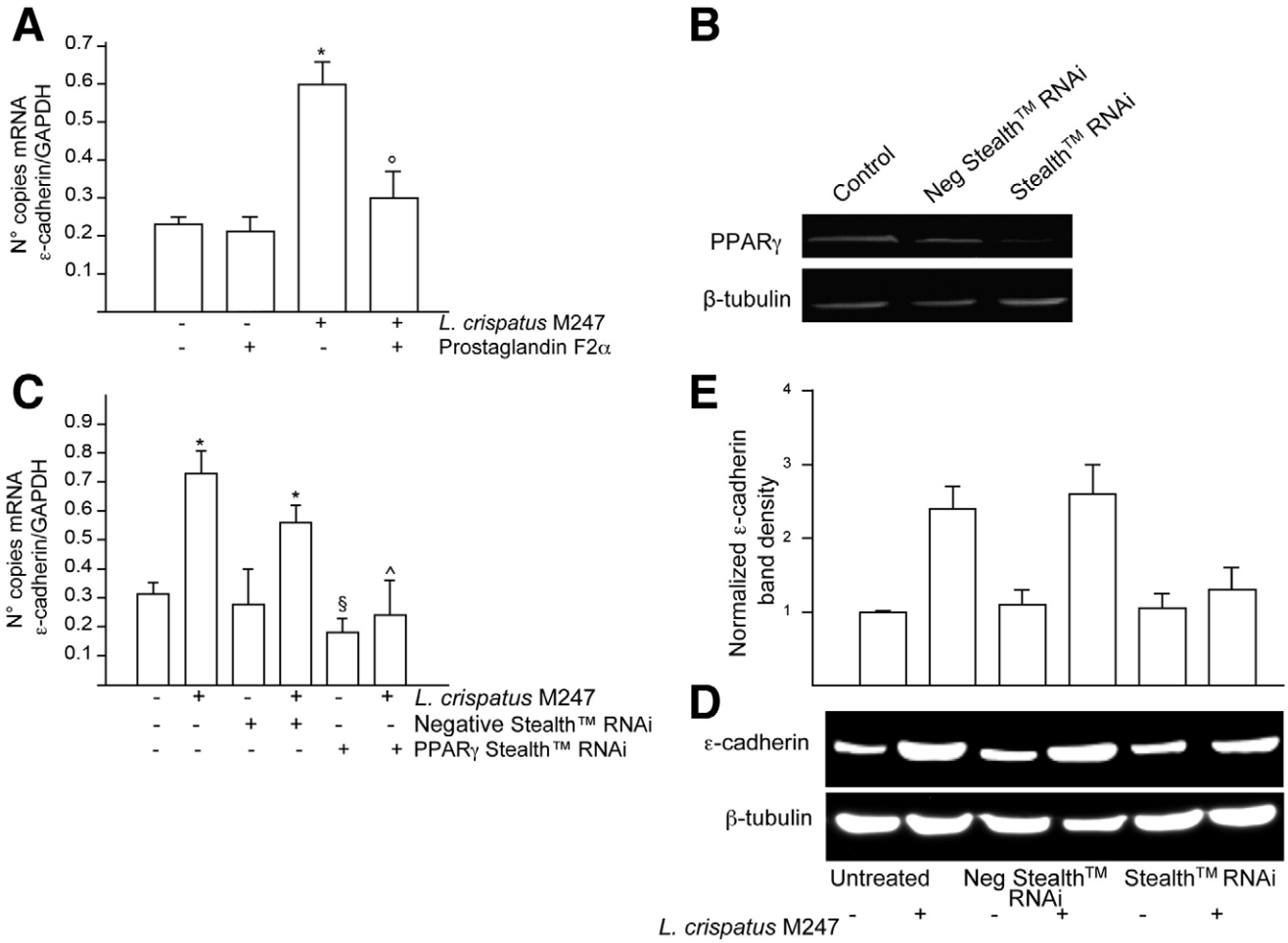

Figure 4. PPAR-γ is involved in *L crispatus* M247 immunomodulatory effects. (*A*) CMT-93 were incubated for 30 minutes in AFM or containing 100 nmol/L prostaglandin F2α before exposure to 10^8/mL CFUs *L crispatus* M247. Then cells were washed and, after 4 hours of incubation in CM, ε-cadherin mRNA was determined by qRT-PCR ($n = 9$). Values are expressed as means ± SEM. $^*P < .01$ versus control; $^\circ P < .01$ versus *L crispatus* M247. (*B–E*) siRNA transfection with Lipofectamine 2000 alone or together with negative Stealth RNAi or PPAR-γ Stealth RNAi MSS207863. After 48 hours, total proteins were extracted and PPAR-γ levels were determined by WB (*B*), or the cells were incubated in AFM alone or supplemented with *L crispatus* M247, and ε-cadherin levels were determined after 4 hours by qRT-PCR (*C*: $^*P < .01$ and $^\S P < .05$ versus control; $\wedge P < .01$ versus *L crispatus* M247 alone) or WB. (*D*) Representative WB. (*E*) Normalized mean of ε-cadherin relative band density, with the density of the control band set to 1.0 (densitometric analysis of 3 experiments). β-Tubulin: internal standard.

duction for up to 8 hours, whereas MU5 showed a significant H_2O_2 release only during the first 3 hours of culture. *L crispatus* M247 induced a time-dependent increase in the oxidation of nonfluorescent H_2DCFDA to fluorescent DCF in cocultured CMT-93 cells, whereas no effect was observed when contact between *L crispatus* M247 and CMT-93 was prevented by incubating epithelial cells with heat-killed bacteria or by coculturing epithelial cells with *L crispatus* MU5. Indeed, the coculture of *L crispatus* M247 with CMT-93 did not affect cell viability as assessed by cell cycle flow cytometry, and apoptosis as measured by annexin V staining (see supporting material). As expected, coculture of CMT-93 cells with *Lactobacillus spp* incapable of producing significant amounts of H_2O_2 (*L fermentum*, *L plantarum,* and *L paracasei*) failed to modify the intracellular ROS, whereas H_2O_2-producers (*L johnsonii* and *L gasseri*) enhanced intracellular fluorescent DCF, albeit to a lesser degree than *L crispatus* M247. Indeed, *L crispatus* M247 adhered more effectively to the epithelial monolayer than *L johnsonii* or *L gasseri*, whereas *L crispatus* MU5 failed to do so at all (see supporting material). In addition, inhibiting intracellular ROS generation by DPI treatment did not affect *L crispatus* M247–mediated H_2DCFDA oxidation (Figure 5). Overall, these data indicate that intimate contact is needed between H_2O_2-producing *Lactobacillus spp* and epithelial cells to enable the small amount of H_2O_2 produced by the bacteria to spread into the epithelial cells.

ROS Are Needed to Induce Immunomodulatory Effects in CMT-93 Cells

To test the role of H_2O_2 in *L crispatus* M247-mediated PPAR-γ activation and transcription activity, we pretreated CMT-93 cells with glutathione (a specific antioxi-

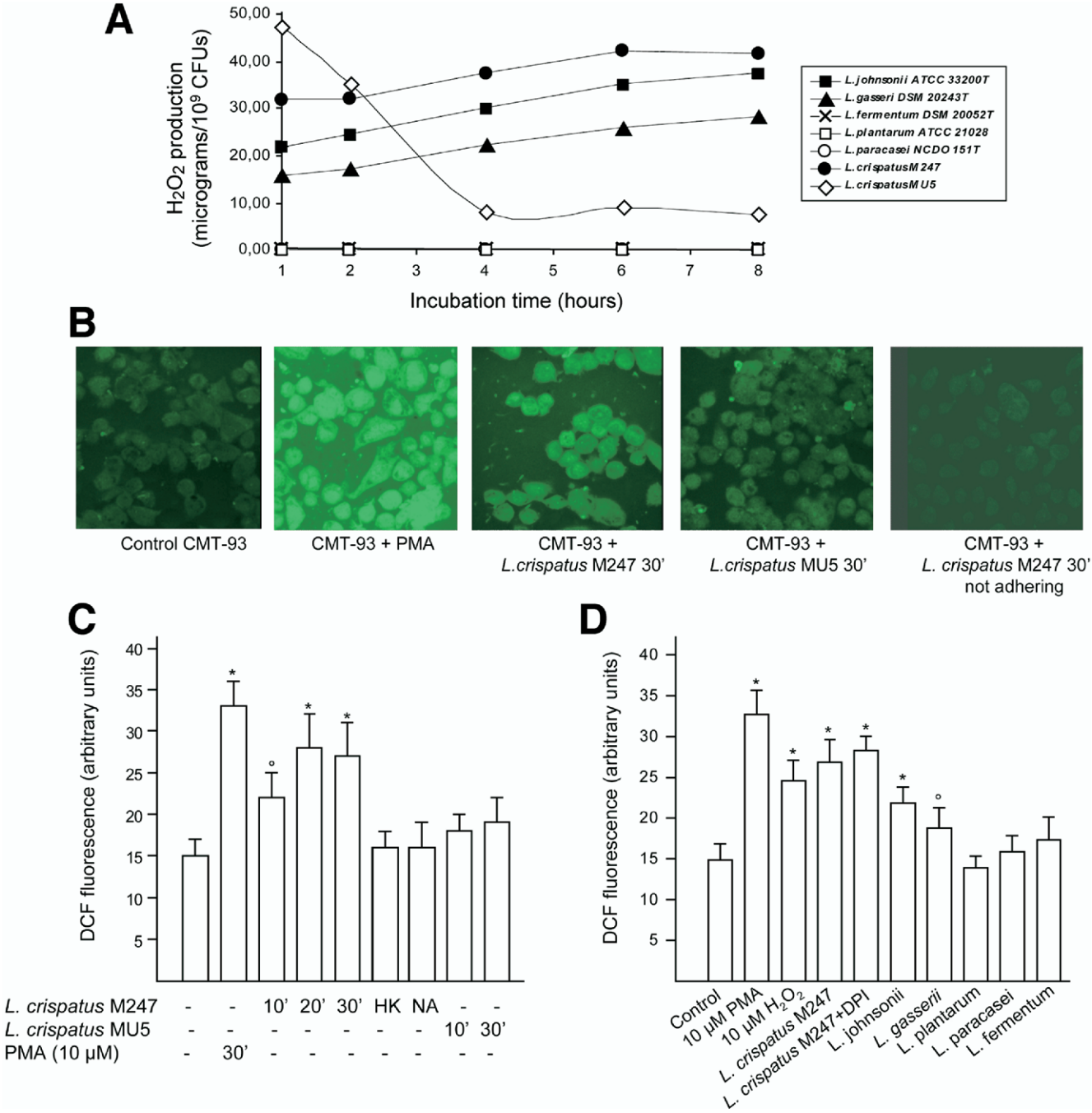

Figure 5. *L crispatus* M247 increases the ROS in colonic epithelial cells. (*A*) Quantification of H_2O_2 production by *Lactobacillus spp* ($n = 6$) incubated for 1–8 hours at 37°C under 5% CO_2 in air. (*B*) ROS-induced H_2DCFDA fluorescence in CMT-93 cells incubated in AFM alone or supplemented with PMA (positive control), *L crispatus* M247 or MU5 assessed by confocal microscopy (×63 magnification). (*C, D*) Intracellular fluorescence was quantified by fluorescence spectrophotometry in cells incubated 30 minutes in the dark with H_2DCFDA with or without DPI (10 μmol/L). Then cells were washed and incubated with the specified stimulus for 10–30 minutes. HK, heat-killed; NA, not adhering. *L crispatus* M247 was placed in the upper compartment of a 2 coculture chamber system. Values are expressed as arbitrary fluorescence units and represent mean ± SEM ($n = 7$). *$P <$ .01 and °$P < .05$ versus control.

dant agent) or catalase (an H_2O_2 scavenger; Figure 6). The presence of glutathione or catalase significantly inhibited the *L crispatus* M247-induced activation of a PPAR-γ–responsive element transfected into CMT-93 cells and PPAR-γ transcriptional activities were almost completely abolished, as shown by qRT-PCR and WB analysis for ε-cadherin and PPAR-γ (data not shown). Furthermore, *L plantarum* producing little H_2O_2 failed to up-regulate PPAR-γ expression or induce the nuclear-cytoplasmic shuttling after coculture with CMT-93 cells, unlike the H_2O_2 producers *L johnsonii* and *L gasseri*, which enhanced PPAR-γ activity (Figure 5, some data not shown).

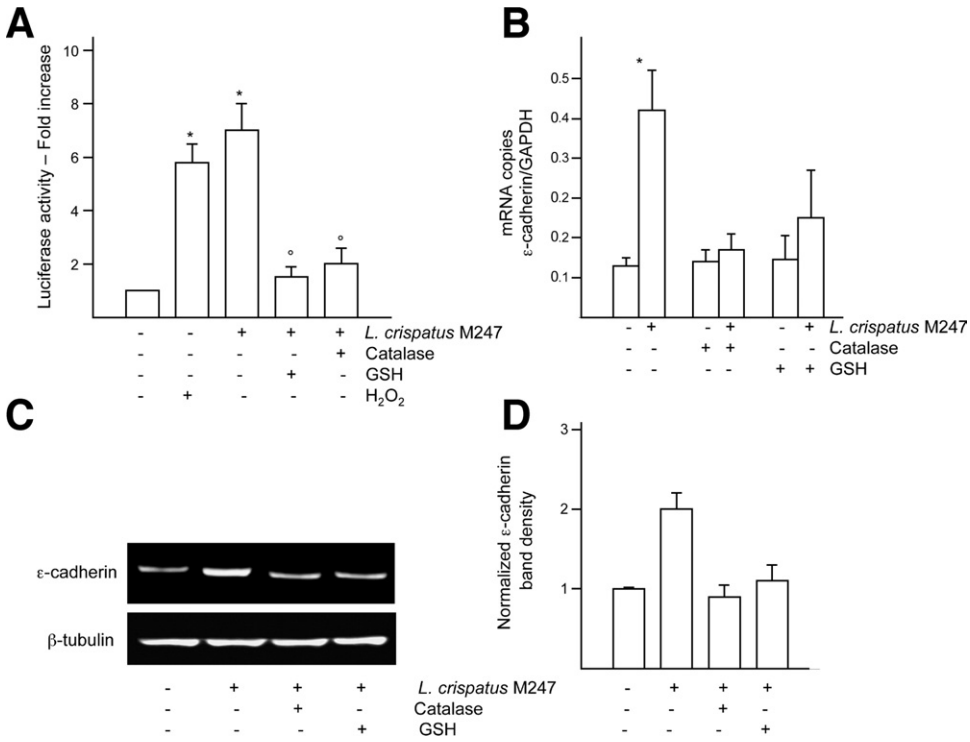

Figure 6. H_2O_2 is needed for *L crispatus* M247-mediated effects in epithelial cells. (*A*) CMT-93 were cotransfected with PPRE-Luc and CMV-βGal. After 24 hours, cells were washed and incubated for 30 minutes in AFM alone or containing GSH or catalase before exposure to *L crispatus* M247 or H_2O_2 (10 μmol/L). After 1 hour, cells were washed and, after 6-hour culture in CM, luciferase and β-Gal activity were quantified. Data are expressed as mean values ± SEM ($n = 8$). *$P < .01$ versus control and °$P < .01$ versus *L crispatus* M247. (*B, C, D*) CMT-93 monolayers were incubated in AFM alone or containing either GSH or catalase. After *L crispatus* M247 coculture for 1 hour, epithelial cells were washed and cultured for another 4 hours in CM before ε-cadherin mRNA level was determined by qRT-PCR (*B*) or WB (*C*). (*D*) Normalized mean of ε-cadherin relative band density, with the density of the control band set to 1.0 (densitometric analysis of 4 experiments). β-Tubulin: internal standard.

L crispatus *M247–Mediated Protective Effects in Colitis Involve PPAR-γ Activity*

PPAR-γ and TLR-2 ligands reduce the severity of colitis and *L crispatus* M247 supplementation up-regulates TLR-2 in the colonic mucosa, so we aimed to establish whether TLR-2 is necessary for *L crispatus* M247–mediated effects in vivo.[7,8,10] After *L crispatus* M247 supplementation in wild-type and TLR-$2^{-/-}$ mice, the effects on the immunomodulatory cytokines (IL-1β and IL-10; data not shown) and PPAR-γ target genes, such as LPL and PPAR-γ itself, in the colonic mucosa were comparable (Figure 7), suggesting that TLR-2 stimulation is not essential to PPAR-γ expression in the colonic mucosa. Indeed, *L crispatus* M247–induced TLR-2 up-regulation in the CMT-93 cells was completely abolished by pharmacologic PPAR-γ inhibition or H_2O_2 neutralization (Figure 7).

Finally, we assessed the effect of *L crispatus* M247 supplementation on DSS colitis in TLR-$2^{-/-}$ mice. Colitis was slightly more severe in the TLR-$2^{-/-}$ mice than in the wild-type animals (Figure 8). *L crispatus* M247 supplementation significantly reduced the severity of colitis in C57/Bl6 mice, but was less influential on colitis outcome in DSS-treated TLR-$2^{-/-}$ mice (Figure 8).[15] In fact, supplementation with *L crispatus* MU5 had no effect at all in DSS-treated wild-type or TLR-$2^{-/-}$ mice (Figure 8).

Discussion

We report on a novel strategy used by *Lactobacillus crispatus* M247 (a probiotic strain) to modulate PPAR-γ expression and activity after adhesion to the IEC through the release of H_2O_2.[7] *Lactobacillus crispatus* M247-derived H_2O_2 acts as a signal transducing molecule that activates PPAR-γ, enabling it to pin down NF-κB signal transduction pathways and to express PRRs in colonic epithelial cells.

PPAR-γ is a key transcriptional regulator needed to preserve mucosal homeostasis.[12–14] Regulating PPAR-γ activity proved effective in treating inflammatory disorders because PPAR-γ interferes with the activity of proinflammatory transcription factors and kinases to inhibit cytokine, chemokine and adhesion molecule production.[27–29] Because commensal gut bacteria can enhance PPAR-γ activity in IEC, it is reasonable to suppose that manipulating the intestinal microflora might affect PPAR-γ expression and activity in the colonic mucosa.[3] We show that *L crispatus* M247 directly increased PPAR-γ transcriptional activity in IEC in vitro and in vivo, resulting in a greater expression of PPAR-γ-target genes, including ε-cadherin, LPL, and PPAR-γ itself.[25,26] We also show that the immunomodulatory effect of *L crispatus* M247 in IEC was lost when PPAR-γ was inhibited either pharmacologically or genetically. These results indicate that the PPAR-γ–dependent pathway plays a prominent role in *L crispatus* M247-mediated responses.

PPAR-γ is a modestly inducible receptor and the cellular environment has an important influence on the expression of this transcription factor, which is classically regulated by diet, hormones, and specific PPAR-γ ligands.[14] Luminal bacteria, possibly through signal trans-

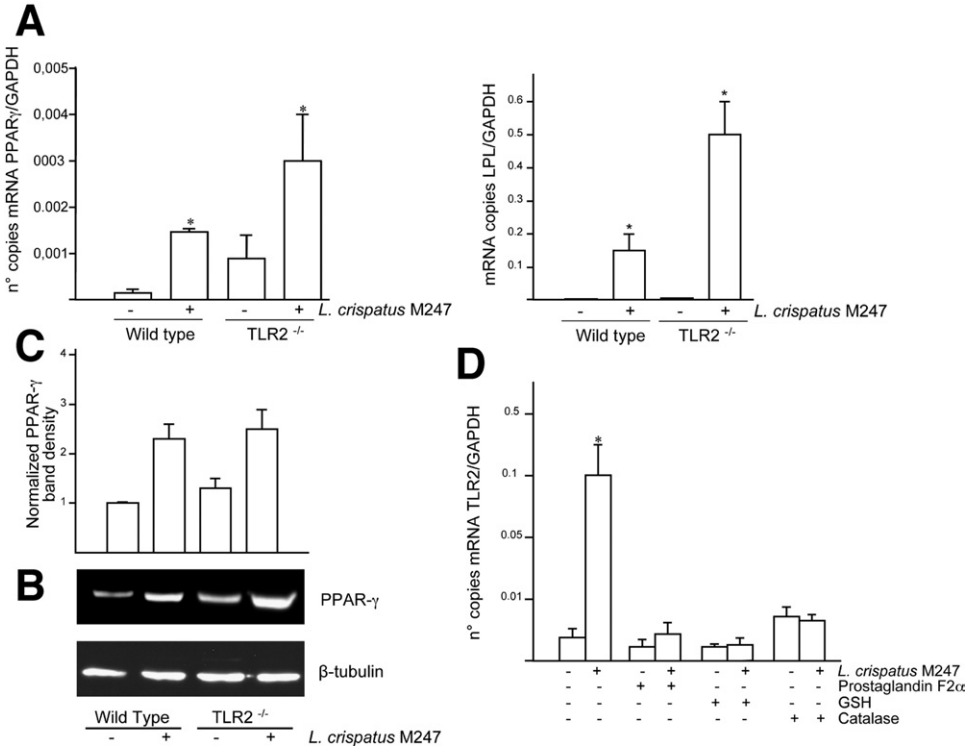

Figure 7. TLR-2 is not needed for *L crispatus* M247–mediated immunomodulatory effects in the colonic mucosa. (*A–C*) Wild-type and TLR-2$^{-/-}$ mice received *L crispatus* M247 or vehicle for 2 weeks ($n = 6–8$ conditions). Then total mucosal RNA (*A*) or proteins (*B*) were extracted and PPAR-γ and LPL mRNA levels were determined by qRT-PCR or WB, respectively. qRT-PCR values were expressed as number of mRNA copies of target gene and were normalized to number of copies of glyceraldehyde 3-phosphate dehydrogenase (*$P < .01$ versus control mice). (*C*) Normalized mean of PPAR-γ relative band density, with the density of the band from wild-type control mice set to 1.0 (densitometric analysis of 4 experiments). β-Tubulin: internal standard. (*D*) CMT-93 monolayers were washed and incubated in AFM alone or containing prostaglandin F2α, GSH, or catalase. Epithelial cells were cocultured with *L crispatus* M247 for 1 hour and TLR-2 mRNA levels were determined by qRT-PCR after 4 hours ($n = 6$ conditions). Values are expressed as means ± SEM. *$P < .01$ versus *L crispatus* M247.

duction pathways involving pattern recognition receptors, can induce PPAR-γ expression in IEC in vivo.[11] Soluble factors derived from microbe metabolism are also known to influence a variety of physiologic activities, however, including PPAR-γ.[30–32] Among the variety of molecules capable of affecting pathogen growth and intestinal mucosa function[33], the probiotic strains also produce H_2O_2, which can affect PPAR-γ expression and activity.[17,18] We demonstrated that *L crispatus* M247 produces significant amounts of H_2O_2 that causes a transient but nontoxic rise in intracellular ROS and induces PPAR-γ nuclear translocation and transcription activities after the adhesion of bacteria to IECs. Furthermore, neutralizing *L crispatus* M247–derived H_2O_2 abolished this *L crispatus* M247–mediated PPAR-γ activation in epithelial cells. The list of bacteria-derived products capable of influencing the gene expression profile in epithelial cells is therefore more complex than currently believed and should also include H_2O_2 generated by adhering *Lactobacilli* that may act as a signal transducing molecule.

The ability to produce H_2O_2 is a well characterized property of several *Lactobacillus spp* and the amounts of H_2O_2 we measured are comparable with those of previous reports on the ability of *L crispatus* to produce considerable quantities of H_2O_2.[34] It is intriguing that *L crispatus* MU5, which has no protective effects in vivo and is incapable of inducing PPAR-γ activation, revealed different H_2O_2 production dynamics. Probiotic-derived H_2O_2 has long been considered only as an antagonistic for controlling the microbial ecosystem associated with the mucosa; a lack of H_2O_2-generating *Lactobacillus* strains is associated with a higher incidence of vaginal infections.[35] A transient increase in intracellular concentrations of H_2O_2 and other ROS are now emerging, however, as key intracellular signal transducing mechanisms.[36,37] Indeed, agents such as CO (which has a protective effect in inflammatory models) stimulate a transient oxidative burst, generating ROS that are involved in the subsequent induction of PPAR-γ and anti-inflammatory activity.[18] To assess whether *L crispatus*-derived H_2O_2 might regulate PPAR-γ activation, we transiently transfected a reporter plasmid containing the luciferase gene regulated by 6 PPAR-γ DNA binding elements, observing an increase in luciferase activity after exposure to known PPAR-γ ligands, such as troglitazone, but also after exposure to *L crispatus* M247. These effects were abolished by ROS neutralization, however. We conclude from these

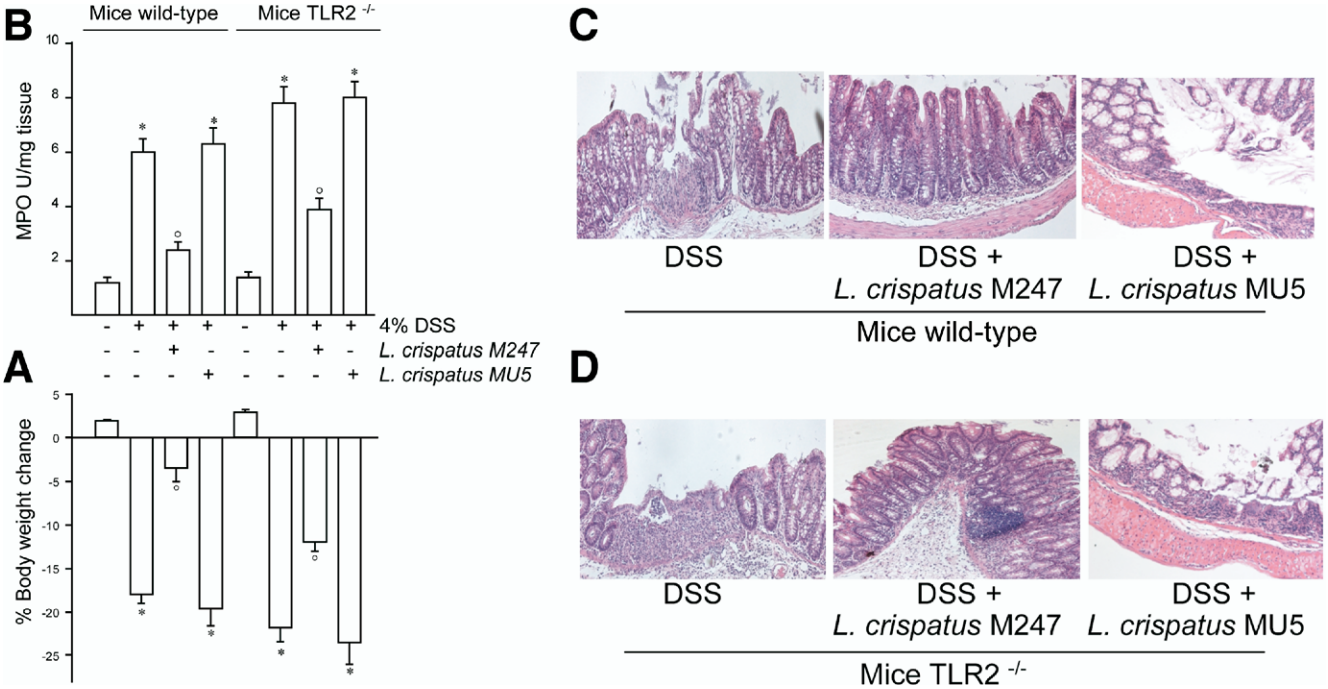

Figure 8. *L crispatus* supplementation partially reduces the severity of DSS colitis in TLR-2$^{-/-}$ mice. Wild-type and TLR-2$^{-/-}$ mice received either *L crispatus* M247, *L crispatus* MU5 or vehicle ($n = 6$ groups) daily, and after 3 days their drinking water was supplemented with 4% DSS. Body weight (% changes) was measured daily for 7 days (*A*). Myeloperoxidase activity (*B*) and histology (*C* and *D*) were analyzed on specimens from the proximal colon. *$P < .01$ versus control; °$P < .01$ versus DSS alone.

experiments that *L crispatus*-derived H_2O_2 enhances PPAR-γ expression and activity.

The ROS burst in epithelial cells in response to *L crispatus* M247 can induce PPAR-γ up-regulation via several mechanisms. We speculate that either a ROS-sensitive intermediary is involved, or that PPAR-γ itself is regulated directly by ROS. Potential targets for ROS-mediated PPAR-γ up-regulation might include protein phosphatase modulation, a reduced PPAR-γ degradation, a differential regulation of scaffold regulatory proteins, or phosphorylation by mitogen-activated protein kinases.[38,39] An independent kinase-driven signaling cascade leading to PPAR-γ activation may include ROS-mediated epidermal growth factor receptor transactivation.[40] Indeed, epidermal growth factor receptor seems to be involved in the protective effects induced by various probiotic strains.[40,41]

Our results also establish a hierarchy in *L crispatus* M247-mediated immunomodulatory effects in vivo. PRRs have a key role in recognizing repetitive structures of nonpathogenic gram-positive and gram-negative microbes, because PRR-derived signals influence a variety of cellular activities. TLR signaling is carefully regulated in the healthy gut, because excessive TLR stimulation can have deleterious effects.[42] Here, we show that administering *L crispatus* M247 modifies PPAR-γ and LPL levels in the colonic mucosa in wild-type and also in Tlr2^{tm1Kir} mice. In the colonic mucosa of UC patients, PPAR-γ expression is impaired, although TLR-4 is up-regulated; the colonic flora shows significant differences by comparison with healthy subjects.[11,43] The limited ability of *L crispatus* M247 to reduce the severity of DSS colitis in Tlr2^{tm1Kir} mice supports the view that probiotic bacteria modulating PPAR-γ expression/signaling can in turn influence the sensitivity of the mucosa-associated immune system to bacteria-derived immunomodulatory substances.[44] By activating regulatory proteins like PPAR-γ in epithelial cells, probiotics thus contribute to maintaining mucosal homeostasis, uncoupling NF-κB–dependent target genes relevant to inflammatory responses and regulating TLR expression in epithelial cells, thereby modifying the enterocytes' relationship with the surrounding environment.[7,12] Indeed, analyzing the promoter region in the mouse genome (Gene Bank accession number AF395910) revealed 3 putative PPAR-γ binding sequences in the regulatory region of the TLR-2 gene that are now undergoing functional analysis.

In conclusion, our study identified a previously unknown probiotic-induced anti-inflammatory mechanism involving PPAR-γ activation and provides new insight into early molecular mechanisms involved in the cross-talk between epithelial cells and commensal flora. Our findings also extend our understanding of the mechanism of action of commensal bacteria on the mucosal surfaces and emphasize the possibility of identifying bacteria-derived bioactive molecules with immunomodulating activities and selecting useful probiotic strains.[33]

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.07.007.

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