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Original Article

Lipopolysaccharides Facilitate Colonic Motor Alterations Associated to the Sensitization to a Luminal Antigen in Rats

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Background/Aims

Enteric dysbiosis is a risk factor for dietary proteins-associated intestinal alterations, contributing to the development of food allergies and the symptomatology of functional gastrointestinal disorders, mainly irritable bowel syndrome (IBS). We explored if a dysbiotic-like state, simulated by intraperitoneal administration of bacterial lipopolysaccharides (LPS), facilitates the sensitization to a luminal antigen, ovalbumin (OVA), in rats.

Methods

Rats were exposed to oral OVA for 1 week, alone or with LPS. Thereafter, colonic histology, goblet cell density, mucosal eosinophils and mucosal mast cell (MMC) and connective tissue mast cell (CTMC) were evaluated. Colonic expression (real-time quantitative polymerase chain reaction) of interleukins, IFN-α1 and integrins was assessed to determine local immune responses. Luminal and wall adhered microbiota were characterized by fluorescence in situ hybridization. Colonic contractility (in vitro) served to assess functional changes associated to OVA and/or LPS.

Results

Neither OVA nor LPS, alone or combined, lead to structural alterations, except for a reduced goblet cell density in OVA-LPS-treated rats. MMC density was unaffected, while CTMC counts increased within the submucosa of OVA-LPS-treated animals. Marginal immune activation (IFN-α1 up-regulation) was observed in OVA-LPS-treated rats. LPS induced a dysbiotic-like state characterized by decreased luminal bacterial counts, with a specific loss of clostridia. LPS facilitated *Clostridium* spp. wall adherence, an effect prevented by OVA. Colonic contractility was altered in OVA-LPS-treated animals, showing increased basal activity and enhanced motor responses to OVA.

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Conclusions
Changes in gut microbiota and/or direct effects of LPS might enhance/facilitate local neuroimmune responses to food antigens leading to motor alterations similar to those observed in IBS.
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Key Words
Food hypersensitivity; Gastrointestinal diseases; Irritable bowel syndrome; Microbiota; Ovalbumin

Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder and is characterized by abdominal pain-discomfort associated with dysmotility and altered bowel habits.[1] Although its underlying pathophysiology remains unclear, recent evidences suggest that IBS is due to a dysregulation of the brain-gut axis, with various peripheral alterations contributing to the exacerbation of the symptomatology. For instance, both changes in gut microbiota and adverse reactions to food allergens have been implicated in the pathogenesis of the disease.[2-5]

Intestinal food allergy-related mechanisms in IBS seem to involve local mucosal responses to dietary antigens with activation of resident mast cells (MCs), rather than classical type-1 hypersensitivity reactions.[4,6] In this line, we have previously demonstrated that long-term exposure to oral ovalbumin (OVA), without adjuvants, results in a non-IgE mediated alteration of colonic motility in rats, an effect related to an excited-activated state of the tissue mucosal mast cells (MMCs). These OVA-mediated changes are reminiscent of those observed in IBS patients and in animal models of the disease.[7-11]

Gut commensal microbiota (GCM) has been implicated in the maintenance of the normal gastrointestinal hyporesponsive state to food antigens. For instance, results obtained in studies in infants with food allergy show a disturbed balance between beneficial and potentially harmful bacteria in the large intestine and that supplementation with probiotics appears to alleviate the allergic inflammation.[12,13] Supporting these observations, results from animal models demonstrate a cause-effect relationship between dysbiotic states with reduced GCM and the development of allergic responses to oral antigens.[14,15] Overall, these data suggest that a disruption of the GCM might cause an impairment of the intestinal tolerogenic mechanisms, increasing the risk of food protein-induced immune activation and the development of food allergy and/or IBS-like alterations.

The aim of the present study was to further explore the impact of dysbiotic states of the colon in the generation of abnormal responses to dietary antigens. For this purpose, we treated rats with low doses of bacterial lipopolysaccharides (LPS).[16] Simultaneously, animals were exposed orally to an allergic protein, OVA. The potential facilitatory effects of LPS towards OVA sensitization were studied in vitro by assessing spontaneous colonic contractility and the contractile responses elicited by the presence of the antigen (OVA). To further understand the potential role of GCM in the functional alterations observed, changes in the colonic microbiota and bacterial wall adherence were determined by fluorescence in situ hybridization (FISH). Finally, to gain insight into the immune nature of the IBS-like responses related to dietary antigens, we assessed local changes in cytokines expression, the potential involvement of eosinophils and the dynamics of colonic MCs populations.

Materials and Methods

Animals

Adult (9-week-old), specific pathogen free (SPF), Sprague-Dawley (SD) male rats were used (Charles River, Les Oncins, France). Animals had free access to water and were fed with a standard diet (145 g/kg protein, 40 g/kg fat, 45 g/kg fiber and 13.4 kJ/g; free of traces of ovalbumin or any other egg derivative; Teklad Global 14% Protein Rodent Maintenance Diet 2014, Harlan Interfauna Iberica S.A.). During all the experiment, rats were maintained under conventional conditions in a light (12 hour/12 hour light-dark cycle) and temperature controlled (20-22°C) room, in groups of 2 per cage. Animals were acclimatized to the new environment for 1 week before starting any experimental procedure. All the experimental protocols were approved by the Ethical Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocols 1010 and 5351, respectively).

Exposure to Oral Ovalbumin and Treatment With Lipopolysaccharides

Rats were exposed to OVA by a daily oral gavage (10 mg/mL, 1 mL/rat, n = 15) during a one week period. A group of rats were used as controls, in which the same exposure protocol was followed but OVA solution was replaced by saline (1 mL/rat, n = 15). In parallel, a subgroup of vehicle- and OVA-exposed rats (n = 8 for each) was treated intraperitoneally with LPS (100 μg/kg/day for 7 days). The rest of the rats (7 vehicle- and 7 OVA- exposed animals) received the vehicle for LPS (1 mL/kg/day for 7 days). Animals were examined for clinical signs and body weight changes on a daily basis (at the time of treatment) and were euthanized by decapitation after 24 hours of the last treatment. At the time of euthanasia, tissue samples from the colon were collected for functional studies (organ bath studies) or either fixed in 4% paraformaldehyde or Carnoy's solution, for immunohistochemical and FISH studies respectively, or frozen in liquid nitrogen and stored at $-80^{o}C$ until analysis.

Organ Bath Studies

Full thickness preparations were obtained from the mid portion of the colon, cut 1 cm long and 0.3 cm wide and hung, oriented to record circular muscle activity, for organ bath studies. Strips were mounted under 1 g tension in a 10-mL organ bath containing carbogenated Krebs solution (95% O_2 and 5% CO_2) maintained at 37 ± $1^{o}C$. The composition of Krebs solution was (in mmol/L): 10.10 glucose, 115.48 NaCl, 21.90 $NaHCO_3$, 4.61 KCl, 1.14 NaH_2PO_4, 2.50 $CaCl_2$, and 1.16 $MgSO_4$ (pH 7.3-7.4). One strip edge was tied to the bottom of the bath and the other one to an isometric force transducer (Harvard VF-1 Harvard Apparatus Inc, Holliston, MA, USA). Output from the transducer was fed to a PC through an amplifier. Data were digitalized (25 Hz) using Data 2001 software (Panlab, Barcelona, Spain). Strips were allowed to equilibrate for about 1h. After this period, contractile responses to carbachol (CCh; 0.1-10 μM), bovine serum albumin (BSA; 0.1%) and OVA (0.1%) were assessed. For CCh, cumulative concentration-response curves, with a 5-minute interval between consecutive doses, were constructed. For BSA or OVA, spontaneous activity was recorded during a 15-minute period after the addition of the protein.

To determine the spontaneous contractile activity, the area under the curve (AUC) was measured (in grams) over a 15-minute period. To assess the effects of CCh, the peak response, from the basal tone, was measured after each concentration tested. For assessing the response to BSA or OVA exposure, the AUC was measured for a 10-minute period before the addition of the peptide and compared with the 10 minutes AUC measured during the 5- to 15-minute period post-addition.

Histology, Immunohistochemistry and Cell Counting

Paraformaldehyde-fixed tissue samples were processed routinely for paraffin embedding, and 5-μm thick sections were obtained for hematoxylin and eosin (H&E), toluidine or alcian blue staining or for immunohistochemistry. All the preparations were viewed with an Olympus CH30RF200 microscope.

H&E-stained colonic sections were used for histological examination of the tissue and also for assessing eosinophil infiltration, as determined by counting the number of eosinophils in 20 non-adjacent fields of colonic mucosa (×600).

MMCs were identified by immunodetection of rat mast cell protease II (RMCP II) using a monoclonal antibody anti-RMCP II (Moredun Animal Health, Edinburgh, UK). The secondary antibody was a biotinylated horse antimouse IgG (BA-2000; Vector Laboratories, Burlingame, CA, USA). Detection was performed with avidin/peroxidase kit (Vectastain ABC kit; Vector Laboratories). Specificity of the staining was confirmed by omission of the primary antibody. Sections were counterstained with 1% toluidine blue solution (pH 0.5) for 20 minutes, which served to identify CTMCs. For MMCs quantification, at least 20 non-adjacent fields (×400) of colonic mucosa were randomly selected and the number of RMCP II-immunopositive cells determined manually. CTMCs were identified by the presence of toluidine blue-stained metachromatic granules in their cytoplasm. Total number of toluidine blue-stained cells in the preparation was determined and then normalized for the surface area of the tissue section. To identify goblet cells, colonic tissue sections were stained with Alcian Blue pH 2.5/Periodic Acid Schiff (AB 2.5/PAS kit; Bio-Optica, Milano, Italy) in order to specifically stain neutral (pink) and acidic (blue) mucins. Colonic goblet cells were counted in 20 longitudinally-oriented villus-crypt units. Length of the villus-crypt unit was determined to obtain goblet cells density (number of cells/mm). In all cases, cell counting was carried out on coded slides to avoid bias.

Rat Mast Cell Protease II Quantification

Protein was extracted from colonic tissue samples using lysis buffer (50 mM HEPES, 0.05% Triton X-100, 0.0625 mM PMSF, and the Mini Complete protease inhibitor Roche) and

RMCP II concentration was determined by ELISA using a commercial kit (Moredun). Total protein was determined using the Bradford assay kit (BIO-RAD, Hercules, CA, USA).

Bacterial Identification by Fluorescence In Situ Hybridization

For FISH, oligonucleotide probes (Biomers, Ulm/Donau, Germany and Tib Molbiol, Mannheim, Germany) with a 5'-Cy3 (carbocyanine) dye were used (5 ng/μL). The bacterial groups characterized and the specific probes used are specified in Table 1.

In situ hybridization of bacteria in the luminal colonic content was performed on glass slides, as previously described by us.[17-19] Samples were hybridized for 16 hours by addition of the hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS at pH 7.2) and thereafter washed with the washing buffer (20 mM Tris-HCl, 0.9 M NaCl at pH 7.2) for 30 minutes. 4',6-diamidino-2-phenylindole (DAPI) served as a control signal in all samples. Hybridized slides were viewed under oil immersion, using a Carl Zeiss Axioskop 40 FL epifluorescence microscope (filter for Cy3) equipped with a digital camera (Zeiss AxioCam MRm; Carl Zeiss MicroImaging GmbH, Jena, Germany) for obtaining digital images (Zeiss AxioVision Release 4.8.1; Carl Zeiss MicroImaging GmbH, Jena, Germany). For quantification of bacteria, 20 randomly selected fields were photographed, the number of hybridized cells counted using the CellC software[20] and the mean value obtained.

To assess bacterial adherence to the colonic wall, sections from Carnoy-fixed tissues were hybridized in the same conditions. Slides were viewed under oil immersion and 20 randomly selected fields were photographed. Analysis of the images was performed manually by 3 independent researchers that observed the pictures and localized hybridized bacteria within the mucus layer or attached to the epithelial surface. A coincidence between 2 out of the 3 observers in bacterial location in at least three out of the 20 pictures observed was required to decide that there was bacterial attachment to the epithelium.[18] All procedures were performed on coded slides, to avoid bias.

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted from frozen colonic samples by homogenization in Trizol reagent (Ambion, Austin, Texas, USA) followed by isopropanol precipitation and quantification by Nanodrop (Nanodrop Technologies, Rockland, DE, USA). For cDNA

Table 1. Probes Used for Fluorescent In Situ Hybridization and Hybridization Conditions

Probe	Sequence (5' → 3')	Target	Hybridization temp (°C)
EUB 338	GCTGCCTCCCGTAGGAGT	Bacteria	50
NON 338	ACATCCTACGGGAGGC	Non bacteria (negative control)	50
EREC 482	GCTTCTTAGTCAGGTACCG	*Clostridium* cluster XIVa	50
ENT-D	TGCTCTCGCGAGGTCGCTTCTCTT	Enterobacteria	50
BIF 164	CATCCGGCATTACCACCC	*Bifidobacterium* spp.	50

Table 2. TaqMan Gene Expression Assays

Protein	Gene symbol	Assay reference
Inflammatory markers		
IL-6	*Il6*	Rn01410330_m1
IL-13	*Il13*	Rn00587615_m1
IL-10	*Il10*	Rn00563409_m1
IL-12 (subunit beta)	*Il12b*	Rn00575112_m1
Interferon alpha-1 (IFN-α1)	*Ifna1*	Rn02395770_g1
Markers of host-microbial interactions		
Integrin-β1	*Itgb1*	Rn00566727_m1
Integrin-α2	*Itga2*	Rn01489315_m1
Resistin-like molecule beta (RELM-β)	*Retnlb*	Rn01439306_m1
Reference gene		
Actin	*Actb*	Rn00667869_m1

synthesis, 1 μg of RNA was reverse-transcribed in a 20 μL reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California, USA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to determine mRNA levels of inflammatory markers, integrins and resistin-like molecule-beta (RELM-beta) in the colon. The TaqMan probes listed in Table 2 were used (Applied Biosystems). The PCR reaction mixture was incubated on a 7900 real-time PCR system (Applied Biosystems) for 40 cycles (95°C for 15 seconds, 60°C for 1 minute). B-Actin expression served as an endogenous control for normalizing the mRNA levels of the target gens. Expression levels were analyzed by the comparative Ct method (2-ΔΔCT) using the vehicle-vehicle group as the calibrator.

Chemicals

OVA (Grade V; A5503), BSA (A9085) and LPS (LPS from *Escherichia Coli* 055:B5; 62326) were purchased from Sigma-Aldrich (St.Louis, MO, USA) and were dissolved in saline solution. Carbachol (Sigma-Aldrich) was dissolved in distilled water at a 10^{-1} M, further dilutions were prepared in distilled water.

Statistical Methods

All data are expressed as mean ± SEM; except for bacterial counts that are expressed as media (interquartile range) ± SD. A robust analysis (one iteration) was used to obtain mean ± SEM for RT-qPCR data. Comparisons between multiple groups were performed by a one-way or a two-way ANOVA, as appropriate, followed when necessary by a Fisher's least significant difference (LSD) *post hoc* test to detect differences between experimental groups. P-values < 0.05 were considered statistically significant.

Results

Animal Monitoring

Exposure to oral OVA alone during 1 week did not affect body weight compared to animals receiving saline. Addition of LPS produced a transitory loss of body weight, evident only during the first day of treatment. LPS-induced body weight loss was similar in vehicle-LPS (5.24 ± 1.01%; $P < 0.05$ vs vehicle-vehicle group) and OVA-LPS-treated groups (4.05 ± 0.77%; $P < 0.05$ vs OVA-vehicle group). Thereafter, the repeated intraperitoneal administration of LPS did not longer affect body weight and similar weight gain was observed across experimental groups. No other clinical signs were observed.

Colonic Histology

Histological examination of the colon revealed no substantial effects associated to treatments, without evidences of tissue damage or inflammatory-like changes. The number of eosinophils infiltrating the colonic mucosa was similar across experimental groups (Fig. 1A). Quantification of goblet cells in AB-PAS-stained sections revealed that LPS treatment was associated to a diminished cell density, as assessed by a two-way ANOVA, reaching statistical significance in OVA-LPS-treated rats (122.7 ± 3.8 cells/mm; $P < 0.05$ vs vehicle-vehicle: 153.8 ± 4.0 cells/mm; Fig. 1B). Animals exposed to OVA alone also showed a tendency for a decrease in the relative abundance of goblet cells (133.8 ± 6.75 cells/mm; $P = 0.050$ vs vehicle-vehicle; Fig. 1B). In vehicle-vehicle-treated animals, combined AB-PAS staining revealed that nearly all the goblet cells of the colonic mucosa contained acidic mucins (blue staining), with very few goblet cells showing mixed or neutral mucins (purple and pink staining, respectively). Relative abundance of acidic, mixed and neutral mucins was not affected by OVA or LPS or their combination.

Mast Cells Counts and Rat Mast Cell Protease II Content

Colonic MMC counts remained unaltered after one week exposure to oral OVA (OVA-vehicle: 5.26 ± 0.90 cells/field; $P > 0.05$ vs vehicle-vehicle: 5.80 ± 2.18 cells/field; Fig. 1C). Addition of LPS had no effect on the colonic density of MMCs (Fig. 1C). Similarly, colonic content of RMCP II was unaffected by OVA (OVA-vehicle: 0.55 ± 0.21 ng/μg protein; $P > 0.05$ vs vehicle-vehicle: 0.56 ± 0.23 ng/μg protein) and remained unaltered after LPS treatment (Fig. 1D).

CTMCs, identified by its metachromatic granules, were mainly localized in the submucosa of the rat colon, with a relatively low density in control conditions (2.73 ± 0.29 cells/field; Fig. 1E and 2). Neither OVA nor LPS, per se, affected CTMCs counts. However, in OVA-LPS-treated animals the counts of CTMCs were increased by 2-fold when compared with the separate treatments or the vehicle-vehicle group ($P < 0.05$; Fig. 1E and 2).

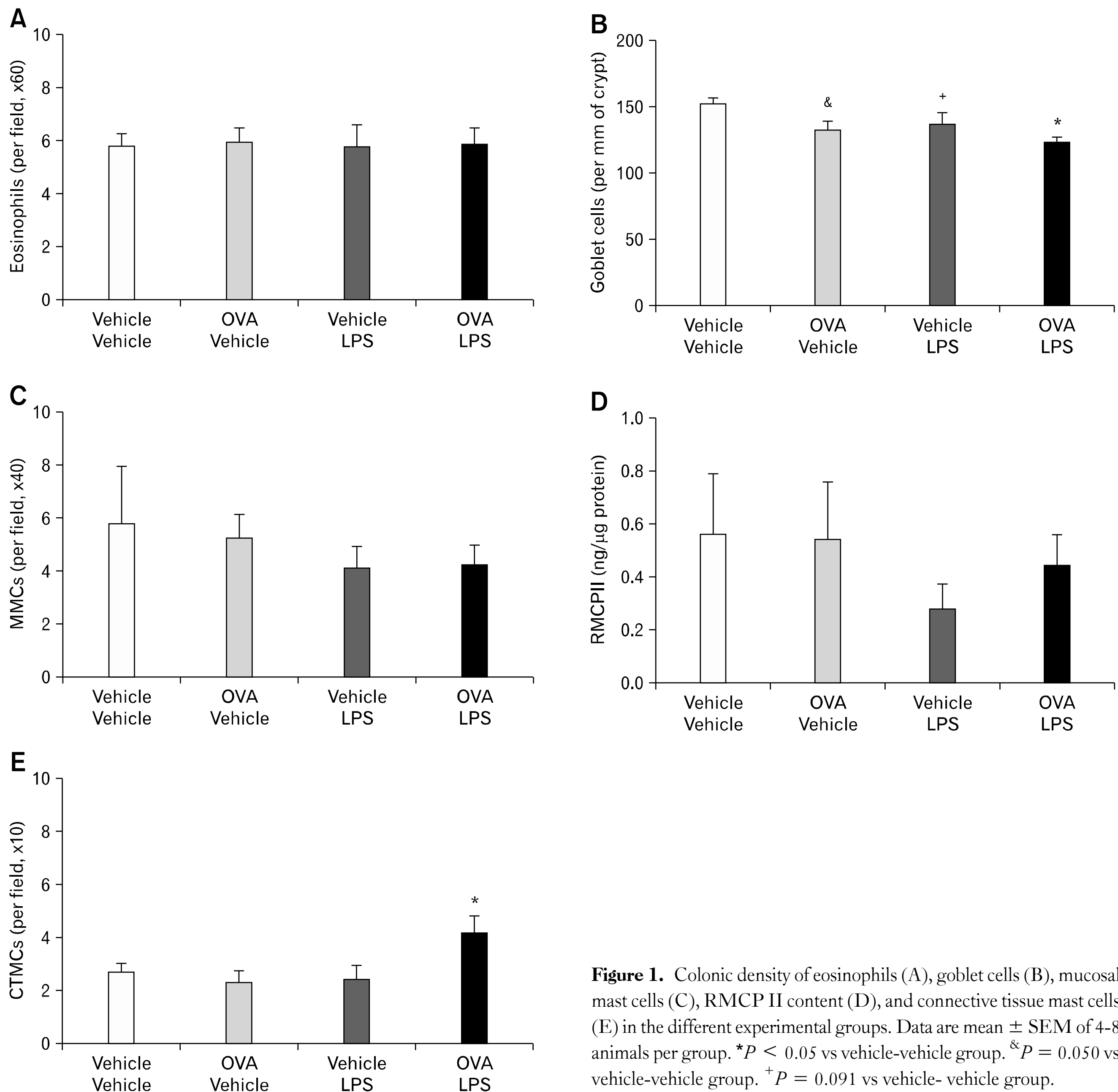

Figure 1. Colonic density of eosinophils (A), goblet cells (B), mucosal mast cells (C), RMCP II content (D), and connective tissue mast cells (E) in the different experimental groups. Data are mean ± SEM of 4-8 animals per group. $^{*}P < 0.05$ vs vehicle-vehicle group. $^{\&}P = 0.050$ vs vehicle-vehicle group. $^{+}P = 0.091$ vs vehicle- vehicle group.

Characterization of Luminal and Wall-adhered Microbiota

In vehicle-vehicle-treated animals, total bacterial counts in the luminal content of the colon, determined by FISH as EUB338-positive cells and confirmed by DAPI staining, were within the margins previously described by us ($3.74 \times 10^{10} \pm 1.16 \times 10^{10}$ cells/mL; Fig. 3).[17,19] Total bacterial counts were not altered by the administration of OVA alone ($2.90 \times 10^{10} \pm 6.10 \times 10^{9}$ cells/mL; $P > 0.05$ vs vehicle-vehicle; Fig. 3). However, treatment with LPS diminished by 50% total bacterial counts vs vehicle-vehicle values, irrespective of the coadmnistration of OVA (LPS-vehicle: $1.75 \times 10^{10} \pm 3.10 \times 10^{9}$ cells/mL; LPS-OVA: $1.62 \times 10^{10} \pm 1.84 \times 10^{9}$ cells/mL; both $P < 0.05$ vs vehicle-vehicle; Fig. 3). This effect was further confirmed by similar results obtained in DAPI-positive nuclei counts (Fig. 3).

Independently of the changes in total bacterial counts, LPS and OVA resulted in states of dysbiosis, affecting selectively the

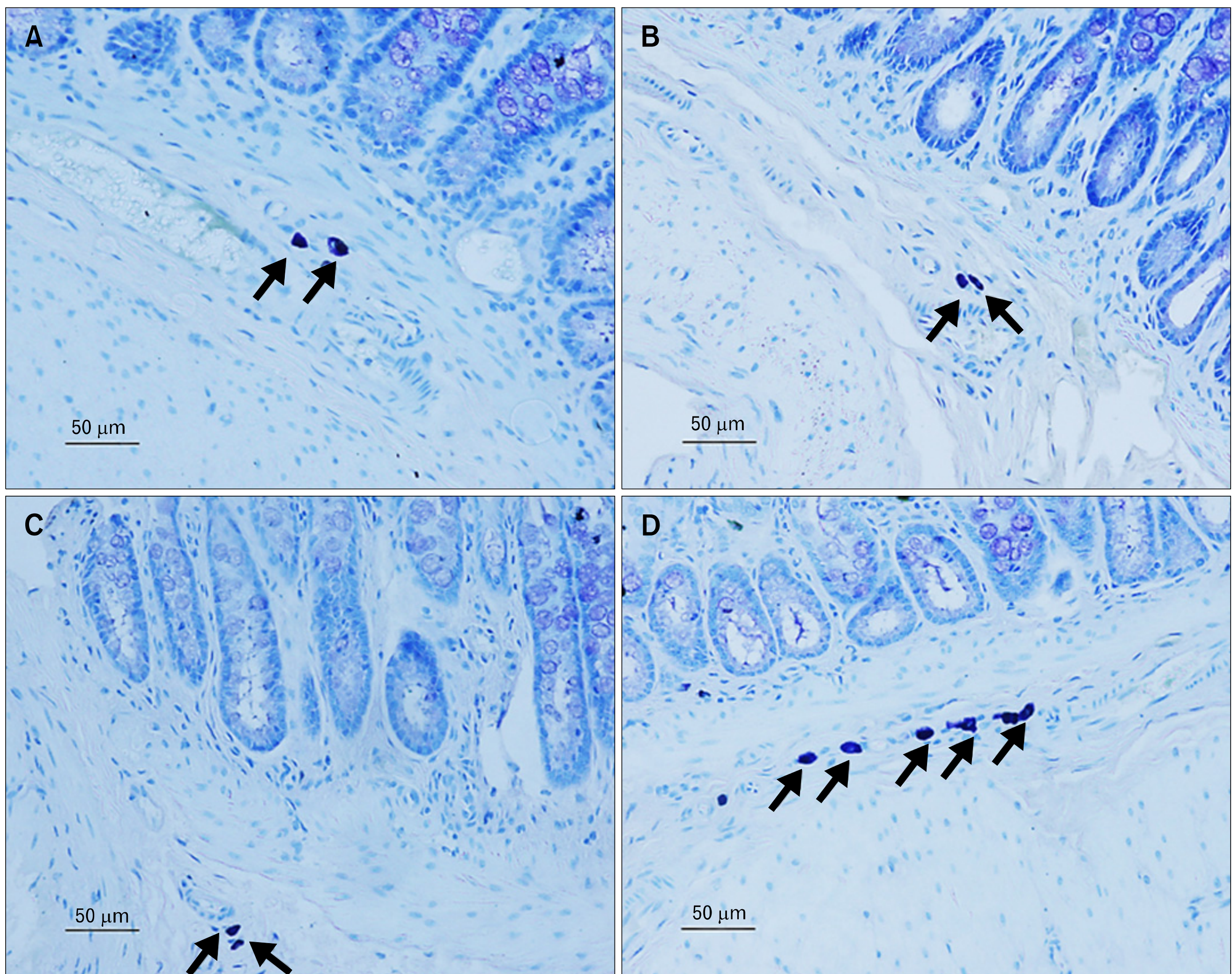

Figure 2. Representative microphotographs showing connective tissue mast cells (CTMCs) in toluidine blue-stained colonic slices from vehicle-vehicle- (A), ovalbumin (OVA)-vehicle- (B), lipopolysaccharide (LPS)-vehicle- (C), and LPS-OVA-treated (D) rats. The arrows indicate CTMCs in the submucosa, as identified by the presence of toluidine blue-stained metachromatic granules in their cytoplasm. Notice the increased density of the CTMC population in the colonic submucosa of LPS-OVA-treated animals (D).

Clostridium cluster XIV or *C. coccoides* group (EREC482 probe). Overall, *Clostridium* spp. was the most abundant strain, irrespective of the treatment considered. Exposure to oral OVA alone or LPS alone decreased *Clostridium* spp. counts in similar proportion (OVA-vehicle: $7.83 \times 10^9 \pm 1.41 \times 10^9$ cells/mL; vehicle-LPS: $7.53 \times 10^9 \pm 1.19 \times 10^9$ cells/mL; $P = 0.072$ and $P = 0.050$ vs vehicle-vehicle, respectively; Fig. 3). The combination of both treatments resulted in a further reduction in *Clostridium* spp. counts to $4.26 \times 10^9 \pm 4.03 \times 10^8$ cells/mL ($P < 0.05$ vs vehicle-vehicle; Fig. 3). Other bacterial groups assessed, namely *Bifidobacterium* spp. (BIF164 probe) and Enterobacteria (ENT-D probe) were not affected by either OVA, LPS or their combination.

In vehicle-vehicle-treated animals, bacterial adherence to the colonic epithelium was relatively low and similar for all the bacterial groups assessed (by 20% incidence; Fig. 3). Treatment with OVA, did not affect the incidence of bacterial wall adherence. However, LPS showed a tendency to favour the adherence of *Bifidobacterium* spp. (incidence: 35%) and *Clostridium* spp. (incidence: 50%) (Fig. 3 and 4). The combination of OVA and LPS completely prevented the adherence of *Clostridium* spp. (0% incidence) without affecting the adherence of *Bifidobacterium* spp. (incidence: 35%).

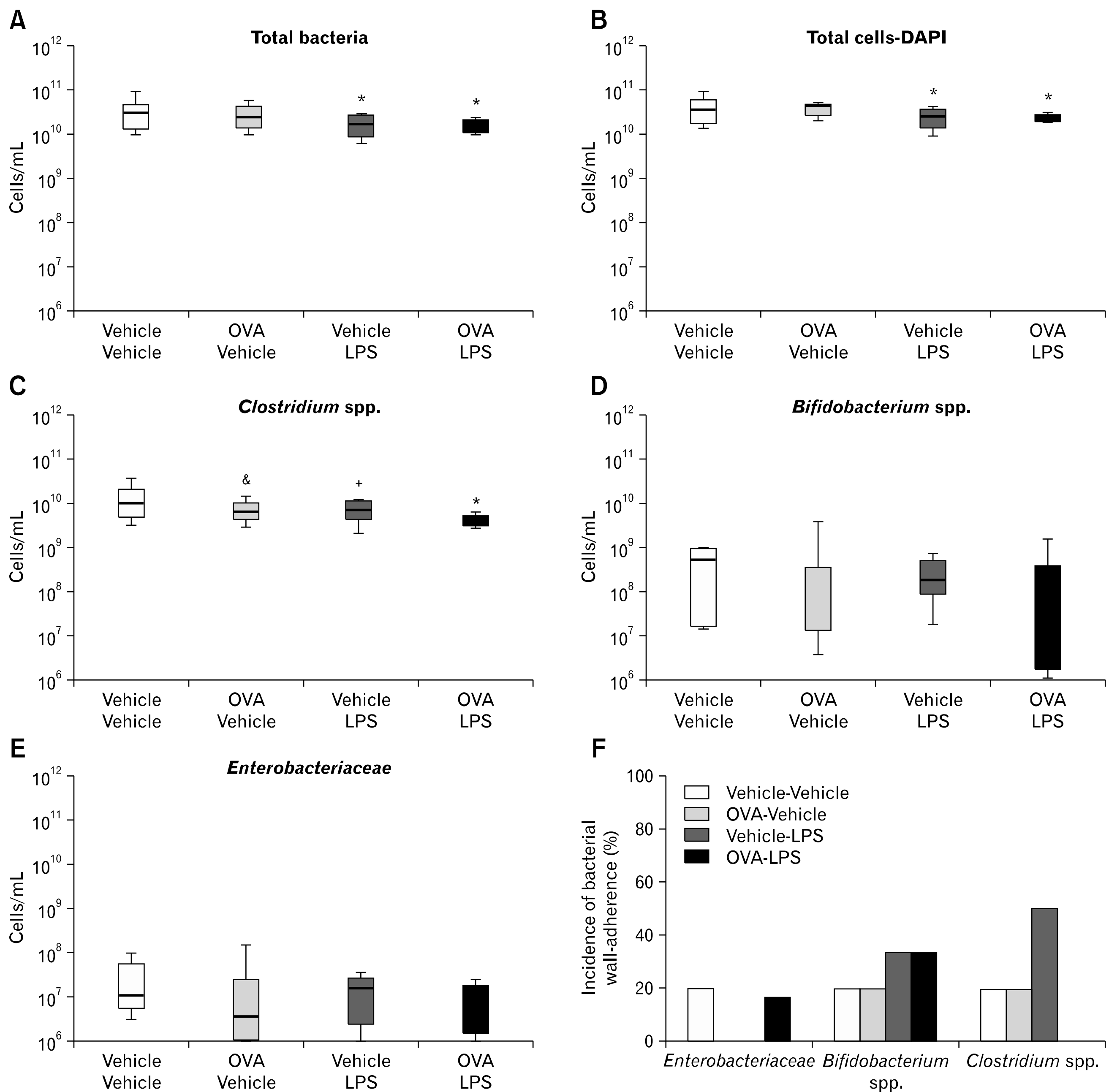

Figure 3. Composition of the luminal microbiota and incidence of bacterial wall adherence, as assessed by fluorescence in situ hybridization. Bacterial counts are median (interquartile range) ± SD, 7-8 animals per group. $^{*}P < 0.05$ vs vehicle-vehicle group. $^{+}P = 0.050$ vs vehicle-vehicle group. $^{\&}P = 0.072$ vs vehicle-vehicle group. Incidence of bacterial wall adherence (bottom right corner): data represent the percentage of animals showing bacterial wall adherence for the different bacterial groups assessed. DAPI, 4',6-diamidino-2-phenylindole; OVA, ovalbumin; LPS, lipopolysaccharide.

Expression of Inflammatory Markers and Markers of Host-bacterial Interactions

In control conditions (vehicle-vehicle-treated animals), colonic mRNA expression of pro-inflammatory (IFN-α1, IL-6, IL-12, and IL-13) and anti-inflammatory markers (IL-10) was relatively low, but within detectable ranges in all samples. Overall, relative expression of cytokines was: IFN-α1 > IL-10 > IL-12β > IL-6/IL-13.

Exposure to oral OVA alone did not affect cytokines ex-

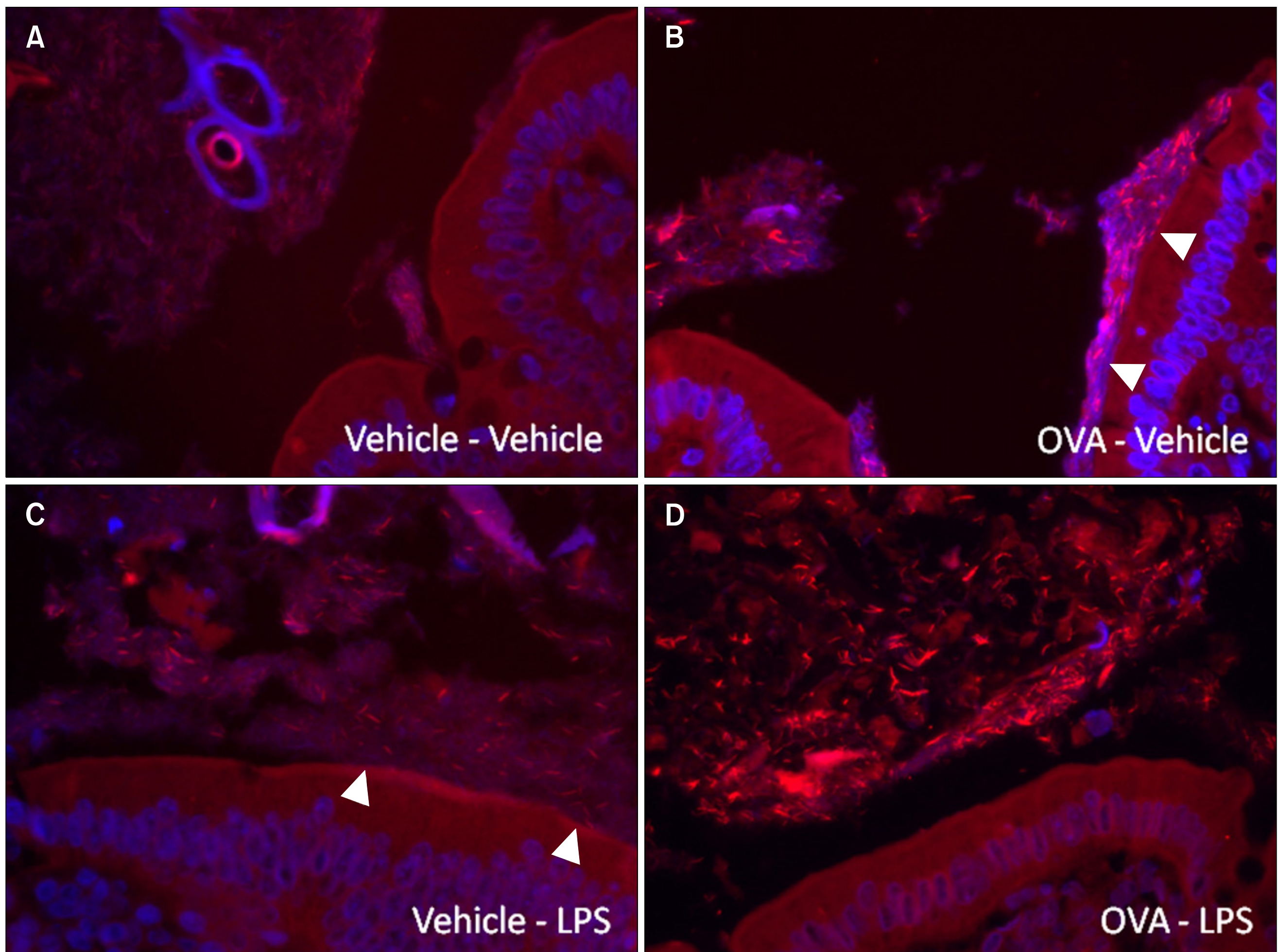

Figure 4. Representative colonic tissue images showing wall adherence of *Clostridium* spp. (EREC 482 probe) in the different experimental groups. White arrowheads indicate hybridized bacteria attached to the colonic epithelium. OVA, ovalbumin; LPS, lipopolysaccharide.

pression; with the exception of IL-12β, which was slightly up-regulated although statistical significance was not reached. Similarly, LPS alone had minor effects on cytokines expression, with only a tendency ($P = 0.060$) to increase colonic IL-6 levels (Fig. 5). However, combined treatment with OVA and LPS resulted in a 2-fold increase in the expression of IFN-α1 (Fig. 5).

In control conditions, markers of host-bacterial interactions, namely integrin-β1, integrin-α2, and RELM-β, were moderately expressed. Relative expression levels were: integrin-β1 > integrin-α2 > RELM-β. OVA tended to down-regulate integrin-α2, particularly in animals co-treated with LPS (Fig. 5).

Colonic Contractility In Vitro

Spontaneous colonic contractile activity was similar in vehicle-vehicle- and OVA-vehicle-treated animals ([AUC in 15 minutes] OVA-vehicle: 39.81 ± 4.88 g; vehicle-vehicle: 34.19 ± 2.39 g; $P > 0.05$; Fig. 6A). A two way ANOVA analysis revealed that the challenge with LPS had a significant effect ($P = 0.043$) enhancing contractile activity both in vehicle-LPS- and OVA-LPS-treated rats (Fig. 6A). However, further *post hoc* test (Fisher's least significant difference) found no differences between specific groups.

In vehicle-vehicle-treated animals, CCh elicited contractile responses in a concentration-dependent manner. Neither OVA nor LPS, alone or in combination, affected the contractile responses to CCh (Fig. 6B).

The spontaneous contractile activity of tissue samples obtained from vehicle-vehicle-treated animals was not affected by the direct addition of OVA to the organ bath. Similarly, in tissues from animals exposed to oral OVA during one week, direct ex-

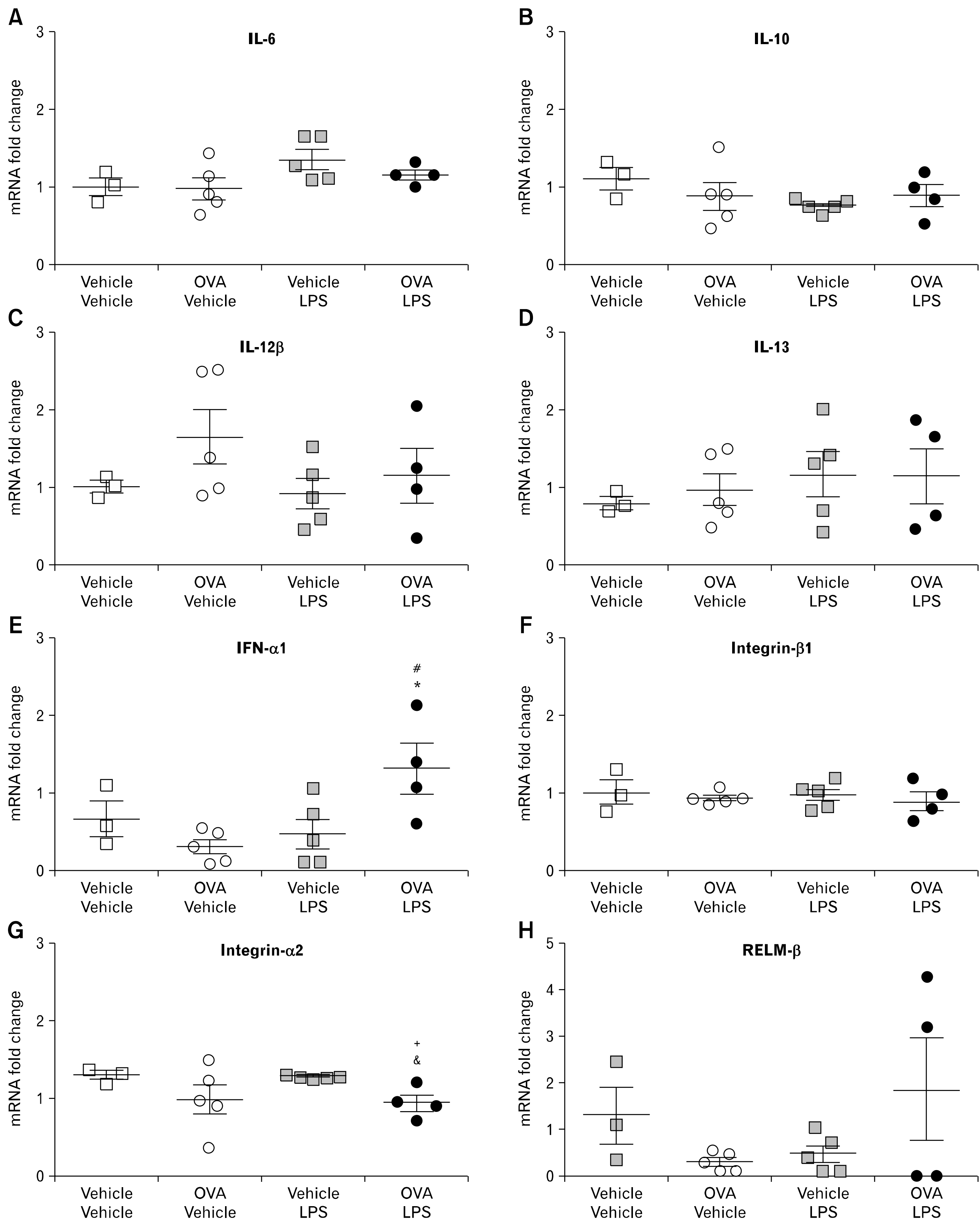

Figure 5. Colonic expression of inflammatory markers and markers of host-bacterial interactions. Each symbol represents an individual animal, the horizontal lines with errors represent the mean ± SEM. *$P < 0.05$ vs vehicle-lipopolysaccharide (LPS) and ovalbumin (OVA)-vehicle group. $^{\#}P = 0.082$ vs vehicle-vehicle group. $^{+}P = 0.091$ vs vehicle-vehicle group. $^{\&}P = 0.075$ vs vehicle-LPS group. RELM, resistin-like molecule.

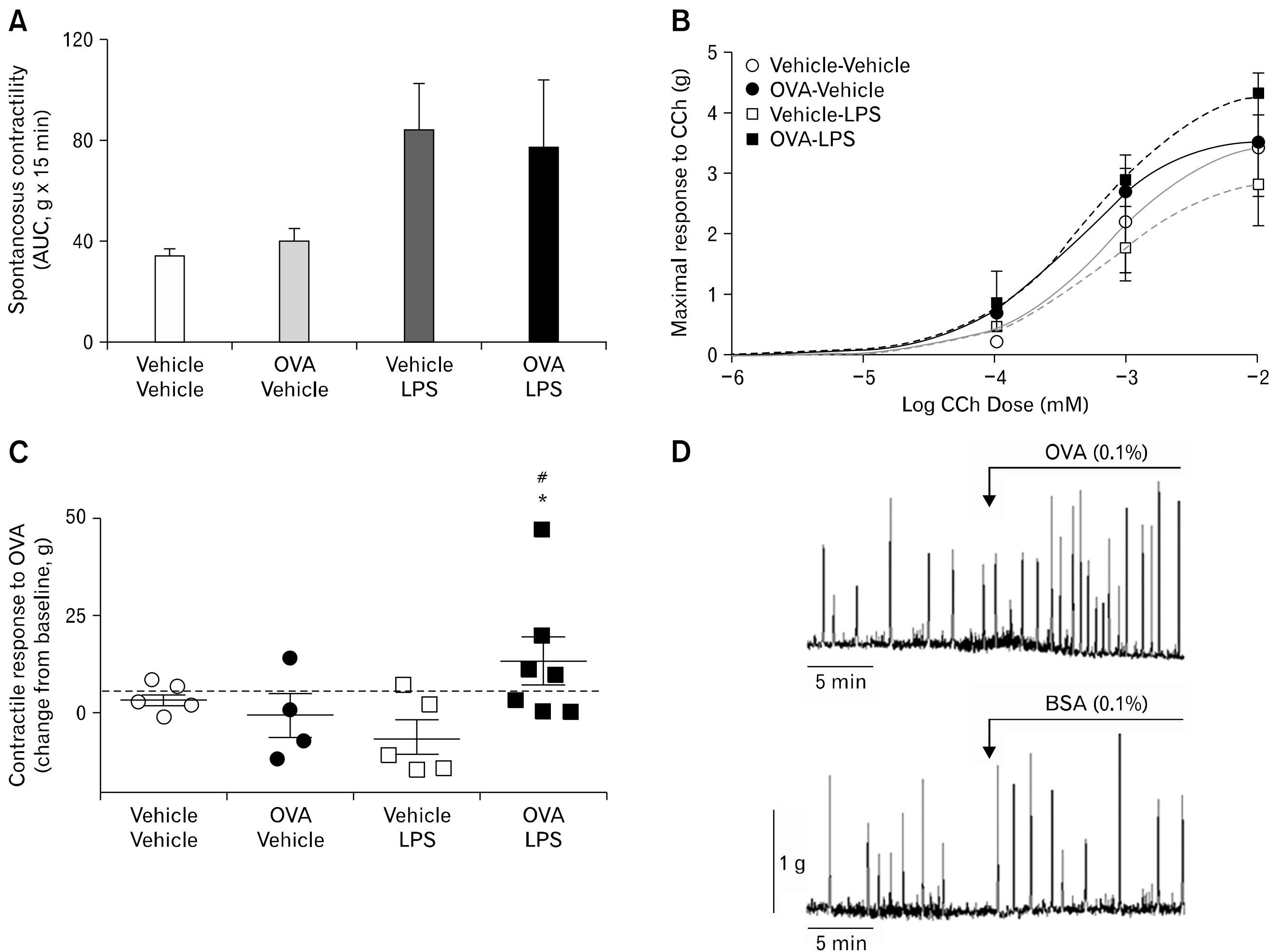

Figure 6. Colonic contractility in vitro. (A) Colonic spontaneous contractile activity (AUC in 15 minutes) in the different experimental groups. Data are mean ± SEM; n = 4-6 per group. (B) Concentration-response curves for carbachol. Data show the maximal response to each concentration of carbachol added to the bath and is represented as mean ± SEM (symbols) and non-linear regression curves. n = 4-6 per group. (C) Colonic contractile responses to ovalbumin (OVA) challenge. Data represent the net change in contractile activity during the 5-15 minute period after addition of OVA to the organ bath (taking as basal the contractile activity during the 10 minutes previous to OVA addition). Each symbol represents an individual animal, the horizontal lines with errors represent the mean ± SEM (n = 4-7 per group). The broken horizontal line corresponds to the mean + 2 × SEM. $^{*}P < 0.05$ vs vehicle-LPS group. $^{\#}P = 0.092$ vs OVA-vehicle group. (D) Representative tracings showing the effects of OVA and bovine serum albumin (BSA) challenge on spontaneous colonic contractility in an OVA-lipopolysaccharide (LPS)-treated animal. Notice the increase in the magnitude and frequency of colonic contractions after addition of OVA to the organ bath, an effected non-observed after the addition of BSA.

posure to OVA was without effect. However, OVA challenge on tissues obtained from OVA-LPS-treated rats resulted in an increase ($P < 0.05$) in the frequency and magnitude of the spontaneous contractile activity (Fig. 6C and 6D). Regardless the experimental group considered, direct addition of a BSA solution to the organ bath did not affect colonic spontaneous contractile activity.

Discussion

Dietary antigens and gut microbiota are potential factors contributing to the pathophysiology of IBS. In this study, we show an interaction between a dietary antigen (OVA) and microbial components that leads to a situation of dysbiosis together with an altered colonic contractility, mimicking some of the features described in IBS patients. Our results indicate that LPS favors the colonic sensitization to OVA, thus suggesting that alterations of

the commensal microbiota and the gut host-microbiotal interactions might be factors facilitating the intestinal (colonic) sensitization against dietary antigens and the development of IBS-like states.

We previously showed that rats exposed to oral OVA, without adjuvants, during a 6-week period, develop a colonic contractility dysfunction, resembling that observed in IBS patients and other animal models of the disease.[7-11,21] However, repeated exposure to low doses of a dietary antigen might lead to a process of oral tolerance.[22,23] Therefore, in the present work, we assessed the effectiveness of a shorter exposure period to high doses of oral OVA in the induction of IBS-like colonic motor alterations. Results obtained show that colonic contractility remained unaltered after 1-week exposure to 10 mg of OVA, thus suggesting that, in contrast to long-term exposure, sensitization to oral antigens induced by few administrations might require the presence of facilitatory elements, such as adjuvants.[24] We also explored if GCM might represent a potential factor favoring the induction of adverse reactions to food antigens. Administration of low-doses of LPS, which mimics a gram-negative bacterial overload, resulted in a specific dysbiosis of the colon. In addition, when combined with oral OVA, LPS induced an enhanced colonic contractility to the direct addition of the antigen to the organ bath, thus indicating a sensitization of the tissue to OVA. These altered responses of the colon are reminiscent of the exacerbated symptomatology reported in a subgroup of IBS patients after the ingestion of certain foods, reinforcing the hypothesis that adverse reactions to dietary components might be a contributing factor to the pathophysiology of the disease.[25] Moreover, results shown here support the view that gut microbiota might facilitate the sensitization process to luminal antigens, thereby representing a factor leading to the development or the exacerbation of symptoms in IBS.

Dietary-related IBS-like responses have been associated, both in patients and animal models, to local mechanisms of the colonic mucosa rather than a systemic reaction, more characteristic of food allergies.[4,6,7] In agreement with these observations, in this study, OVA-induced altered contractility in OVA-LPS-treated rats was neither related to the presence of circulating specific IgEs (unpublished results) nor to the altered expression of pro- (IL-6 and IL-13) or anti-allergic (IL-12 and IL-10) cytokines.[26,27] However, these animals showed a specific up-regulation of IFN-α1, similar to that observed in IBS-like states.[28,29]

Colonic motor changes after long term exposure to oral OVA are related to an excited-activated state of MMCs.[21] Moreover, MMCs have been directly implicated in the pathophysiology of IBS, although their exact role remains unclear.[30-32] Here, no changes in MMC density or their excited-activated state was observed after a 1-week OVA exposure, with or without LPS. However, CTMC counts were increased in the submucosa of OVA-LPS-treated animals, while unaffected in animals treated only with OVA or LPS. Although the potential involvement of CTMCs in IBS has been less studied, several observations suggest that they could act also as effector cells leading to functional alterations within the gut. For instance, we have described a similar increase in CTMCs in the jejunum of *Trichinella spiralis*-infected rats, an accepted model of post-infectious IBS that also courses with dysmotility.[33] Moreover, CTMC counts are increased in mice with food allergy[34,35] and the degranulation of CTMCs excites the intestinal smooth muscle in vitro.[36] Although further in deep studies are required, these observations suggest that submucosal CTMC could be taking part in the altered contractile responses observed in OVA-LPS-treated rats.

Gut microbiota is a dynamic component of gastrointestinal homeostasis. Epidemiological and clinical data support the hypothesis that significant perturbations of the GCM can disrupt the mechanisms of oral tolerance leading to allergic responses.[37] In agreement, in the current study, low doses of LPS facilitated the sensitization to oral OVA at the same time that induced changes in GCM (an overall decrease in luminal bacterial counts with a specific dysbiotic state characterized by a loss of clostridia). At the same time, LPS favored the adherence of *Clostridium* spp. to the colonic wall. A cause-effect relationship between adherence and luminal microbiota can be suggested from these changes. It is feasible to speculate that increased host-bacterial interactions, driven by the adherence of clostridia, might trigger a local response that results in the dysbiotic-like state observed when assessing the luminal microbiota. The apparent mismatch between adherence and luminal bacterial counts is similar to that observed previously in other models of dysbiosis and suggests that, besides the absolute number of bacteria, other factors modulate host-bacterial interactions.[18] Nevertheless, we did not observe changes in the overall expression of adhesion molecules or RELM-beta, important components facilitating host-bacterial interactions within the gut. From the present studies, we cannot state that the observed changes in the microbiota are relevant in the facilitatory effects of LPS towards OVA sensitization. However, this hypothesis is supported by previous results that demonstrate a direct relationship between altered states of GCM and increased susceptibility to sensitization to oral dietary antigens.[14]

Abnormal intestinal responses to dietary antigens have been related in part to an altered epithelial barrier function.[38] Indeed, altered gut microbiota is able to favor sensitization towards dietary antigens throughout changes in epithelial permeability.[38] Here, although not directly assessed, it is feasible to assume the presence of an altered barrier function as the doses and pattern of administration of LPS were similar to other studies demonstrating an increased epithelial permeability.[39,40] In addition, adherence of *Clostridium* spp. to the colonic epithelium, favored by LPS, could be a contributing factor in the alteration of the barrier function and the subsequent sensitization to OVA. Surprisingly, in OVA-LPS-treated rats no adherence of *Clostridium* spp. was observed (0% incidence). This might be associated to the presence of colonic hypercontractility generated by the presence of OVA in a state of LPS-induced enhanced sensitization. In these conditions, increased colonic motility might difficult the interaction between luminal bacteria and the epithelium, preventing bacterial adherence. In agreement with this hypothesis, we observed that direct addition of OVA to the organ bath only increased colonic contractile activity in tissues from OVA-LPS-treated animals. Moreover, this effect on bacterial dynamics might be potentiated by the enhanced mucus release present in OVA-LPS-treated rats, as suggested by the reduced density of goblet cells.[41] Thus, in OVA-LPS-treated animals, the mucus discharge could facilitate the trapping of bacteria and prevent their attachment to the epithelial surface, while the altered motor responses would assist in their subsequent expulsion.[42]

It is worthy to mention that LPS, per se, might be directly responsible for some of the changes in colonic contractility observed in the present work. Indeed, low doses of LPS have been reported to enhance intestinal motility through mechanisms implicating the enteric nervous system and the modulation of the production of several neuroimmune mediators (including IL-1β, cyclooxygenase, and constitutive nitric oxide synthase) in mice and rats.[43-45] Although not directly addressed, similar mechanisms might be contributing to the changes observed in the present study, with direct effects of LPS on local neuroimmune responses leading to alterations in smooth muscle contractility.

In summary, this study shows an interaction between luminal dietary antigens and components of the gut microbiota (LPS) leading to potential alterations in colonic motor activity. In particular, we show that a dysbiotic situation favours the sensitization against a luminal dietary antigen, namely OVA, and the generation of abnormal motor responses against that antigen. Similar mechanisms might contribute to the pathophysiology of IBS, where a significant proportion of patients show an altered microbiota and sensitivity to certain food components. Alterations of the microbiota might enhance/facilitate the local neuroimmune responses to specific food antigens leading to motor alterations reminiscent to those observed in IBS. Nevertheless, direct effects of LPS (independent of any microbial-related change) on local neuroimmune responses modulating colonic contractility and/or sensitization to OVA cannot be discarded.

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