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Endotoxicity of Lipopolysaccharide as a Determinant of T-Cell—Mediated Colitis Induction in Mice

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BACKGROUND & AIMS: The intestinal microbiota is an important determinant of the mucosal response. In patients with inflammatory bowel diseases, the mucosal immune system has inappropriate interactions with the intestinal microbiota. We investigated how the composition of the intestinal microbiota affects its endotoxicity and development of colitis in mice. **METHODS:** Germ-free C57BL/6J-Rag^{1tm1Mom} (*Rag1*$^{-/-}$) mice were colonized with 2 different types of complex intestinal microbiota. Colitis was induced in *Rag1*$^{-/-}$ mice by transfer of CD4^{+}CD62L^{+} T cells from C57BL/6J mice. Colonic tissues were collected and used for histologic analysis and cell isolation. Activation of lamina propria dendritic cells and T cells was analyzed by flow cytometry. **RESULTS:** After transfer of CD4^{+}CD62L^{+} T cells, mice with intestinal Endo^{lo} microbiota (a low proportion of *Enterobacteriaceae*, high proportion of *Bacteroidetes*, and low endotoxicity) maintained mucosal immune homeostasis, and mice with highly endotoxic Endo^{hi} microbiota (a high proportion of *Enterobacteriaceae* and low proportion of *Bacteroidetes*) developed colitis. To determine whether the effects of Endo^{hi} microbiota were related to the higher endotoxic activity of lipopolysaccharide (LPS), we compared LPS from *Enterobacteriaceae* with that of *Bacteroidetes*. Administration of *Escherichia coli* JM83 (wild-type LPS) to the mice exacerbated colitis, and *Escherichia coli* JM83 + htrBPG (mutated LPS, with lower endotoxicity, similar to that of *Bacteroidetes*) prevented development of colitis after transfer of the T cells to mice. **CONCLUSIONS:** The endotoxicity of LPS produced by the intestinal microbiota is a determinant of whether mice develop colitis after transfer of CD4^{+}CD62L^{+} T cells. This finding might aid the design of novel biologics or probiotics to treat inflammatory bowel disease.

Keywords: Mouse Model; IBD; Prevention; Bacteria.

The mammalian gastrointestinal tract harbors a dense and diverse community of an estimated 10—100 trillion micro-organisms[1–3] consisting of 500—1000 different species, of which the vast majority are bacteria.[2,4] It is well accepted that in inflammatory bowel disease (IBD), the mucosal immune system reacts inappropriately toward the commensal microbiota.[5] No particular microbial species has been consistently linked to IBD pathogenesis or prevention; however, some symbiotic bacterial species have been shown to prevent inflammatory host responses.[2,6–9]

Numerous animal models have been generated to experimentally investigate human IBD,[10] including erosive models of acute colitis (eg, dextran sodium sulfate [DSS]-induced colitis), spontaneous models of chronic colonic, and/or small bowel inflammation induced by targeted gene deletion (eg, interleukin [*IL*]*10*$^{-/-}$ mice) or induction by disruption of T-cell homeostasis (eg, *Rag1*$^{-/-}$ mice).[10] As chronic colitis results from a dysregulated immune response to components of the normal intestinal microbiota, it is reasonable to suggest that the T-cell—dependent models are significantly more relevant to human disease than are the erosive models of acute colitis, if one wishes to investigate the immunologic mechanisms inducing, perpetuating, or preventing chronic colitis.[10] Microbe-associated molecular pattern, such as lipopolysaccharide (LPS) or flagellins, are recognized by different pattern recognition receptors. However, there is a dichotomic role for Toll-like receptor (TLR) in intestinal inflammation.[11] For example, *IL2*$^{-/-}$ *MyD88*$^{-/-}$ mice develop colitis independent of TLR signaling, and *IL10*$^{-/-}$ *MyD88*$^{-/-}$ mice remain healthy,

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Abbreviations used in this paper: **clp, colonic lamina propria; DC, dendritic cell; DSS, dextran sodium sulfate; IBD, inflammatory bowel disease; IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MUT, mutant; rDNA, ribosomal DNA; TLR, Toll-like receptor; WT, wild type.**

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indicating an inflammation promoting effect of MyD88-dependent TLR.[12] Additionally, in the DSS model of acute inflammation, which is based on disruption of tight junction proteins and severe disturbance of the intestinal barrier, the depletion of the intestinal microbiota through antibiotics resulted in aggravation of acute colitis, which was ameliorated by feeding of LPS.[13] In the Crohn's disease–like murine T-cell–induced chronic colitis, the roles of LPS and of pattern recognition receptor signaling remain unclear.

The goal of our study was to show the influence of qualitatively different TLR4 signals on development of chronic T-cell–driven colitis. We demonstrate that the endotoxicity of the intestinal microbiota given by the composition of the intestinal bacterial communities determines either the maintenance of intestinal homeostasis or the induction of colitis in genetically predisposed hosts. Importantly, T-cell–transferred *Rag1*$^{-/-}$ mice, with low endotoxic microbiota due to a high number of bacteria of the anaerobic *Bacteroidetes* group, were protected from induction of transfer colitis, and *Rag1*$^{-/-}$ mice, with high endotoxic microbiota due to a high number of commensal *Enterobacteriaceae*, develop colitis. The low endotoxic *Escherichia coli* JM83 +*htrB*$_{Pg}$ strain (*E coli*$_{MUT}$) with alterations in the acylation pattern promoted intestinal homeostasis, and feeding with the high endotoxic *E coli* JM83 K-12 wild-type (*E coli*$_{WT}$) stain resulted in severe intestinal inflammation. This was, in particular, supported by feeding experiments with isolated LPS from both the WT and mutant (MUT) strain.

The current results shed new light on the previously unrecognized role of LPS toxicity in the maintenance of intestinal immune homeostasis and suggest novel treatment options to shape mucosal immunity in patients with IBD.

Material and Methods

Mice

For the experiments, inbred C57BL/6J mice and C57BL/6J-Rag^{1tm1Mom} (*Rag1*$^{-/-}$)[14] mice were used. Germ-free mice were colonized with different complex intestinal microbiota by co-housing with Endo^{lo} or Endo^{hi} colonized C57BL/6 mice bred and kept in isolated ventilated cages. Mice were free of *Helicobacter hepaticus*, norovirus, and rotavirus. Endo^{lo} mice harbor microbiota with a high proportion of *Bacteroidetes* and low proportion of *Enterobacteriaceae*, and Endo^{hi} *Rag1*$^{-/-}$ mice harbor a high proportion of *Enterobacteriaceae* and low proportion of *Bacteroidetes*. *Rag1*$^{-/-}$ mice were transplanted with 5×10^5 splenic CD4$^+$CD62L$^+$ T cells at 8–10 weeks of age.[15–20] Endo^{hi} *Rag1*$^{-/-}$ mice were analyzed after manifestation of colitis 4–6 weeks after T-cell transfer, Endo^{lo} *Rag1*$^{-/-}$ mice 6 weeks after T-cell transfer. All animal experiments were reviewed and approved by the responsible Institutional Review Board.

Growth of Bacteria, Isolation of LPS, and Analytical Methods

E coli strains (*E coli* JM83 [*E coli*$_{WT}$] and *E coli* JM83 +*htrB*$_{Pg}$ [*E coli*$_{MUT}$][21]) were grown in Luria Bertani medium until log phase. Where indicated, 100 μg/mL ampicillin and isopropyl-β-D-thiogalactopyranoside (1 mM) was added. The LPS were extracted according to Galanos et al,[22] in the yields of 2.6% (WT) and 2.9% (MUT). Fatty acid analyses[23] and high-resolution electrospray ionization Fourier transform ion cyclotron mass spectrometry[24] were performed as published. Mice were challenged with 10^8 viable bacteria or with 160 μg purified LPS (6.4 μg/g mouse).

See the Supplementary Materials for information on cell culture experiments, analysis of fecal samples by culture methods and quantitative polymerase chain reaction, isolation of DNA from fecal samples, preparation of amplicon pool and 454-sequencing, bioinformatic analysis, isolation of lp dendritic (DC) and T cells, intracellular cytokine staining, flow cytometry, histology, and statistics.

Results

Low Endotoxicity of the Intestinal Microbiota in Rag1$^{-/-}$ Mice Is Associated With Mucosal Immune Homeostasis

In a model of IBD, we investigated whether the endotoxicity of complex intestinal microbiota influenced the incidence or severity of colitis. Therefore, *Rag1*$^{-/-}$ mice, raised in a germ-free facility, were colonized with 2 types of complex intestinal microbiota with different endotoxicity. We used microbiota with a low TLR4-activating effect (Endo^{lo}) (Figure 1*A*) characterized by low numbers of *Enterobacteriaceae* (including *E coli*) and high numbers of *Bacteroidetes* (including *Bacteroides vulgatus* or *Porphyromonas sp*) (Figure 1*B* and *C*) and, in addition, a high TLR4-activating microbiota (Endo^{hi}) (Figure 1*A*) characterized by high numbers of *Enterobacteriaceae* and low numbers of *Bacteroidetes* as revealed by culture techniques (Figure 1*B*) and quantitative polymerase chain reaction (Figure 1*C*). Analysis of the intestinal microbiota by 454 sequencing of the 16S ribosomal DNA (rDNA) amplicons containing the variable regions V3–V6 revealed 70.3% of *Bacteroidetes* and 22.94% of *Firmicutes* in the Endo^{lo} *Rag1*$^{-/-}$ mice. Proteobacterial (including *E coli*) 16S rDNA amplicons were below the detection limit. In Endo^{hi} *Rag1*$^{-/-}$ mice, 0.19% of the analyzed 16S rDNA amplicons belonged to *Proteobacteria* (*Enterobacteriaceae*, including *E coli*, are a family within this phylum), 32.42% to *Bacteroidetes* and 61.84% to *Firmicutes* (including, eg, the classes *Bacilli* with the order of *Bacillales* and *Lactobacillales*, *Clostridia*, or *Erysipelotrichia*) (Figure 1*D*). All mice described in this study were raised in these colonies to assure early perinatal colonization with the complex microbiota defined here.

On transfer of T cells from healthy specific pathogen-free C57BL/6 mice the Endo^{hi} *Rag1*$^{-/-}$ mice developed severe colitis within 6 weeks, lost significant amounts of weight, and exhibited pronounced inflammation of colonic mucosa and submucosa. In contrast, T-cell–transferred Endo^{lo} *Rag1*$^{-/-}$ mice remained healthy for 6 weeks, as indicated by both weight gain during the course of the experiment and missing histologic signs of inflammation (Figure 2*A*–*C*).

DCs in the colonic lamina propria (clp) of T-cell–transferred Endo^{hi} *Rag1*$^{-/-}$ mice showed significantly higher expression of major histocompatibility complex (MHC) class II and CD40 as compared with the lp DC

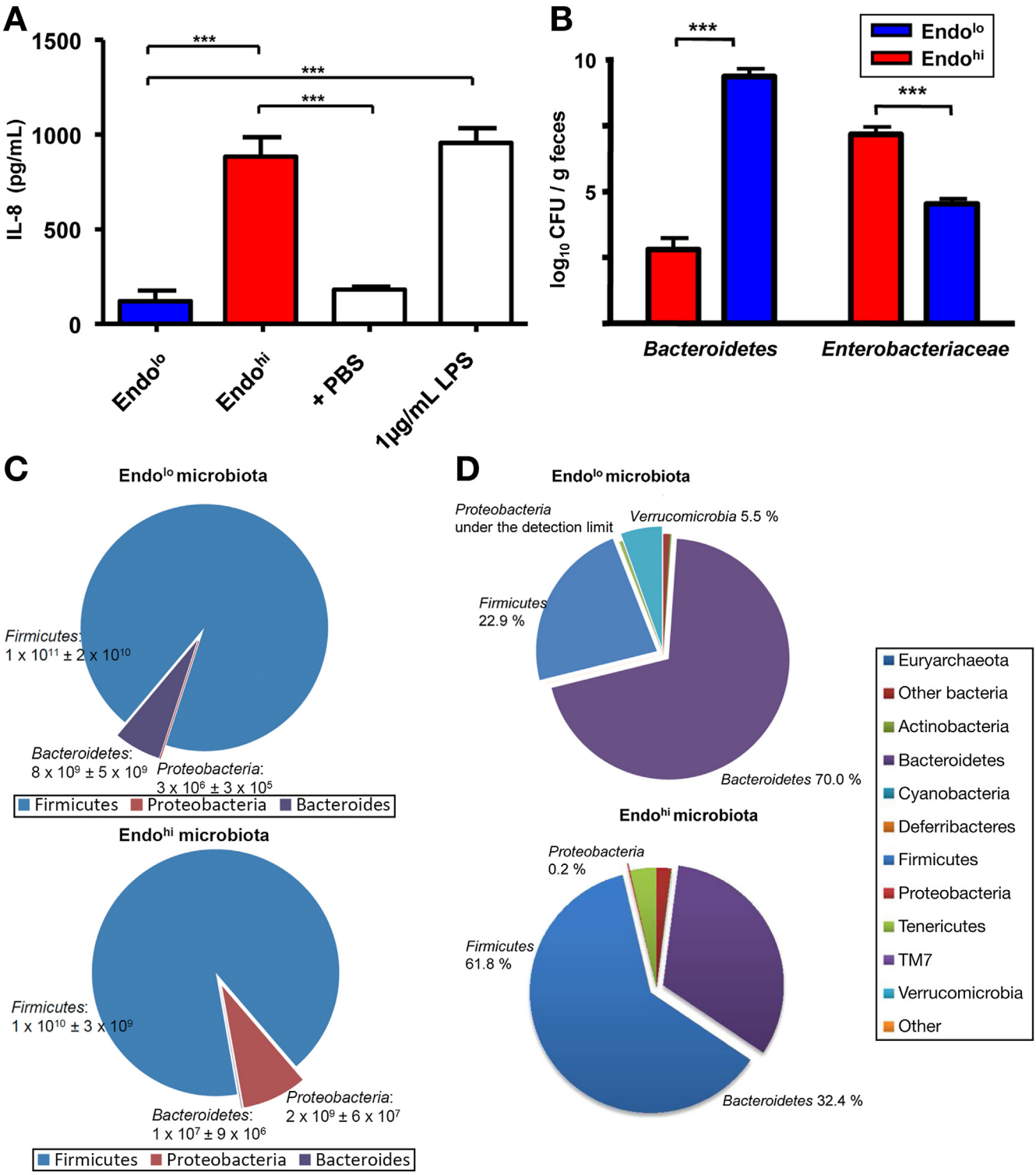


Figure 1. Endo^{lo} and Endo^{hi} microbiota exhibit differences in the endotoxicity and composition. Fecal samples from Endo^{lo} or Endo^{hi} *Rag1*$^{-/-}$ mice were collected and analyzed by stimulation of TLR4-overexpressing cells, culturing methods, quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis and 454 sequencing before T-cell transfer. (*A*) Concentrations of IL-8 in supernatants of feces stimulated TLR4-transfected HEK293 cells. Data are means of 3 independent experiments (±SEM), statistical analyses were performed using analysis of variance with Bonferroni's post test. (*B*) Serial dilutions of fresh fecal homogenates were plated on optimal and selective agar plates and incubated under anaerobic and aerobic conditions. The colony-forming units (CFU) per plate were counted and expressed as $\log_{10}$ CFU/g feces; detection limit 50 CFU; ($n \geq 5$ mice per group). (*C*) Fecal samples were analyzed by quantitative RT-PCR; ($n \geq 5$). (*D*) Relative abundance of phyla of intestinal bacteria. Fecal samples were analyzed via 454 sequencing of the 16S rDNA amplicons; ($n \geq 5$ mice per group). All statistical analyses were performed using Student *t* test. ***$P < .001$.

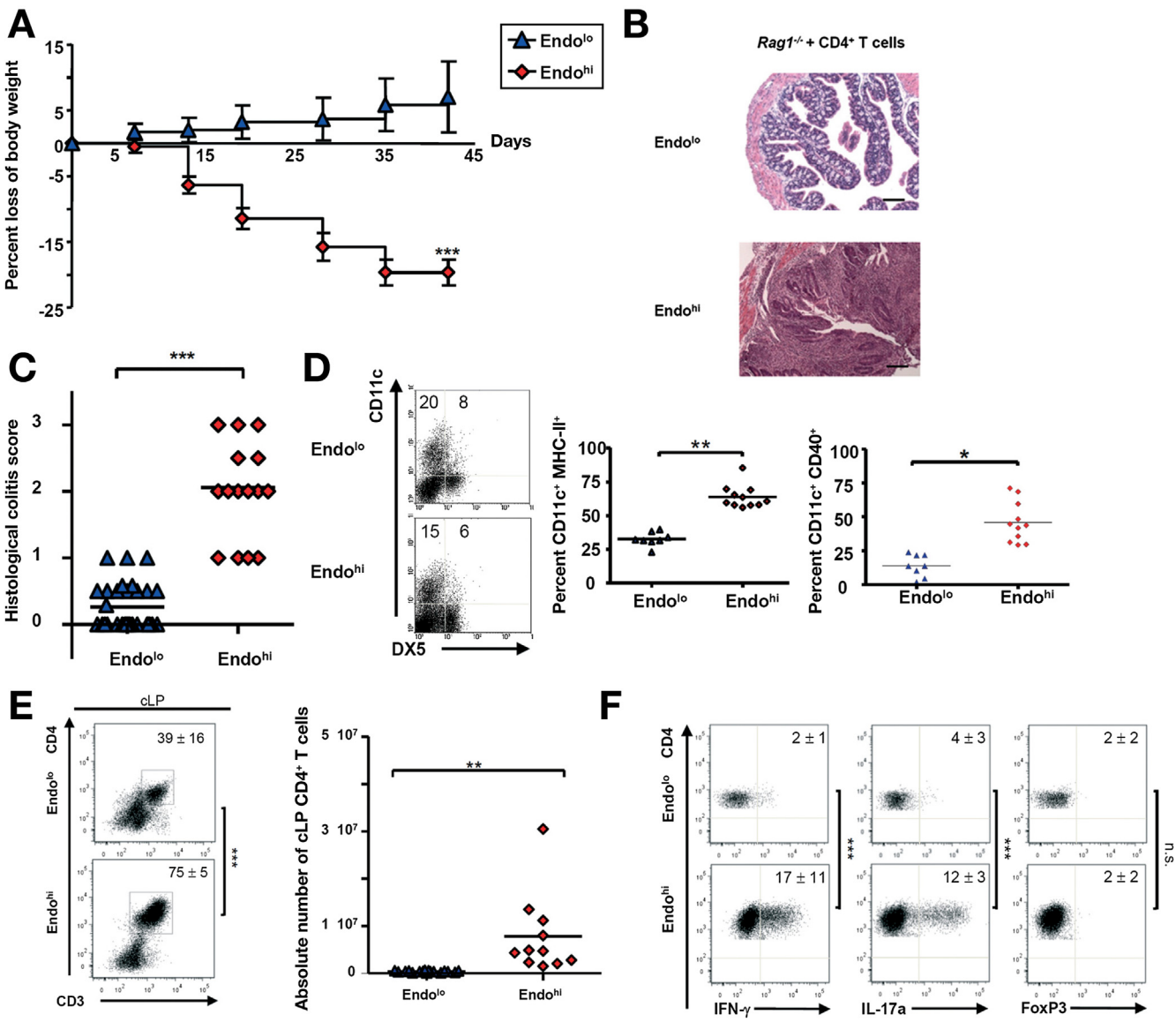


Figure 2. T-cell–induced colitis depends on the endotoxicity and composition of intestinal microbiota. Endo^{lo} and Endo^{hi} *Rag1*$^{-/-}$ mice were transferred with CD4^{+}CD62L^{+} T cells. (*A*) The body weight was monitored weekly and the mean percent of loss of body weight (±SD) was calculated; (n ≥ 6). (*B*) Representative H&E-stained colonic sections. Scale bar = 100 μm (n ≥ 15). (*C*) Colon histology score. Colon sections were scored blinded. Each symbol represents 1 animal. (*D*) Percentage of CD11c^{+}, CD11c^{+}MHCII^{+}, and CD11c^{+}CD40^{+} cells in the clp (±SD) (n ≥ 9). (*E*) T-cell repopulation in clp (±SD) (n ≥ 20). (*F*) Intracellular cytokine and FoxP3 expression in clp CD4^{+}CD3^{+} T-cells (±SD) (n ≥ 9). All statistical analyses were performed using Student *t* test. *$P < .05$; ***$P < .001$.

from Endo^{lo} *Rag1*$^{-/-}$ mice (Figure 2*D*). Intestinal inflammation was associated with a significant increase in CD4^{+}CD3^{+} T cells in the clp as compared with healthy T-cell–transferred Endo^{lo} *Rag1*$^{-/-}$ mice (Figure 2*E*). This corresponded to the total numbers of clp T cells (Figure 2*E*).

Additionally, clp T cells from diseased Endo^{hi} *Rag1*$^{-/-}$ mice produced significantly more interferon gamma and IL-17a than T-cells from healthy Endo^{lo} *Rag1*$^{-/-}$ mice. However, there was no significant difference in the percentage of FoxP3^{+} regulatory T cells (Figure 2*F*). The absolute number of T cells differed significantly due to the higher total amount of T cells present in Endo^{hi} *Rag1*$^{-/-}$ mice (Supplementary Table 1).

These observations suggest that the endotoxicity and composition of the intestinal microbiota are crucial for maintaining the mucosal immune homeostasis or induce inflammation. Endo^{lo} microbiota promotes intestinal immune homeostasis and Endo^{hi} microbiota results in a T_H1/T_H17a-driven colitis in *Rag1*$^{-/-}$ mice after the adoptive transfer of naïve T cells.

Structure of LPS Is Essential for Colitogenicity of E coli

Variations in the biologic activity of LPS from various organisms have been ascribed to differences in the structure

of LPS.[21,25] From these reports, we hypothesized that the different LPS structures might account for differences in the anti- or pro-inflammatory potential of Endo^{lo} and Endo^{hi} microbiota. Therefore, we used a commensal *E coli* JM83 K-12 (*E coli*$_{WT}$) WT strain and a MUT strain, *E coli* JM83 + *htrB*$_{Pg}$ (*E coli*$_{MUT}$), which had been published to contain in the lipid A the fatty acid 16:0 instead of 12:0.[21] In a previous study, this minor lipid A modification significantly affected host cell signalling.[21]

We isolated and purified LPS from both *E coli*$_{WT}$ and *E coli*$_{MUT}$ and characterized its fatty acid composition; both contained the typical *E coli* LPS fatty acids, however, strain *E coli*$_{MUT}$ possessed additional 16:0. Additional investigations by high-resolution electrospray ionization Fourier transform ion cyclotron mass spectrometry proved the presence of the same hexa-acetylated lipid A molecules in both strains (Supplementary Figure 1). In addition, *E coli*$_{MUT}$ contained a major portion of lipid A, in which 12:0 had been exchanged to 16:0.

To verify the altered stimulatory capacity of LPS$_{MUT}$ compared with LPS$_{WT}$, we used TLR4-overexpressing human embryonic kidney cells (HEK293). Stimulation of cells with the modified LPS$_{MUT}$ resulted in a significantly reduced IL-8 secretion 4 hours after stimulation, as compared with LPS$_{WT}$ (Figure 3*A*).

To investigate whether *E coli*$_{MUT}$ and *E coli*$_{WT}$ actually contribute to mucosal immune homeostasis or colitis in our model, Endo^{lo} mice were pretreated with metronidazole and Endo^{hi} mice with streptomycin, and then fed with *E coli*$_{WT}$. Streptomycin was administered to suppress putative colitogenic *Enterobacteriaceae* and to reduce endogenous *E coli* to permit colonization of administered *E coli*$_{WT}$. Metronidazole was administered to disrupt the endogenous possibly protective bacteria of the phylum of *Bacteroidetes* and to assess the anti-inflammatory effect of *E coli*$_{MUT}$ (Supplementary Figure 2). As indicated in Figure 3*B* and *C*, Endo^{lo} and Endo^{hi} *Rag1*$^{-/-}$ mice treated with *E coli*$_{WT}$ developed disease upon T-cell transfer and showed high colony-forming units of *Enterobacteriaceae* (Supplementary Figure 3). Histology revealed that T-cell–transferred Endo^{lo} and Endo^{hi} *Rag1*$^{-/-}$ mice treated with *E coli*$_{WT}$ showed inflammation of mucosa and submucosa and significantly enhanced histologic score (Figure 3*B* and *C*).

In contrast, metronidazole-conditioned *E coli*$_{MUT}$-treated Endo^{lo} or streptomycin-conditioned *E coli*$_{MUT}$-treated Endo^{hi} *Rag1*$^{-/-}$ mice did not develop colitis, although in Endo^{lo} *Rag1*$^{-/-}$ mice, the application of metronidazole reduced the number of protective endogenous *Bacteroidetes* (Supplementary Figure 3). More strikingly, treatment of Endo^{hi} *Rag1*$^{-/-}$ mice with *E coli*$_{MUT}$ resulted in protection from disease (Figure 3*B* and *C*), although these animals harbored high colony-forming units of *Enterobacteriaceae* in their feces (Supplementary Figure 3). As a control, we treated *Rag1*$^{-/-}$ mice with only streptomycin or metronidazole before T-cell transfer. Upon T-cell transfer, streptomycin-treated Endo^{hi} *Rag1*$^{-/-}$ mice developed severe colitis, and metronidazole-treated Endo^{lo} *Rag1*$^{-/-}$ mice did not (Supplementary Figure 4). Based on these data, we conclude that the observed effect is a result of treatment with bacteria, but not the administration of antibiotics.

The relative abundance of phyla in the *E coli*$_{WT}$ and *E coli*$_{MUT}$ treated Endo^{lo} or Endo^{hi} *Rag1*$^{-/-}$ mice was determined by 454 16S rDNA amplicon sequencing (Supplementary Figure 5, Supplementary Table 2).

As expected, the treatment of Endo^{lo} or Endo^{hi} *Rag1*$^{-/-}$ mice with *E coli*$_{MUT}$ resulted in decreased expression of lp DC MHC class II and CD40 as compared with *E coli*$_{WT}$ (Figure 3*D*).

T-cell numbers in the lp of metronidazole-treated, *E coli*$_{WT}$-fed Endo^{lo} *Rag1*$^{-/-}$ mice were significantly higher compared with metronidazole-treated, *E coli*$_{MUT}$-fed Endo^{lo} *Rag1*$^{-/-}$ mice, which did not show accumulation of T cells in the clp (Figure 3*E*). This was in line with the total numbers of lp T cells (Figure 3*E*).

In streptomycin-treated, *E coli*$_{WT}$-fed Endo^{hi} *Rag1*$^{-/-}$ mice, the percentage of T cells was increased significantly, and treatment with streptomycin plus *E coli*$_{MUT}$ resulted in a significant reduction in lp T-cell frequency (Figure 3*E*), which could also be seen for the total numbers of lp T cells (Figure 3*E*).

Lamina propria T cells from *E coli*$_{WT}$-treated Endo^{lo} *Rag1*$^{-/-}$ mice produced significantly less interferon gamma than *E coli*$_{MUT}$-treated Endo^{lo} *Rag1*$^{-/-}$ mice, although the IL-17a production did not differ. Strikingly, Endo^{lo} *Rag1*$^{-/-}$ mice administered with *E coli*$_{MUT}$ had significantly more FoxP3^{+} lp T cells as compared with *E coli*$_{WT}$-treated Endo^{lo} *Rag1*$^{-/-}$ mice (Figure 3*F*). Interferon gamma and IL-17a secretions were similar in *E coli*$_{WT}$-treated and *E coli*$_{MUT}$-treated Endo^{hi}.

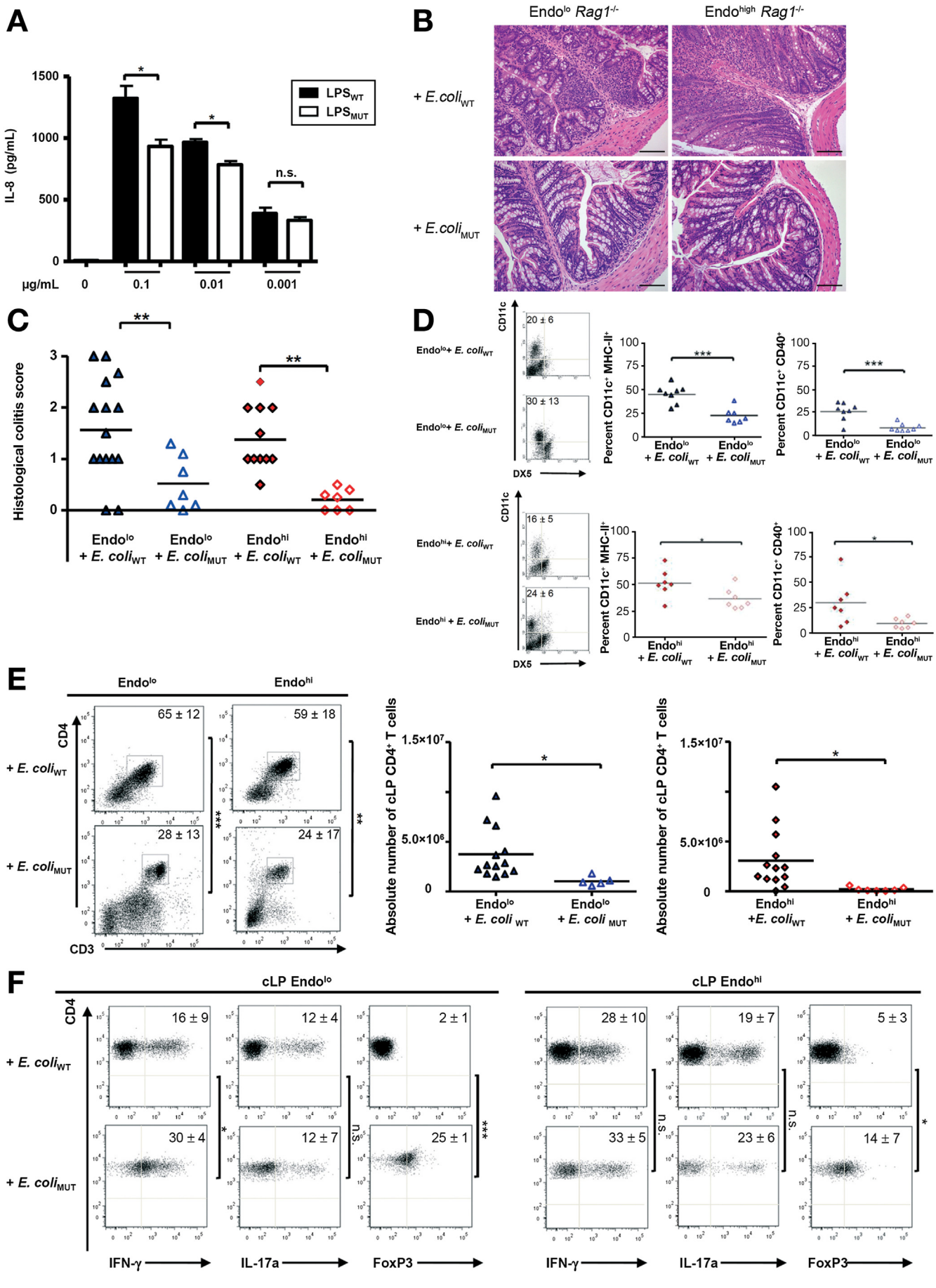
Rag1$^{-/-}$ *Mice*

Again administration of *E coli*$_{MUT}$ resulted in a significantly increased expression of FoxP3 in Endo^{hi} *Rag1*$^{-/-}$ mice, as compared with *E coli*$_{WT}$-feeding (Figure 3*F*).

From these results, we conclude that administration of *E coli*$_{WT}$ or *E coli*$_{MUT}$, thereby shifting the intestinal microbiota, is associated with altered activation and maturation of DC and a change in the T-cell polarization pattern. Depending on the change in the endotoxicity and composition (Endo^{lo} vs Endo^{hi}), the intestinal microbiota might promote intestinal homeostasis or trigger inflammation.

Feeding of LPS$_{MUT}$ Promotes Mucosal Immune Homeostasis

Up to this point, we demonstrated that the differences in the LPS of *E coli* were essential for the ability of *E coli* to induce or prevent colitis, as shown by feeding experiments with *E coli*$_{WT}$ inducing inflammation and *E coli*$_{MUT}$ preventing disease. To demonstrate conclusively that LPS of *E coli*$_{WT}$ and *E coli*$_{MUT}$ mediated the pro- or anti-inflammatory effect, we investigated whether the feeding of purified WT LPS from *E coli*$_{WT}$ (LPS$_{WT}$) or mutant LPS (LPS$_{MUT}$) from *E coli*$_{MUT}$ could confirm these results (Supplementary Figure 2). Therefore, we challenged Endo^{lo} and Endo^{hi} *Rag1*$^{-/-}$ mice with purified LPS$_{WT}$ or LPS$_{MUT}$.


A
IL-8 (pg/mL)
1500
1000
500
0
LPS$_{WT}$
LPS$_{MUT}$
*
*
n.s.
µg/mL 0 0.1 0.01 0.001
B
Endo^{lo} Rag1$^{-/-}$
Endo^{high} Rag1$^{-/-}$
+ E.coli$_{WT}$
+ E.coli$_{MUT}$
C
Histological colitis score
**
**
Endo^{lo} + E. coli$_{WT}$
Endo^{lo} + E. coli$_{MUT}$
Endo^{hi} + E. coli$_{WT}$
Endo^{hi} + E. coli$_{MUT}$
D
CD11c
DX5
20 ± 6
30 ± 13
16 ± 5
24 ± 6
Percent CD11c$^+$ MHC-II$^+$
Percent CD11c$^+$ CD40$^+$
***
*
E
Endo^{lo}
Endo^{hi}
CD4
CD3
65 ± 12
59 ± 18
28 ± 13
24 ± 17
Absolute number of cLP CD4$^+$ T cells
1.5×10^7
5.0×10^6
0
F
cLP Endo^{lo}
cLP Endo^{hi}
16 ± 9
12 ± 4
2 ± 1
30 ± 4
12 ± 7
25 ± 1
28 ± 10
19 ± 7
5 ± 3
33 ± 5
23 ± 6
14 ± 7
n.s.
IFN-γ
IL-17a
FoxP3

Treatment of Endo^{lo} *Rag1*$^{-/-}$ mice with LPS_{WT}, but not with LPS_{MUT}, resulted in induction of colonic inflammation (Figure 4*A*), as indicated by an increased histology score (Figure 4*B*). In addition, LPS_{WT}-fed Endo^{hi} *Rag1*$^{-/-}$ mice showed increased colonic inflammation as compared with LPS_{MUT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice (Figure 4*A* and *B*). The histology of the inflamed mucosa resembled the pathology of Endo^{hi} mice (Figure 2*B* and *C*). Dose–response experiments clearly demonstrated that the protection of Endo^{hi} mice from inflammation followed a LPS_{MUT} dose response (Supplementary Figure 6).

The relative abundance of phyla in intestinal microbiota of LPS_{WT}- and LPS_{MUT}-treated Endo^{lo} or Endo^{hi} *Rag1*$^{-/-}$ mice was determined (Supplementary Figure 7, Supplementary Table 3) by 454 sequencing of the 16S rDNA amplicons. However, it remains unclear whether the changes in the composition of the microbiota due to administration of LPS are a cause or consequence of the altered host immune response along with the development of colitis, and whether this change is an epiphenomenon or shows a causal effect.

Feeding LPS_{WT} to Endo^{lo} *Rag1*$^{-/-}$ mice resulted in significantly more activated lp DC in terms of CD40 and MHC class II expression as compared with LPS_{MUT}-treated Endo^{lo} *Rag1*$^{-/-}$ mice (Figure 4*C*). Lamina propria DC of LPS_{MUT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice showed significantly lower expressions of CD40 than LPS_{WT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice and comparable low amounts of MHC class II (Figure 4*C*). Feeding LPS_{WT} to Endo^{lo} *Rag1*$^{-/-}$ mice resulted in significantly more lp $CD4^+$ T cells as compared with treatment with LPS_{MUT} (Figure 4*D*). Total numbers of lp T cells of LPS_{WT}-treated Endo^{lo} *Rag1*$^{-/-}$ mice were significantly higher than in LPS_{MUT}-treated Endo^{lo} *Rag1*$^{-/-}$ mice (Figure 4*D*). In LPS_{WT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice, the number of $CD4^+$ T cells was significantly increased. In line with histologic scoring, the absence of colitis in LPS_{MUT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice was associated with a significantly decreased frequency of lp T cells (Figure 4*D*). This was consistent with the total numbers of lp T cells (Figure 4*D*).

Interferon gamma and IL-17a production in lp T cells of LPS_{WT}-treated Endo^{lo} *Rag1*$^{-/-}$ mice was significantly higher as compared with LPS_{MUT}-treated Endo^{lo} *Rag1*$^{-/-}$ mice; no significant difference in expression of FoxP3 could be observed (Figure 4*E*). Lamina propria T cells of LPS_{WT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice showed significantly higher expression of interferon gamma and IL-17a as compared with LPS_{MUT}-treated Endo^{hi} *Rag1*$^{-/-}$ mice. However, no significant differences in FoxP3 expression of lp T cells was observed (Figure 4*E*).

In summary, these data show that changes in the lipid A structure can convert a pro-inflammatory *E coli* strain into an anti-inflammatory *E coli* strain, and that the proportion of LPS with different lipid A structures within the intestinal microbiota might have a critical influence on development of colitis in a genetically predisposed host in the context of a specific microbiota.

Discussion

Recent studies have examined the function of the intestinal microbiota in the pathogenesis of inflammatory intestinal diseases in genetically predisposed hosts and the prospects of preventing inflammation by selective alteration of the intestinal microbiota.[26–29]

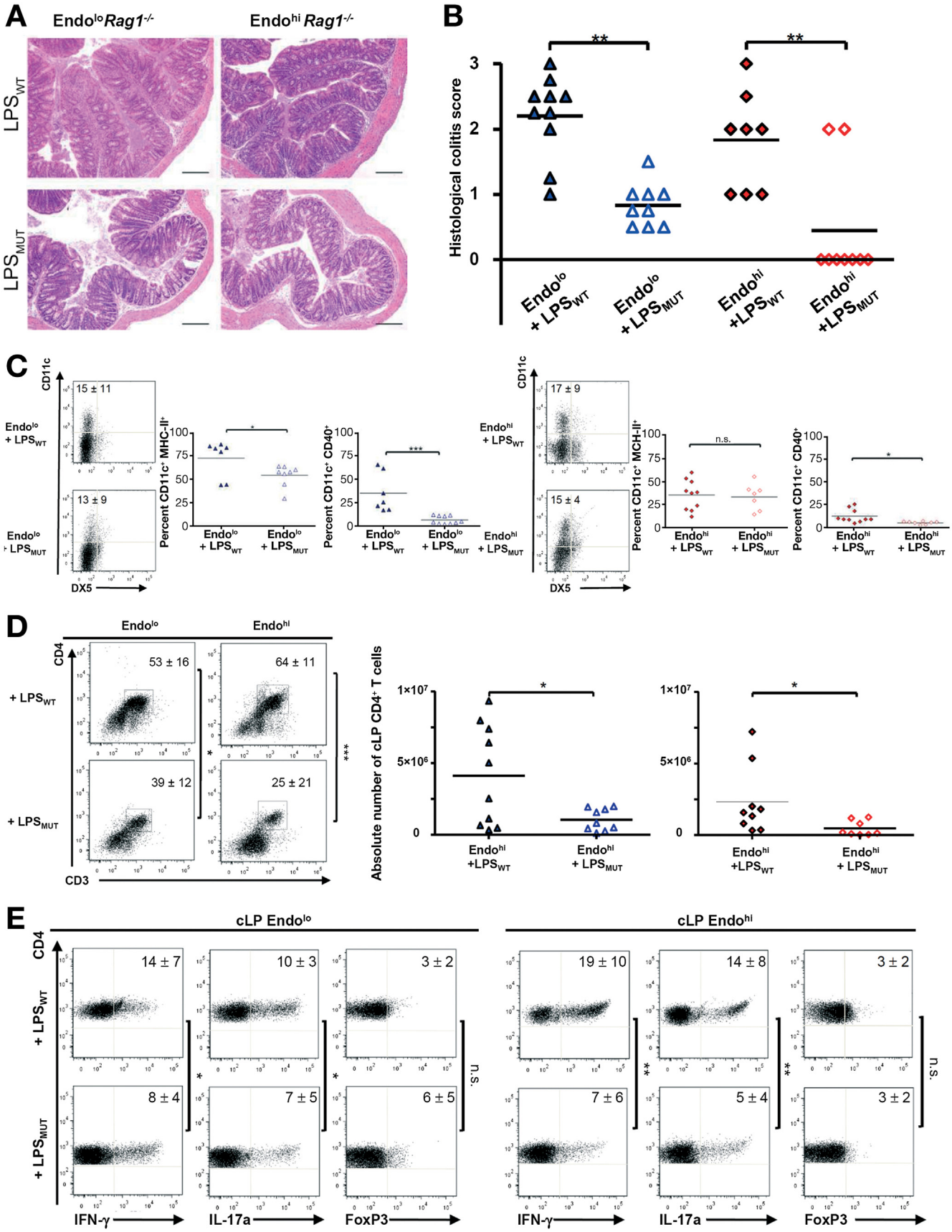
We identified LPS as a microbial factor that, according to its composition/structure, can either promote or prevent the development of bowel inflammation in the $CD4^+$ T-cell transfer model of colitis in *Rag1*$^{-/-}$ mice. We demonstrated that probably by structural changes in the lipid A, the colitogenic potential of a commensal *E coli* strain can not only be abolished, but also converted into a protective commensal strain that prevents development of T-cell–induced colitis.

Several animal studies demonstrated that the intestinal microbiota shapes homeostasis of the intestinal mucosal immune system,[29–34] and that a distinct composition of the intestinal microbiota is associated with promotion of bowel inflammation.[29–31,35] However, it is not yet clear whether a specific microbiota composition or a specific microbial compound might initiate the inflammatory process or perpetuate the chronic inflammation. To clarify this, we used *Rag1*$^{-/-}$ mice transferred with T cells that develop colitis in the presence of a specific complex microbiota or specific pathobionts (eg, *Helicobacter hepaticus*[36]), but remain healthy under germ-free conditions.

In our model, we observed that an intestinal microbiota exhibiting low endotoxicity and harboring a high proportion of *Bacteroidetes* (Endo^{lo}) was associated with prevention of T-cell–induced colitis, supporting the idea that low endotoxic microbiota or bacteria of the *Bacteroidetes* group might inhibit mucosal pro-inflammatory host responses. In contrast, the high endotoxicity and high proportion of *Enterobacteriaceae* in Endo^{hi} *Rag1*$^{-/-}$ mice was clearly associated with development of intestinal inflammation. According to the reports with different animal models, such associations cannot be generalized, but might rather reflect the pathogenesis of different models of disease.[30,31,37]

The recognition of microbe-associated molecular patterns, such as LPS, which are ubiquitous to all microbes of a

Figure 3. Structure of LPS is essential for colitis induction, DC, and T-cell activation. (*A*) TLR4-HEK293 cells were stimulated with different concentrations of *E coli*$_{WT}$ LPS (LPS_{WT}) or *E coli*$_{MUT}$ LPS (LPS_{MUT}) (0.001–0.1 μg/mL) and IL-8 concentrations in supernatants were measured 4 hours after stimulation. Data are means of 3 independent experiments (±SEM). (*B*) Endo^{lo} mice were conditioned with metronidazole, Endo^{hi} mice with streptomycin and fed with 1×10^8 *E coli*$_{WT}$ or *E coli*$_{MUT}$. After 7 days, naïve $CD4^+CD62L^+$T cells were transferred. Representative H&E-stained colonic sections. Scale bar = 100 μm ($n \geq 7$). (*C*) Colon histology score. Colonic sections were scored blinded. Each symbol represents 1 animal. (*D*) Percentage of clp $CD11c^+$, $CD11c^+MHCII^+$, and $CD11c^+CD40^+$ cells (±SD) ($n \geq 7$). (*E*) T-cell repopulation in clp (±SD) ($n \geq 7$). (*F*) Intracellular cytokine and FoxP3 expression in clp $CD4^+CD3^+$ T cells (±SD) ($n \geq 7$). All statistical analysis were performed using the Student *t* test. $^{***}P < .001$; $^{*}P < .05$.


A
Endo^lo Rag1^-/-
Endo^hi Rag1^-/-
LPS_WT
LPS_MUT
B
Histological colitis score
Endo^lo + LPS_WT
Endo^lo + LPS_MUT
Endo^hi + LPS_WT
Endo^hi + LPS_MUT
C
CD11c
DX5
Percent CD11c+ MHC-II+
Percent CD11c+ CD40+
Percent CD11c+ MCH-II+
n.s.
D
CD4
CD3
Absolute number of cLP CD4+ T cells
E
cLP Endo^lo
cLP Endo^hi
IFN-γ
IL-17a
FoxP3

given class, is essential for the induction of, and plays an instructive role in the development of, the adaptive immune response.[38–40] There are several studies describing either a pro-inflammatory or even a protective role for TLR4/MyD88 signaling pathways in the development of intestinal inflammation.[15,41] In *IL-10*$^{-/-}$ mice, induction of colitis seems to depend completely on intact TLR/MyD88 signaling pathways, and *IL-2*$^{-/-}$ mice develop colitis, even in the absence of TLR/MyD88.[12] Yet, studies with acute DSS colitis report on a protective role of TLR/MyD88 signaling, stating that the recognition of microbe-associated molecular patterns by TLR is required for intestinal homeostasis.[13] We, however, have shown that high endotoxic TLR4 activation is associated with colitis induction. Because the DSS model of acute inflammation is based on disruption of tight junction proteins and the intestinal barrier, and the T-cell transfer model mimics a chronic T_H1/T_H17-driven colitis, it is unclear to what extent results of the 2 models are comparable.

LPS is a lipoglycan or glycolipid located in the outer membrane of Gram-negative bacteria composed of an amphipathic lipid A component and, in S-form LPS, by a polysaccharide comprising the core region and the O antigen.[42] It was shown that LPS binding induces the formation of a receptor multimer complex composed of 2 copies of the TLR4/MD2/LPS complex.[43] Depending on the structure, the endotoxic activity of lipid A shows marked variation in different bacteria, for example, the lipid A of *Porphyromonas gingivalis* or *Bacteroides* species is considerably weaker than that of *E coli*, resulting in altered immune responses.[44–46] An opinion paper recently suggested that the TLR4 dimer might undergo conformational changes at different cellular locations due to environmental factors, such as pH. Different dimerization modes might, in turn, lead to recruitment of different sets of adaptors, which determine signaling output.[47]

We hypothesized that *E coli* might cause colitis due to the acylation pattern of its lipid A. If the acyl-transferase encoding gene *htrB* of *P gingivalis* is also expressed in *E coli* JM83 WT, the strain expresses an additional 16:0 in its lipid A.[21] This *E coli*$_{MUT}$ possesses a reduced ability to activate TLR2/4 expressing HEK293 cells and monocytes, but a WT activity to stimulate endothelial cells.[21] Our compositional and electrospray ionization Fourier transform ion cyclotron mass spectrometry investigations additionally confirmed this change in lipid A structure, suggesting that the mutation has changed the toxicity of the LPS. Nevertheless, our data demonstrate that the changes in the lipid A of *E coli* resulted in a decreased ability to induce colitis in *Rag1*$^{-/-}$ mice. Even more, *E coli*$_{MUT}$ or the isolated LPS_{MUT}, protected Endo^{hi} mice from developing colitis.

In a previous work, we reported that low or high concentrations of LPS induce differential DC activation and maturation resulting in differential T-cell activation and polarization.[48] Here we show that the expression of activation and maturation markers of lp DC significantly differs in Endo^{hi} and Endo^{lo} *Rag1*$^{-/-}$ mice, in *E coli*$_{MUT}$ or *E coli*$_{WT}$ and in LPS_{WT} or LPS_{MUT} challenged mice, respectively, according to our in vitro studies. Consistent with this, we found increased expression of T_H1/T_H17 cytokines in Endo^{hi}-, *E coli*$_{WT}$-, and LPS_{WT}-treated *Rag1*$^{-/-}$ mice, but not in the Endo^{lo}-, *E coli*$_{MUT}$-, or LPS_{MUT}-challenged *Rag1*$^{-/-}$ mice. Accordingly, treatment of mice with *E coli*$_{MUT}$ resulted in increased expression of FoxP3 and treatment with LPS_{MUT} in a reduced expression of IL-17a in the colonic lp T cells.

The delivery of purified LPS or LPS by viable bacteria might result in different LPS availability at different intestinal and cellular components,[49] and can contribute to the differences in the IL-17a and FoxP3 expression on treatment with LPS or viable bacteria. However, we cannot rule out that an additional factor of viable *E coli* might account for this effect.

Currently, it seems to be highly likely that the intestinal microbiota plays a critical role in the accumulation and functional maturation of intestinal regulatory T cells.[50] We demonstrated that commensal bacterial species, depending on the structure of LPS, can induce or prevent expansion and polarization of intestinal T cells. However, as discussed by Chung et al,[33] many questions remain about the causes of differential T-cell response. The different maturation states of lp DC might be induced directly by the commensal bacteria or be due to a secondary effect induced by differences in the local chemokine and cytokine milieu, possibly resulting in a difference in the downstream T-cell proliferation.

Both the administration of bacteria (*E coli*$_{WT}$ and *E coli*$_{MUT}$) and treatment with LPS_{WT} or LPS_{MUT} resulted in alterations in the composition of the intestinal microbiota. Analysis of the intestinal microbiota by deep sequencing techniques implies that phylogenetic groups of bacteria like *Firmucutes* or *Verrucomicrobia* might also be involved in the regulation of the intestinal immune homeostasis. However, additional functional studies need to clarify the biologic relevance of this finding and whether the shift of the intestinal microbiota by LPS administration is a direct effect, or represents a secondary effect due to changes in the local environment in terms of, for example, nutrition, altered cytokine and chemokine patterns, or induction of defensins. In addition, an influence of the altered intestinal microbiota, and therefore the modified endotoxicity of the intestinal microbiota on the maintenance of intestinal homeostasis or induction of inflammation, would be conceivable.

Figure 4. Feeding of LPS_{MUT} promotes intestinal homeostasis. Endo^{lo} mice were conditioned with metronidazole, Endo^{hi} mice with streptomycin and fed with 160 μg LPS from *E coli*$_{WT}$ or *E coli*$_{MUT}$ once by oral gavage and received 60 μg/mL LPS in drinking water during the whole experiment. After 7 days, mice were transferred with 5×10^5 naïve CD4$^+$ CD62L$^+$ T cells. (*A*) Representative colonic sections. All sections were stained with H&E. Scale bar = 100 μm (n $\geq$ 8). (*B*) Colon histology score. Colonic sections were scored blinded. Each symbol represents 1 animal (n $\geq$ 8). (*C*) Percentage of CD11c$^+$ cells, CD11c$^+$MHCII$^+$, and CD11c$^+$CD40$^+$ cells in the clp ($\pm$SD) (n $\geq$ 8). (*D*) T-cell repopulation in lp ($\pm$SD) (n $\geq$ 8). (*E*) Fluorescence-activated cell sorting analysis of intracellular cytokine and FoxP3 expression in lp CD4$^+$CD3$^+$ T cells ($\pm$SD) (n $\geq$ 8). All statistical analysis were performed using Student *t* test. ***$P < .001$; *$P < .05$.

Our data suggest that the ratio of high endotoxic and low endotoxic LPS is crucial for the regulation of the intestinal immune balance. A predominance of high endotoxic LPS might promote a T_H1/T_H17 response, subsequently supporting intestinal inflammation, and a predominance of low endotoxic LPS might induce an altered activation of the innate immune system, resulting in DC semi-maturation and either induction of regulatory T cells or prevention of a T_H1/T_H17 response, associated with intestinal immune homeostasis. Zwitterionic polysaccharide A of *Bacteroides fragilis* has been identified as a microbial symbiosis factor acting on the adaptive immune system.[32,51] We propose LPS as a key microbial symbiosis factor that, depending on its structure, can induce or prevent bowel inflammation by shaping the innate immunity via TLR4-dependent signalling mechanisms.[52]

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.11.033.

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