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# Strategic Compartmentalization of Toll-Like Receptor 4 in the Mouse Gut<sup>1</sup>

Cesar F. Ortega-Cava,<sup>2\*</sup> Shunji Ishihara,\* Mohammad A. K. Rumi,\* Kousaku Kawashima,\* Norihisa Ishimura,\* Hideaki Kazumori,\* Jun Udagawa,<sup>†</sup> Yasunori Kadowaki,\* and Yoshikazu Kinoshita\*

Pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs), are involved in the innate immune response to infection. TLR4 is a model for the TLR family and is the main LPS receptor. We wanted to determine the expression of TLR4 and compare it with that of TLR2 and CD14 along the gastrointestinal mucosa of normal and colitic BALB/c mice. Colitis was induced with 2.5% dextran sodium sulfate (DSS). Mucosa from seven segments of the digestive tract (stomach, small intestine in three parts, and colon in three parts) was isolated by two different methods. Mucosal TLR4, CD14, TLR2, MyD88, and IL-1 $\beta$  mRNA were semiquantified by Northern blotting. TLR4 protein was determined by Western blotting. TLR4/MD-2 complex and CD14 were evaluated by immunohistochemistry. PRR genes were constitutively expressed and were especially stronger in colon. TLR4 and CD14 mRNA were increased in the distal colon, but TLR2 mRNA was expressed more strongly in the proximal colon, and MyD88 had a uniform expression throughout the gut. Accordingly, TLR4 and CD14 protein levels were higher in the distal colon. TLR4/MD-2 and CD14 were localized at crypt bottom epithelial cells. TLR4/MD2, but not CD14, was found in mucosal mononuclear cells. Finally, DSS-induced inflammation was localized in the distal colon. All genes studied were up-regulated during DSS-induced inflammation, but the normal colon-stressed gut distribution was preserved. Our findings demonstrate that TLR4, CD14, and TLR2 are expressed in a compartmentalized manner in the mouse gut and provide novel information about the *in vivo* localization of PRRs. *The Journal of Immunology*, 2003, 170: 3977–3985.

The first line that is exposed to the gut luminal environment is the monolayer of epithelial cells. Gut epithelial cells are known to function as a living barrier against pathogenic agents present within the external environment of the gut lumen; additionally they must permit the passage of necessary nutrients. Epithelial cells work in close relation with other cell populations present in the lamina propria, such as the macrophages, dendritic cells, and lymphocytes that form the intestinal mucosal immune system (1–3). The innate immune system has evolved to perform nonclonal cell recognition of conserved products of microbial metabolism present on broad classes of organisms (4). These microbial products are referred to as pathogen-associated molecular patterns (PAMPs),<sup>3</sup> and the best examples include LPS of Gram-negative bacteria and peptidoglycan of Gram-positive bacteria. PAMPs are recognized by receptors of the innate immune system called pattern recognition receptors (PRRs) (5).

The group of PRRs includes the mammalian homologues of *Drosophila* Toll receptors, Toll-like receptors (TLRs). TLRs are transmembrane proteins characterized by an extracellular domain

with leucine-rich repeats and an intracellular domain homologous to the IL-1R, or Toll/IL-1R (TIR). Activated TIR binds to a homologous domain in the adaptor protein MyD88 and starts a cascade that finally leads to activation of NF- $\kappa$ B and production of its related proinflammatory cytokines (6). TLR4 is the prototype of the TLR family and the main receptor for Gram-negative bacterial LPS. For efficient LPS-induced signaling, TLR4 requires association with MD-2, a secreted glycoprotein, and CD14 (4, 7). Another member of the TLR family is TLR2. Several molecules have been reported to activate innate immune responses through TLR2, including peptidoglycan, zymosan, and bacterial lipopeptides (4).

It has previously been reported that mouse intestinal epithelial cell lines express some PRRs (8), but nothing is known about the *in vivo* expression of these receptors. Since the intestinal bacterial content varies throughout the gut (9), it is probable that the expression of these receptors will also be different. Furthermore, since PRRs do not distinguish between PAMPs from commensal bacteria and pathogenic bacteria (10), mechanisms should exist to regulate the exposure of these receptors to gut luminal content. In the present study we wanted to determine the localization of TLR4 in the mouse gut mucosa and compare it with that of TLR2 and CD14 in normal mice and in mice with dextran sodium sulfate (DSS)-induced colitis. DSS-induced colitis is a model of epithelial barrier dysfunction; its possible pathogenesis includes direct cytotoxicity by DSS or interference with the normal interaction among epithelial cells, intestinal lymphocytes, and the extracellular matrix (11–13).

## Materials and Methods

### Animal model

Six-week-old male specific pathogen-free BALB/c mice (Nihon Clea, Tokyo, Japan) were used in the study. Mice were placed in appropriate facilities of the Animal Institute of Shimane Medical University and were handled according to the institute guidelines for animal experimentation

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<sup>3</sup> Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; DSS, dextran sodium sulfate; PRR, pattern recognition receptor; T<sub>a</sub>, annealing temperature; TIR, Toll/IL-1R; TLR, Toll-like receptor.

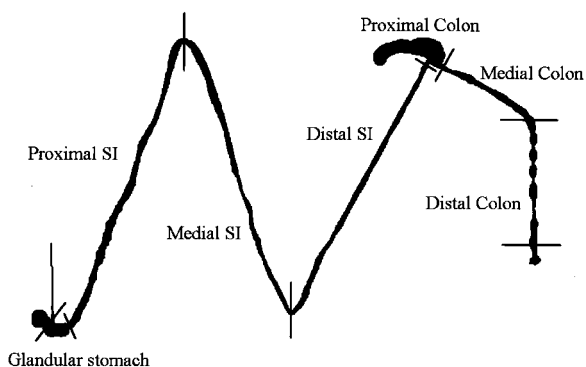
with the approval of the ethics committee for animal experimentation of the university. Animals were housed under constant environmental conditions with circadian light-dark cycles and were permitted an initial adaptation period of 1 wk.

### Experimental design

Initially a single group of normal mice was used to screen TLR4, MD-2, and TLR2 expression and distribution in normal mouse gut mucosa using RT-PCR ( $n = 5$ ). Then these results were confirmed by Northern blotting and extended to the MD-2, TLR2, CD14, and MyD88 genes; for this purpose, five normal animals were sacrificed per group, and the experiment was performed twice ( $n = 10$ ). Another group of normal mice was used for Western blot studies ( $n = 5$ ). Two additional groups of mice were used to study the localization of the TLR4/MD-2 complex and CD14 by immunohistochemistry ( $n = 11$ ). Thereafter, to determine whether the distribution of TLR4, TLR2, CD14, MyD88, and IL-1 $\beta$  in mouse gut mucosa varies during acute colonic inflammation, we induced DSS colitis in a group of mice. These animals received 2.5% (w/v) DSS (5 kDa; Wako Pure Chemical Industries, Osaka, Japan) solution as drinking water for 7 days. In a previous pilot study we determined that 2.5% DSS is the appropriate concentration to cause colitis with a clinical disease activity index (14) between 1.7 and 3 without extensive erosive epithelial loss, a necessary condition for the study. All mice were assessed using the disease activity index in a blinded fashion. Mice were sacrificed 1 day after the end of the DSS administration week. The experiment was performed three times, and each group included five animals ( $n = 15$ ). The first two groups were used for Northern blotting, and the last group was used for Western blotting and immunohistochemistry. Subsequently, to investigate the expression of the target genes with time during DSS colitis, a comparative placebo and DSS group design was performed. In this experiment control and DSS mice were sacrificed at 8 h and 1, 4, 9, and 14 days after the end of the DSS period. The experiment was performed twice with five animals per group ( $n = 20$ ), and in both cases samples were used for Northern blot analysis. Furthermore, we determined the expression of TLR4, TLR2, CD14, and MyD88 in isolated gut epithelium. For this purpose the standard DSS protocol was performed with a group of mice. An appropriate time control group was used. Animals were sacrificed 24 h after the end of DSS administration, and gut epithelial cells were isolated by the EDTA-vibration method (15). This experiment was performed once, and samples were used for Northern blotting ( $n = 6$ ). The purity of epithelial cells isolates was evaluated in a group of normal mice ( $n = 5$ ). A total of 77 mice were used.

### Sample collection

The animals were sacrificed by an overdose of diethyl ether anesthesia. The intestine was dissected and placed on an ice-cold surface, opened from the anus to the stomach, and rinsed with ice-cold normal saline. The distal 0.5 cm, including the anus, was discarded. The digestive tract was divided into seven segments: glandular stomach, three segments of small intestine (proximal, medial and distal), and three segments of colon (proximal, medial, and distal; Fig. 1). For mRNA or protein studies the mucosa was



**FIGURE 1.** Segmentation of the mouse gut. The digestive tract was dissected, fixed with pins on an ice-cold surface, and photographed. Segments in an oral to anal direction are: glandular stomach (from the line of epithelial transition to the pylorus); proximal, medial, and distal small intestine (with equal length); and proximal, medial, and distal colon (the proximal colon is the cecum and the rest of the colon was divided into two segments of equal length). SI, small intestine. The lines indicate points of division.

segmentally scraped with a sharpened spatula; the mucosal scrapings and the remaining tissue (submucosa, muscular, and serosa) were separately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until further processing. The same investigator always performed the scraping. For immunostaining studies the gut was rinsed immediately after being opened, segmentally divided, immersed in embedding medium (Tissue-Tek OCT compound; Sakura Finetechnical, Tokyo, Japan), frozen on dry ice, and stored at  $-70^{\circ}\text{C}$ . In other animals colonic epithelial cells were isolated using an EDTA-vibration method that results in  $<5\%$  contaminating nonepithelial cells (15). Briefly, the unopened proximal and distal segments of the colon were placed on an ice-cold surface and flushed with chilled ( $4^{\circ}\text{C}$ ) calcium- and magnesium-free PBS. The colon segments were everted, their ends were closed with silk, and they were filled with PBS at  $4^{\circ}\text{C}$ . These colon bags were incubated in 3 mM EDTA in PBS, pH 7.4, at  $37^{\circ}\text{C}$  and gently shaken every 5 min. After 20 min the separated cells were centrifuged at  $4^{\circ}\text{C}$  and washed twice with PBS. Cells were frozen at  $-70^{\circ}\text{C}$  until being processed for mRNA extraction.

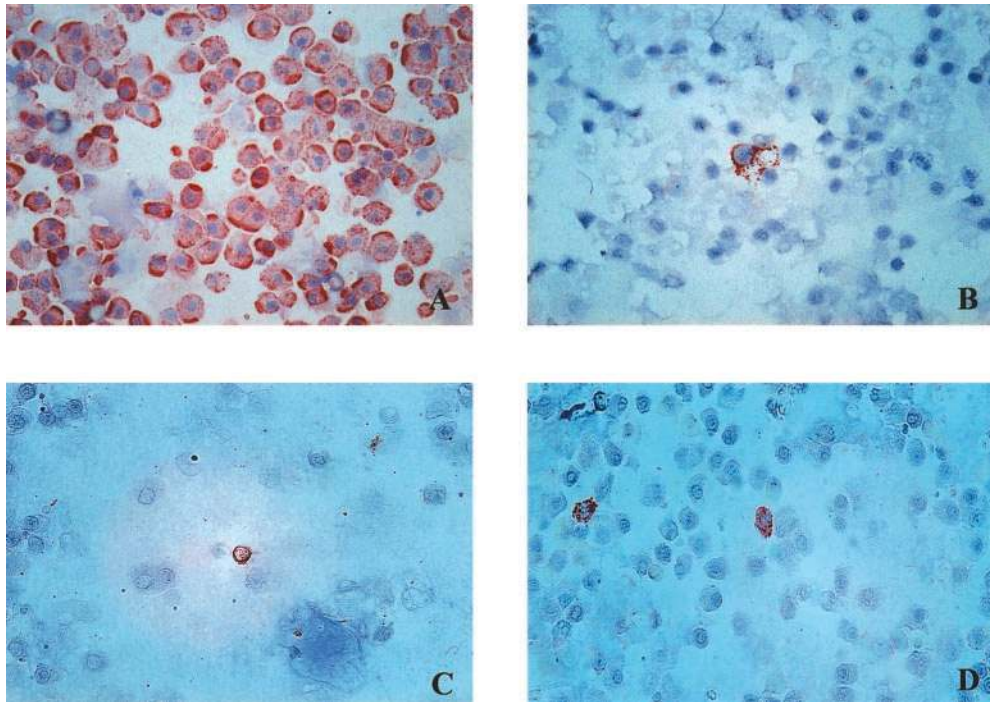
### Histology and immunohistochemistry

As MD-2 is an important protein required for TLR4 function, a rat mAb against mouse TLR4/MD2 complex (MTS510; eBioscience, San Diego, CA) was used to perform immunohistochemistry. CD14 was marked by a polyclonal goat anti-mouse CD14 Ab (sc-6999; Santa Cruz Biotechnology, Santa Cruz, CA). Frozen samples from each segment of the gut were taken as previously described, and 6- $\mu\text{m}$ -thick sections were prepared. Sections were fixed in cold acetone for 20 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxidase in methanol for 30 min at room temperature, followed by incubation with blocking normal serum for 30 min. Subsequently, sections were incubated for 2 h at room temperature with primary Ab at 1/100 dilution and then processed with the corresponding commercial immunoperoxidase staining kit (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Sections were counterstained with hematoxylin. Negative controls were performed by omitting the primary Ab. The efficiency of one of the investigators (C.F.O.) in scraping the mucosa without going beyond the muscularis mucosa was determined to be  $97 \pm 2.33\%$  (mean  $\pm$  SD) by two different observers. Briefly, formalin-fixed scraped gastrointestinal specimens were embedded in paraffin, sectioned, and stained with H&E for microscopic examination in a blinded fashion. The purity of epithelial isolation by scraping was evaluated on cytocentrifuge cell preparations. Scraped mucosa was digested with 1 mg/ml collagenase, followed by washes with 3 mM EDTA in PBS. Cells from different gut segments were pooled, and a suspension of  $10^6$  cells/ml was prepared. Aliquots of 50  $\mu\text{l}$  were cytocentrifuged (DC8 cell settling chamber; Neuroprobe, Cabin John, MD) on coated slides and immunostained with the same method as that used for frozen tissue sections. Epithelial cells, dendritic cells, B lymphocytes, and macrophages were labeled with wide spectrum rabbit anti-cytokeratin (DAKO, Carpinteria, CA), hamster anti-CD11c (HL3; BD Pharmingen, San Diego, CA), rat anti-CD45R (RA3-6B2; BD Pharmingen), and rat anti-F4/80 (CI:A3-1; Caltag, Burlingame, CA) anti-mouse Abs, respectively. A total of 24 digital images from the samples of each animal were evaluated at a magnification of  $\times 400$ . Cells were counted by a trained observer, and positive cells were expressed as a percentage. Cytocentrifuge preparations showed that  $94.2 \pm 2.1\%$  were epithelial cells,  $0.56 \pm 0.1\%$  were CD11c $^{+}$  cells,  $1.21 \pm 0.1\%$  were CD45R $^{+}$  cells, and  $2.48 \pm 0.4\%$  were F4/80 $^{+}$  cells (mean  $\pm$  SD; Fig. 2).

### RT-PCR

The first screening of TLR expression was performed by RT-PCR. Gut mucosa was scraped as previously described. The chain of lymph nodes located in the mesentery of the ileocecal junction was dissected and taken as a positive control (16). The tissue was homogenized in liquid nitrogen, and total RNA was isolated by the guanidine thiocyanate-phenol-chloroform method (Isogen; Nippon Gene Co., Tokyo, Japan). Ten micrograms of RNA from glandular stomach mucosa and from proximal and distal segments of small intestine and colon was reverse transcribed with oligo(dT) primer in a 50- $\mu\text{l}$  reaction (ProSTAR First Strand RT-PCR Kit; Stratagene, Cedar Creek, TX). Two microliters of cDNA was amplified with the GeneAmp PCR system 9600 (PE Applied Biosystems, Foster City, CA). Amplification was performed in a 25- $\mu\text{l}$  reaction with 10 pmol of each primer with 200  $\mu\text{M}$  of each dNTP, 1.5 mM  $\text{MgCl}_2$ , and 0.75 U of DNA polymerase (AmpliTaq Gold; PE Applied Biosystems) for 30 standard PCR cycles. Sense and antisense primers and the corresponding annealing temperatures ( $T_a$ ) were: TLR4, 5'-TCC TGG CTA GGA CTC TGA TCA T-3' and 5'-CAT GGC ATG GCT TAC ACC ACC T-3' ( $T_a = 60^{\circ}\text{C}$ ); MD-2, 5'-AAC AGT GGT TCT GCA ACT CCT C-3' and 5'-CAA TAC CAT TCT CTT TCG AGG G-3' ( $T_a = 54^{\circ}\text{C}$ ); and TLR2, 5'-GAC





**FIGURE 2.** Immunocytochemical evaluation of the purity of epithelial isolates. Scrapped gut epithelia from five mice were digested with collagenase and washed with EDTA-PBS. Cells were randomly fixed on glass slides by centrifugation and immunostained as specified in *Materials and Methods*. Cells were imaged, and positive cells were counted and expressed as percentage (mean  $\pm$  SD). *A*, Epithelial cells stained with wide spectrum rabbit anti-cytokeratin ( $94.2 \pm 2.1\%$ ). *B*, Hamster anti-CD11c was used to label dendritic cells in isolated cells ( $0.56 \pm 0.1\%$ ). *C*, Cytocentrifuge cell preparations stained with rat anti-CD45R as pan B cell marker ( $1.21 \pm 0.1\%$ ). *D*, Macrophage localization using rat anti-F4/80 ( $2.48 \pm 0.4\%$ ). Representative images are shown (magnification,  $\times 400$ ).

AGC TAC CTG TGT GAC TCT CC-3' and 5'-ACA AGT AAT AGA ACG CGT CAA A-3' ( $T_a = 50^\circ\text{C}$ ). Five microliters of PCR product was electrophoresed on a 1.5% agarose gel with 0.5% ethidium bromide and photographed under UV light. As an internal control, a  $\beta$ -actin cDNA sequence was amplified with the primers 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'-CGG ACT GTT ACT GAG CTG CGT TTT A-3' ( $T_a = 56^\circ\text{C}$ ). In all cases amplification included a nontemplate control reaction to check carryover contamination.

#### Northern blotting

RNA was extracted as previously described, and 20  $\mu\text{g}$  was electrophoresed in 1% agarose gel containing 0.66 mmol/liter formaldehyde, transferred to a supported nitrocellulose membrane (Hybond-c Extra; Amersham International, Little Chalfont, U.K.), and UV linked. To evaluate gut mucosal distribution of target genes, the samples were arranged in a single-mouse gut display. Alternatively, the samples from normal and DSS mice from the comparative experiments were displayed in the same membrane to permit comparison. Twin membranes containing the fixed RNA from each mouse were elaborated; one membrane was used to sequentially evaluate TLR4 and MyD88, and the other was used to measure TLR2 and CD14. To assess whether the presence of minimal leukocyte contaminants in the scrapped mucosal epithelium could be detected or was negligible by Northern blot, we compared the scrapped mucosa and the remnant tissue (submucosa, muscular, and serosa) contents of CD45 mRNA. For this purpose RNA from mucosa and remnants were blotted onto the same membrane and hybridized with a CD45 probe. cDNAs corresponding to TLR4 (292 b), TLR2 (595 b), CD14 (413 b) (15), MyD88 (660 b) (17), IL-1 $\beta$  (418 b), CD45 (184 b) (15), and  $\beta$ -actin (349 b) were used as probes. To elaborate probes, PCR amplification was conducted using normal mouse first-strand cDNA as a template. Primers for amplification of TLR4, TLR2, and  $\beta$ -actin were the same as those for RT-PCR. Other sense and antisense primers were: CD14, 5'-GGA AGC CAG AGA ACA CCA TCG-3' and 5'-GGA TTC TAT TCG GAG CCC TGC-3'; MyD88, 5'-ATC CGA GAG CTG GAA ACG-3' and 5'-GAT TAT ACC AAC CCT TGC-3'; IL-1 $\beta$ , 5'-AGC ACA TCA ACA AGA GCT TCA G-3' and 5'-TGT CCT GTG TAA TGA AAG ACG G-3'; and CD45, 5'-CTT CAG AGC CTC GTA CCA GC-3' and 5'-GAT TTG CCC TTC TGG ACA CA-3'. The amplified cDNA was cloned by ligation into a plasmid vector (TA Cloning Kit Dual

Promoter; Invitrogen, Carlsbad, CA), subsequently transformed into competent cells, and cultured (One Shot Competent cells; Invitrogen). The plasmid was extracted (Qiagen Plasmid Midi Kit; Qiagen, Valencia, CA), restricted with *EcoRI* (Takara, Shiga, Japan), and purified (Qiaex II Gel Extraction Kit; Qiagen). The probe sequence was confirmed by sequencing on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems).  $^{32}\text{P}$ -labeled probes (Megaprime DNA labeling systems; Amersham International) were hybridized with mRNA at  $42^\circ\text{C}$ . Membranes were washed twice at room temperature in  $2\times$  SSC containing 0.2% SDS for 5 min and once at  $50^\circ\text{C}$  in  $0.1\times$  SSC containing 0.2% SDS for 10 min. Signals were detected and measured with a bioimage analyzer system (BAS 2000 II; Fujix, Tokyo, Japan). All signals were standardized to the intensity of  $\beta$ -actin.

#### Western blotting

Mucosal scrapings were completely lysed by syringing in 20 mM Tris, pH 7.6, containing 0.1% SDS, 1% Triton-X, 1% deoxycholate, 100  $\mu\text{g}/\text{ml}$  of the protease inhibitor PMSF, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates were centrifuged at  $20,000\times g$  for 20 min at  $4^\circ\text{C}$ . The protein concentration was estimated by the Bradford method (Bio-Rad, Hercules, CA). One hundred micrograms of protein per lane was loaded and processed for SDS-PAGE fractionation (10% Multigel; Daiichi Pure Chemicals, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham International). After blocking the membrane for 2 h in 5% skim milk (Difco, Detroit, MI) in TBS (20 mM Tris and 150 mM NaCl, pH 7.4), it was incubated for 24 h with goat anti-mouse TLR4 polyclonal Ab (M-16; Santa Cruz Biotechnology) at a 1/500 dilution at  $4^\circ\text{C}$ , reacted with peroxidase-conjugated rabbit anti-goat Ig (DAKO) at a 1/1500 dilution at room temperature for 2 h, and washed three times in TBST. The resulting signals were imaged by ECL (Amersham Pharmacia Biotech, Arlington Heights, IL). The immunoblot was washed and reprobbed with goat anti- $\beta$ -actin Ab (Santa Cruz Biotechnology) as an internal control.

#### Statistical analysis

Data were expressed as the mean  $\pm$  SE. Differences between the control group and the experimental group were tested by the Mann-Whitney *U* test

( $p < 0.05$  was considered the level of significance). Analysis was performed with statistical software (SPSS for Windows, release 10.0.1; SPSS, Chicago, IL).

## Results

### RT-PCR of TLR2, TLR4, and MD-2

The first approach was to screen TLR expression by RT-PCR. Interestingly, TLR expression appeared different throughout the various segments of the gastrointestinal tract. TLR4 and TLR2 showed different distributions in the normal mouse digestive tract. TLR4 was mostly expressed in the stomach and in the distal segment of the colon, whereas TLR2 was strongly expressed in the proximal segment of the colon, whereas TLR2 was strongly expressed in the proximal segment of the colon. MD-2, on the other hand, was uniformly expressed throughout the gastrointestinal tract (data not shown).

### Northern blotting: TLR4 is preferentially expressed in the distal colon

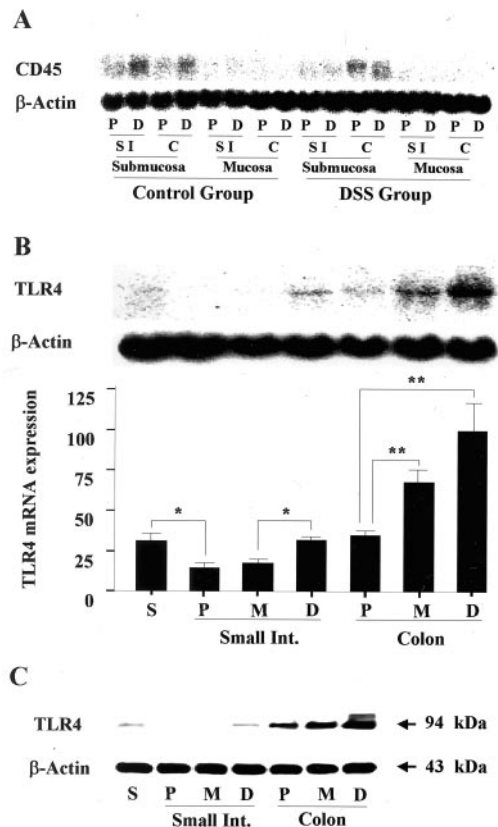
Hybridization of RNA from scrapped mucosal epithelium and remnant submucosal tissue with a CD45 probe determined that CD45 transcripts were detectable in the submucosal fraction, but not in the scrapings (Fig. 3A). Northern blotting confirmed that TLR4 was constitutively and strongly expressed in the normal distal colon compared with the other gastrointestinal segments. Quantification showed that TLR4 was expressed three times more strongly in the distal colon than in the rest of the colon, small intestine, and stomach (Fig. 3B).

### TLR4 protein is increased in the distal colon mucosa, and TLR4/MD-2 complex is expressed in strategically located mucosal cells in normal mice

Western blotting with an Ab against TLR4 showed reactive protein bands at the size of TLR4 (93.5 kDa) (18) in stomach, distal small intestine, and proximal, medial, and distal colon of normal mice, with the strongest signal in the distal colon. Protein levels in stomach and distal small intestine were weaker compared with that in proximal colon, differing from the mRNA pattern (Fig. 3C). Thus, both protein and mRNA of TLR4 were most abundant in the distal colon. The next step was to determine which cells in the gut mucosa express TLR4. The task was performed using a specific Ab against the TLR4/MD-2 complex. Immunohistochemical analysis with this Ab showed in all animals that TLR4/MD-2 was expressed by selected cell populations along the mouse gut mucosa. TLR4/MD-2 complex was mainly found in two different mucosal cell types: epithelial cells from the intestinal crypts and mesenchymal cells within the lamina propria. The epithelial cells located in the bottom of the crypts in distal small intestine and colon segments strongly expressed TLR4/MD-2 in their apical surface. A stronger expression was noted in the colon segments. The mesenchymal cells were mononuclear cells that had cytoplasmic granular structures stained by the mAb (Fig. 4, A–D).

### Distribution of CD14 correlates with that of TLR4

Since a tight functional relationship between TLR4 and CD14 is necessary for cells to respond to LPS, we decided to determine whether there are similarities in the segmental gastrointestinal expression of these receptors. As expected, Northern blotting showed that CD14 gene expression was higher in the colon than in the small intestine and stomach. CD14 expression was especially strong in the distal colon. Thus, TLR4 and CD14 have a similar expression pattern in the gut (Fig. 5A). In addition to mRNA studies, CD14 protein was localized by immunohistochemistry in the gut of normal animals. Epithelial cells located in the bottom of stomach pits and in the crypts of distal small intestine and colon



**FIGURE 3.** TLR4 determination in normal mice gut. **A**, Northern blotting evaluation of leukocyte contaminants in scrapped mucosa. RNA was extracted from scrapped mucosa and submucosal remnants (submucosa, muscular, and serosa) of proximal (P) and distal (D) small intestine (SI) and colon (C). RNA from normal ( $n = 3$ ) and DSS animals ( $n = 3$ ) was blotted, probed with CD45 cDNA, and imaged. CD45 mRNA was detected in the submucosal remnants, but not in the scrapped mucosa. **B**, TLR4 gene expression in the normal gut mucosa of BALB/c mouse. Mucosa from glandular stomach (S) and proximal (P), medial (M), and distal (D) segments of small intestine and colon was scrapped, and mRNA was extracted. A Northern blot demonstrating the stronger expression of TLR4 in the distal segment of the colon is shown. The graph includes data from two independent experiments ( $n = 10$ ). Bars represent the mean  $\pm$  SE. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . **C**, TLR4 protein in the normal gut scrapped mucosa ( $n = 5$ ) also has a restricted pattern, as shown by Western blotting. A goat polyclonal anti-mouse TLR4 Ab was used as a probe. Distal colon had the strongest signal. All blots were performed as specified in *Materials and Methods*. Typical results are presented.  $\beta$ -Actin was used as an internal control.

segments were positively stained. It was noted that CD14 staining of colonic epithelial cells was stronger than that in the epithelium of small intestine and stomach. CD14<sup>+</sup> mononuclear cells were not observed in the gut lamina propria (Fig. 6, A and B).

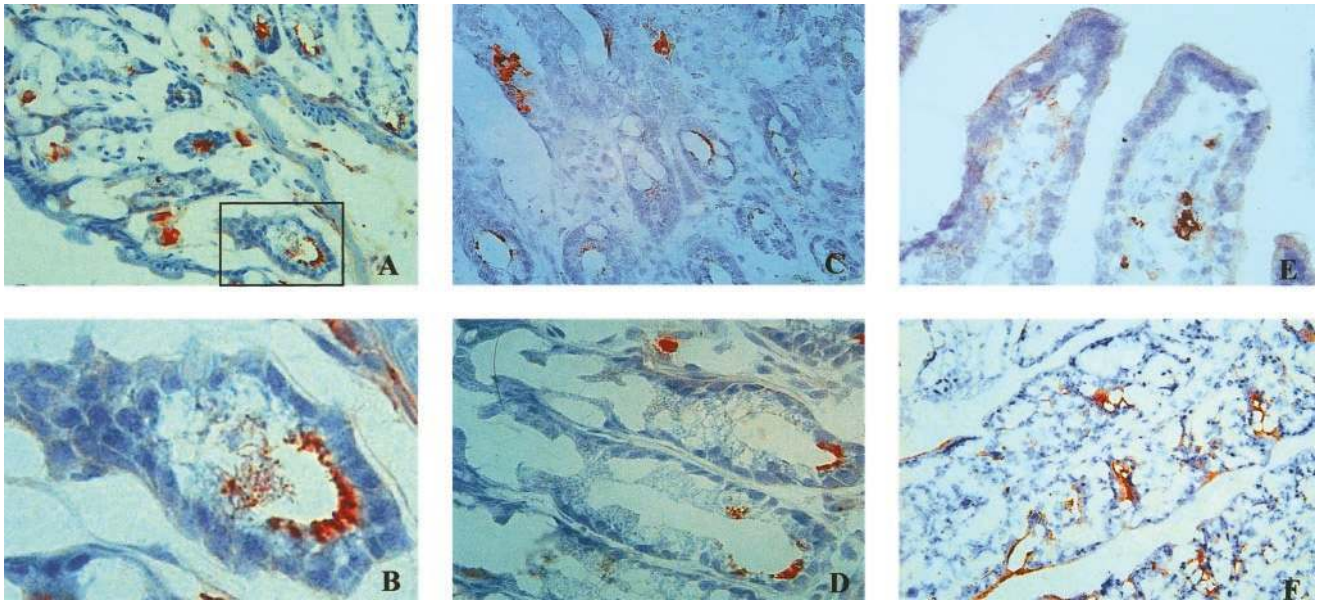
### TLR2 gene is localized in the proximal colon

Interestingly, the distribution of TLR2 was different from that of TLR4 as determined by Northern blotting. The TLR2 gene was predominantly expressed in the proximal colonic segments (Fig. 5B).

### MyD88 is uniformly expressed throughout the gastrointestinal tract

As MyD88 is an important adaptor molecule that participates in the intracellular signaling of multiple PRRs, we decided to determine its expression and distribution in the gut mucosa of normal BALB/c mice. Semiquantitative Northern blotting showed that the





**FIGURE 4.** TLR4/MD-2 protein complex is present in gut mucosal epithelial cells and lamina propria mononuclear cells in normal ( $n = 6$ ) and DSS animals ( $n = 5$ ). Gut frozen sections were reacted with anti-TLR4/MD-2 Ab as described in *Materials and Methods*. *A*, Panoramic view of distal colon (magnification,  $\times 200$ ). The marked area is amplified in *B*. *B*, Crypt amplification showing apical staining of the epithelial cells (magnification,  $\times 400$ ). *C*, Small intestine villi with negative epithelial cells but positive lamina propria mononuclear cells, and crypts bearing positive epithelial cells (magnification,  $\times 400$ ). *D*, Longitudinal section of normal colon crypts showing that only bottom epithelial cells bear TLR4/MD-2 (magnification,  $\times 400$ ). *E*, Small intestine during DSS inflammation showing positive luminal surface epithelium (magnification,  $\times 400$ ). *F*, The colonic surface epithelium is also positive during DSS colitis, but stronger signals compared with small intestine can be noted (magnification,  $\times 200$ ). Microscopy original magnifications are noted.

expression of MyD88 does not vary greatly throughout the mouse gastrointestinal tract (Fig. 5C).

#### *DSS-induced inflammation is focalized in the distal colon*

Next, we addressed the question of whether the expression intensity or gut distribution of the genes studied is modulated during inflammation induced by DSS. Disease activity index scores showed that colitis developed uniformly in all mice. The localization of inflammation in the DSS colitis model was determined by measuring IL-1 $\beta$  expression in the different gastrointestinal segments. Northern blotting showed that inflammation-related IL-1 $\beta$  gene expression was localized in the distal colon segment (Fig. 7A). Additional time-correlation studies showed that peak expression of IL-1 $\beta$  occurred in the distal colon during the first day after the end of DSS administration and decreased progressively. Four days after the end of DSS administration, IL-1 $\beta$  expression was weak, but still detectable by Northern blotting (Fig. 7B).

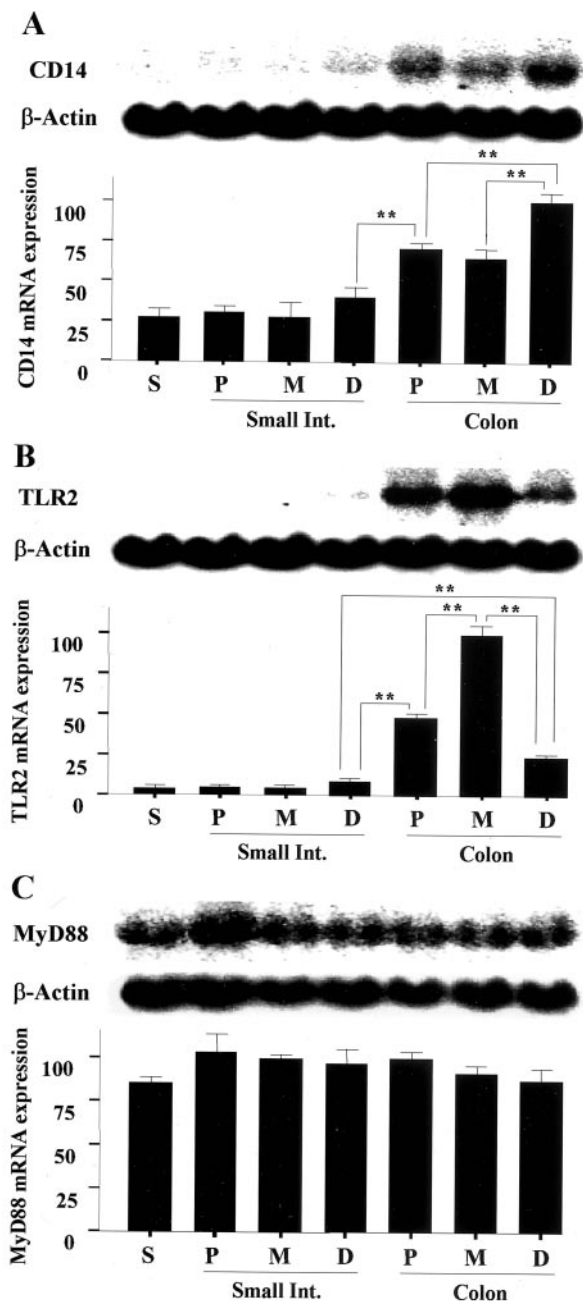
#### *Pattern recognition receptors are up-regulated during DSS colitis*

First, the expression and gut mucosal distribution of TLR4, CD14, TLR2, and MyD88 were assessed in one group of animals 1 day after stopping DSS administration. We found that the normal segmental expression patterns of all investigated molecules were preserved during inflammation. TLR4 and CD14 had stronger signals in the distal colon segment, whereas TLR2 was strongly expressed in the proximal segments even in animals with colonic inflammation (Fig. 7A). Next, the intensity of expression during DSS colitis of all molecules studied was compared with that in normal mice. Northern blotting showed that all genes were up-regulated during inflammation. Interestingly, CD14 had very strong signals in inflamed gut. The up-regulation of all genes was evident until day 4 after stopping DSS administration (Fig. 7B). Moreover, mucosal epithelium from the proximal and distal segments of the colon was

isolated by the EDTA-vibration method, and gene expression of TLR4, CD14, TLR2, and MyD88 was determined by Northern blotting. The expression of the studied genes was similar to that found in scraped mucosa (Fig. 7C). Western blot showed that TLR4 protein during DSS colitis was expressed in the stomach mucosa and in the gut as an increscent signal starting in the small intestine medial segment and progressing to its strongest signal in the distal colon. TLR4 protein was not detected in proximal small intestine. In general, protein expression extended more proximally in the small intestine during DSS colitis compared with that under normal conditions (Fig. 7D). TLR4/MD2 and CD14 immunohistochemistry of the inflamed gut showed basically similar segmental distributions as in normal tissue, but stronger staining compared with normal samples was observed. An increased area of epithelium lining the crypt and on the luminal surface was noted positive to TLR4/MD2 (Fig. 4, *E* and *F*). In addition, an increased intensity of CD14 staining was noted during DSS inflammation (Fig. 6, *C* and *D*).

## Discussion

In the present in vivo study segmentation of the gut permitted us to demonstrate for the first time that PRRs are differentially expressed along the mouse gastrointestinal tract. A few studies have addressed the compartmentalization of the immune response in the mouse gut, but none of them measured PRRs (19, 20). We have now shown that TLR4, CD14, and TLR2 are constitutively expressed in the mouse gut mucosa and that this expression varies along the gut. Interestingly, differences were found not only between small intestine and colon, but also between different segments of the colon. Our tentative explanation is that the focalized expression of PRRs in the mouse colon mucosa reflects its adaptation to the flora normally present at the luminal side of the epithelium. In concordance with this, it has previously been shown that various bacterial species, especially those of the autochthonous



**FIGURE 5.** A, CD14 mRNA distribution in the normal mouse gut mucosa as shown by Northern blotting. CD14 is more strongly expressed in the distal colon segment, similarly to TLR4. B, Localization of TLR2 in the normal gut mucosa is different from that of TLR4. The intensity of TLR2 expression in the medial and proximal segments of the colon was stronger than that in the rest of the gut. C, Expression of MyD88 in the normal gut mucosa is uniformly distributed. MyD88 expression did not vary markedly throughout the gut. In all experiments mouse gut mucosa was segmentally scraped, and RNA was extracted, electrophoresed, and transferred to a nitrocellulose membrane. mRNA was hybridized with a  $^{32}\text{P}$ -labeled cDNA probe. Signals were standardized to the intensity of  $\beta$ -actin. Typical results are shown. S, glandular stomach; P, proximal; M, medial; D, distal. The bar graph shows results from 10 mice as the mean  $\pm$  SE. \*\*,  $p < 0.01$ .

flora, do not occur randomly in the gut, but, rather, exhibit marked selectivity in their anatomic localization (21). Moreover, although it is clear that the large intestine is the most heavily colonized region of the mouse gut, factors such as local immunity that control distribution of the flora are not completely understood (9).

We found that the mRNA expression of TLR4 and CD14, both important receptors for Gram-negative LPS, is constitutively stronger in the distal segment of the colon. TLR4 and CD14 were also effectively translated into protein, as determined by Western blot and/or immunohistochemistry. It seems logical that genes encoding proteins with similar bacteria-detecting functions would have similar expression patterns along the gut. The functional interaction of TLR4 and CD14 is well known, and CD14 represents the major binding site for LPS on the cell surface. Binding of LPS to CD14 is essential for subsequent interaction with the TLR4/MD-2 complex. This is supported by the inability of TLR4/MD-2 to interact with LPS when CD14 is blocked by anti-CD14 Abs (22).

Moreover, our data show that TLR4 protein in the normal mouse gut mucosa was expressed in a pattern similar to that of mRNA, with increased protein levels in the colonic segments, especially in the distal colon. Minor variations in the expression levels observed in normal small intestinal segments can be explained by mechanisms of translation control present in epithelial cells (23, 24).

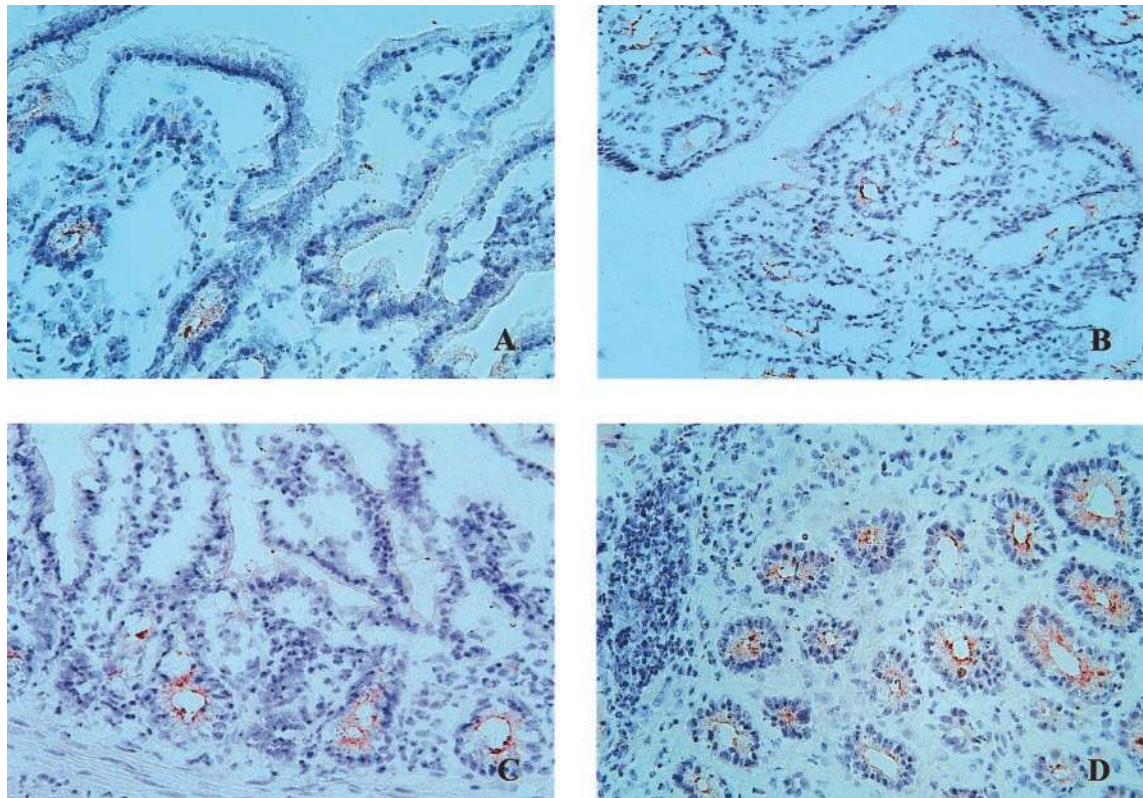
TLR2 was also highly expressed in the colon compared with the rest of the gut. In contrast to TLR4, TLR2 was strongly expressed in the medial and proximal segments of the colon. TLR2 has been shown to react with an extraordinary array of other molecules, including peptidoglycan, zymosan, lipoproteins, modulin, and LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*. Some of this broad ligand specificity of TLR2 is explained by its dimerization with TLR6 or TLR1 (4, 25). Inhibition of either TLR2 or TLR6 blocks the responses of mouse macrophages to zymosan, peptidoglycan, and phenol-soluble modulin (25, 26). These data support the idea that the contrasting gut localizations of TLR2 and TLR4 that we found may be related to their different roles in the gut.

MyD88 is the intracellular adaptor molecule for the TIR family and is critical for TLR signaling and activation of NF- $\kappa$ B and mitogen-activated protein kinase (27, 28). Although the presence of a MyD88-independent pathway for TLR4 signaling and activation of NF- $\kappa$ B has been reported, it would not be capable of inducing TNF- $\alpha$  production, which is completely impaired in MyD88 knockout mice (29, 30). Our results show that MyD88 is widely and uniformly distributed along the gut mucosa, in agreement with its function as an intracellular adaptor for multiple and varied receptors.

We determined the mRNA expression of TLR4 and other PRRs in mucosal homogenates from each gut segment. Complementing this approach, the mucosal cells that bear the functional complex TLR4/MD-2 were localized by immunohistochemistry with an Ab that selectively recognizes TLR4 associated with MD-2 (31, 32). The TLR4/MD-2 complex was mainly found in two different cell populations of the mucosa, crypt epithelial cells and lamina propria mononuclear cells. On the other hand, CD14 was detected only in mucosal epithelial cells. TLR4/MD-2 and CD14 were mainly positioned in the apical side of epithelial cells located in the bottom of the crypts. We speculate that these findings may reflect a strategy of the mucosal innate immune system to simplify the task of sensing the gut bacterial content. Changes in crypt microflora at different depths have previously been described (33). In addition, it has been reported that villous tip epithelium can accumulate more LPS than crypt epithelium (34). As a consequence of exposure to LPS, down-regulation of TLR4/MD-2 and CD14 may occur, resulting in tolerance to LPS in the villous epithelium (35, 36). This theory is compatible with epithelial cell maturation along the maturing crypt-villous axis, since the expression of some genes has been shown to be altered during epithelial differentiation (37).

TLR4/MD-2 Ab also reacted with mononuclear cells present in the gut mucosa lamina propria, staining some granular organelles.





**FIGURE 6.** CD14 immunohistochemistry in normal and DSS-inflamed gut. Tissue sections from gut segments were immunostained with goat anti-CD14 Ab as described in *Materials and Methods*. *A* and *B*, Normal small intestine and normal colon, respectively. *C* and *D*, DSS-inflamed small intestine and colon, respectively. In all cases CD14 was detected only in epithelial cells. Increased expression of CD14 protein in epithelial cells during DSS inflammation can be noted. Representative images are shown (magnification,  $\times 200$ ;  $n = 10$ ).

These cells were heterogeneously dispersed, and most of them are probably lamina propria macrophages containing positively stained phagosomes. Several TLRs, including TLR1, TLR2, and TLR6, have been shown to be recruited to mouse macrophage phagosomes, where they sample the contents independently of their nature (26, 38). Although TLR4 is expressed in mouse macrophages (35, 39) and is probably also recruited to phagosomes, there is no previous report of the TLR4/MD-2 complex in this location.

The expression of PRRs during DSS-induced colitis was also determined. It was found that in scraped gut mucosa the segmental distribution of TLR4, CD14, TLR2, and MyD88 was the same as that in normal mice, but the expression of these genes was higher in colon with DSS-induced inflammation. When the epithelium was isolated by EDTA-vibration and used as a material for study, similar results were obtained. Thus, DSS-induced inflammation occurs in the gut segment showing high expression of TLR4 and CD14; no such correlation was observed for TLR2. Therefore, TLR4 and CD14, but not TLR2, may have a role in the development of DSS-induced colitis. Indeed, the evidence that suppression of intestinal flora and LPS production in the gut with antibiotics prevents DSS colitis in mice supports the involvement of TLR4 in the induction of DSS colitis (40, 41).

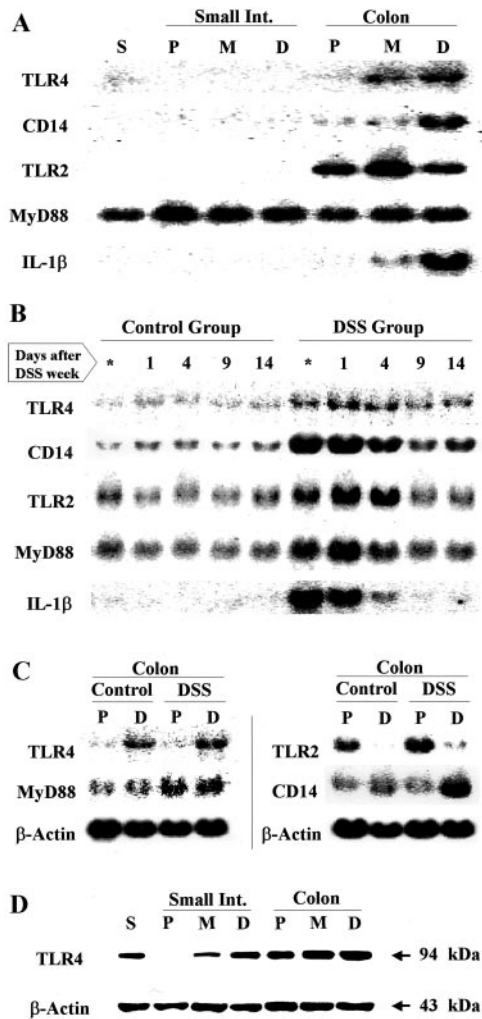
Similar to normal conditions, during DSS colitis TLR4 and CD14 proteins were strongly localized in the epithelium of distal colon, and protein levels of both PRRs were increased compared with normal levels. These results are in agreement with previous *in vivo* studies. CD14 transcripts and protein were present in normal colonic epithelial cells, and CD14 levels were up-regulated preceding the development of colitis in IL-2-deficient mice (15). In addition, mouse renal tubular epithelial cells express CD14 (42,

43) as well as TLR4, TLR2, and MD-2 (18, 43), and in a mouse model of renal ischemia TLR4 and TLR2 were found to be up-regulated in the renal tubular epithelium during inflammation (18). Collectively, the data strongly support the idea that TLR4 and CD14 are down-regulated and similarly distributed in the gut epithelium as a strategy properly tuned to sense their ligands in normal tolerant condition and that both receptors are up-regulated as part of the inflammatory response.

In mouse epithelial cell lines, CD14 and TLR4 were positive in the small intestine cell line m-IC<sub>12</sub> (8), while the rectum carcinoma cell line CMT93 was CD14 negative (44). In humans, the colon cancer cell lines Caco-2, HT-29, T84, and SW620 as well as the gastric cancer cell line MKN45 were reported to express TLR4 and TLR2, but not CD14 (44–47). On the contrary, other research groups (48, 49) reported that CD14 was positive in Caco-2, HT29, and SW-480. Thus, publications about the expression of PRRs in immortalized neoplastic epithelial cells are contradictory, probably reflecting the difference from normal *in vivo* conditions and/or the different sensitivities of the detection methods in relation to the number of CD14 transcripts per cell (36).

By restriction of PRRs to the crypt bottom in normal conditions, crypts may function as a microanatomical niche where the exposure of cells with high expression of PRRs to commensal bacterial ligands from the lumen could be controlled. This system seems to be overwhelmed during DSS inflammation according to our observations, and increased expression of PRRs occurs on most gut epithelial cells. Other studies in animals have also shown increased expression of TLR4 and CD14 in epithelial cells under acute inflammatory conditions (15, 18), and in humans it has been reported that TLR4 expression is increased in the intestinal epithelium during inflammatory bowel disease (44). Collectively, the data support the importance of





**FIGURE 7.** Evaluation of PRRs during DSS colitis. **A**, Gut segmental distribution of TLR4, CD14, TLR2, and MyD88 during DSS colitis is similar to normal distribution, as shown by Northern blotting. IL-1 $\beta$  expression demonstrates that inflammation is localized in the distal colon. Colitis was induced by DSS given for 7 days. Mice were sacrificed 1 day after stopping DSS. The experiment was performed twice with the same results ( $n = 10$ ). **B**, Distal colon mucosa expression of TLR4, CD14, TLR2, and MyD88 in DSS mice compared with normal mice. All genes were up-regulated during DSS colitis. Mice ( $n = 20$ ) were sacrificed at 8 h (\*) and on the indicated days after the DSS week. **C**, TLR4, TLR2, CD14, and MyD88 expression in proximal and distal colon epithelia of normal and DSS mice. The DSS group ( $n = 3$ ) and the control group ( $n = 3$ ) were sacrificed 1 day after the DSS week. The epithelium was isolated by the EDTA-vibration method applied to everted gut bags. Results were the same as those obtained by scraping. **D**, Western blot showing TLR4 protein expression in DSS mice ( $n = 5$ ). Similar to normal conditions, the protein level was higher in the distal colon. TLR4 expression was extended more proximally in the small intestine during colitis compared with normal. A goat polyclonal anti-mouse TLR4 Ab was used. Blots and epithelium isolation were performed as specified in *Materials and Methods*.  $\beta$ -Actin was used as an internal control. Representative autoradiograms are shown. S, glandular stomach; P, proximal; M, medial; D, distal.

PRR restriction in epithelial cells as a mechanism of tolerance under normal conditions.

In conclusion, we have demonstrated that the PRRs TLR4, CD14, and TLR2, are constitutively expressed in mouse intestinal mucosa. These receptors show different expression patterns along the gut segments, but all are focalized in the colon. TLR4 and CD14 gene and protein gut expression patterns showed important

similarities. This study also provides evidence that the gene expression of these receptors and the protein levels of TLR4 and CD14 are up-regulated during DSS-induced inflammation. Additionally, highly important restrictions on epithelial TLR4/MD-2 complex and CD14 expression are evidenced along the crypt axis. We also show that TLR4/MD-2, but not CD14, is expressed in mucosal mononuclear cells. Collectively, our findings provide novel information about the *in vivo* strategic localization of PRRs in the mouse gut.

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