

# Identification of Card15/Nod2 mRNA in Intestinal Tissue of Experimentally Induced Colitis in Rats

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(Received 24 January 2006/Accepted 7 March 2006)

**ABSTRACT.** Card15/Nod2 has been suggested to be an intracellular pathogen-associated molecular pattern (PAMPs) recognition molecule, which contains a leucine-rich repeat region similar to the Toll-like receptors (TLRs). Card15/Nod2 gene variants play an important role in the susceptibility to Crohn's disease. In this study, we examined the kinetics of Card15/Nod2 expression in intestinal tissue during inflammation in the 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-treated rat experimental colitis model. At 2 and 4 days after TNBS administration, the mononuclear cells remarkably infiltrated the mucosal layer and tunica muscularis, which was followed by a gradual decrease to resting levels at 14 days after TNBS administration. Card15/Nod2 mRNA expression increased and peaked at 4 days after the TNBS administration, followed by a gradual decrease in accordance with the amelioration of the inflammatory response. Expressions of Tlr2, Tlr4 and Myd88 were also upregulated in the inflamed colonic region, and in an *in situ* hybridization study, a positive signal for Card15/Nod2 was observed in the crypt of the epithelial cell layer and in the infiltrated cells of the submucosal and myenteric regions. These results suggest that in addition to the TLR recognition systems, Card15/Nod2 may contribute to the inflammatory process not only in the epithelial and submucosal layers but also in the tunica muscularis.

**KEY WORDS:** Card15/Nod2, colon, intestinal inflammation, TNBS.

*J. Vet. Med. Sci.* 68(7): 701–708, 2006

Crohn's disease is a chronic, relapsing inflammatory disease of the gastrointestinal tract most commonly affecting the terminal ileum and colon. Crohn's disease is considered to be a complicated genetic disorder resulting from the complex interplay between several genetic and environmental risk factors.

Recently, a susceptibility locus for Crohn's disease on chromosome 16 was identified, (IBD (inflammatory bowel disease) 1 locus) [9], and shortly after, the Card15 (caspase recruitment domain-containing protein 15)/Nod2 (nucleotide oligomerization domain 2) gene was identified within the same locus and determined to be the specific causative gene [10]. The Card15/Nod2 gene encodes an intracellular protein with homology to the Apaf-1/CARD4 superfamily, and contains 2 N-terminal CARD domains, one centrally located NBD (nucleotide-binding domain), and leucine-rich repeats (LRRs) in the C-terminal. Thus, it has been suggested that Card15/Nod2 may be involved in pattern recognition of bacterial pathogens [12, 21, 23]. Card15/Nod2 is expressed intracellularly in antigen-presenting cells (APCs), such as macrophages or dendritic cells, and recognizes and reacts to the muramyl dipeptide (MDP) components of peptidoglycan (PGN) [6, 11]. Card15/Nod2 enters into CARD-CARD interactions with the serine threonine kinase, RICK, which results in the modulation of NF- $\kappa$ B activation [15, 21]. Thus, it has been speculated that disruption of this cascade might be associated with Crohn's disease, since bacte-

rial surveillance by APCs is expected to play a pivotal role in intestinal immune function.

Card15/Nod2 is highly expressed in the cells of myeloid lineage and lymphatic organs, and to a lesser extent in the other organs, including intestinal tissue [7, 12, 21, 23]. Using epithelial cell lines, Card15/Nod2 expression can be induced by the following stimulation with proinflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$  [7]. Card15/Nod2 can be detected especially in epithelial cells in the intestinal tissue of Crohn's disease patients, and its expression has been linked to the production of antimicrobial peptides known as cryptdins from the crypts [16, 22]. It has been reported that the Card15/Nod2 null mutant mice are susceptible to bacterial infection via oral delivery [15], which suggests that the Card15/Nod2 protein is a critical mediator of bacterial immunity within the intestine, thereby providing a possible mechanism for Card15/Nod2 mutations in Crohn's disease. However, to our knowledge, there are no reports that have demonstrated a change in the expression of the Card15/Nod2 gene in intestinal inflammation models.

Administration of 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) induces intestinal inflammation with the features of transmural mononuclear inflammation and a predominant Th1-type activity of the mucosal leukocyte, which are consistent with those observed in Crohn's disease [3, 20]. In this study, we attempted to define the profile of Card15/Nod2 expression during the inflammatory process of TNBS-treated colitis by examining the Card15/Nod2 expression in rat colon through the use of semi-quantitative RT-PCR and *in situ* hybridization analyses.

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## MATERIALS AND METHODS

**Induction of colitis model:** Experiments and animal care were carried out in compliance with guidelines outlined by the Guide to Animal Use and Care of the University of Tokyo. Male Sprague Dawley rats (6-wk-old, 160–180 g; Charles River Japan) were anesthetized with 40 mg/kg i.p. pentobarbital sodium. The abdomen was opened by midline laparotomy, and the proximal colon was gently exteriorized. TNBS colitis was induced by injecting 100 mg/kg TNBS (Tokyo Kasei Kogyo, Tokyo, Japan) in a 50% ethanol solution into the colonic lumen 2–4 cm distal to the cecal-colonic junction [14]. The nontreatment rat proximal colon was used as control in this experiment. Rats were sacrificed at 2, 4, 7 and 14 days following TNBS treatment under pentobarbital anesthesia, with the proximal colon isolated and used for the RNA analysis. For *in situ* hybridization and determination of the colitis developmental stage of the rat, the proximal colon was excised and immersed in Bouin's solution for 12–24 hr, followed by processing and embedding in paraffin.

**Histology and microscopic damage score:** To examine the pathohistological features of the TNBS colitis model, morphometric analysis was performed using a previously described protocol [19]. The thickness of the muscle layer was determined at three points around the circumference, separated by 60 degrees. The average of the 6 measurements was used for statistical analysis. The degree of inflammation for the microscopic tissue sections was scored as follows: 0, no leukocyte infiltration; 1, low level of leukocyte infiltration; 2, moderate level of leukocyte infiltration; 3, high vascular density and thickening of the colon wall; and 4, transmural leukocyte infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall. Grading was done in a blind manner.

**Semi-quantitative reverse transcription polymerase chain reaction:** Animals were sacrificed under pentobarbital anesthesia at various times after TNBS treatment. The inflamed region of the proximal colon was identified and removed, opened along the mesenteric border, and then pinned mucosal side down in Hank's saline. Both the smooth muscle layer and mucosal layer were gently separated, put into the TRIzol reagent (Life Technologies Ltd.) to isolate total RNA according to the manufacturer's protocol. Total RNA was converted to cDNA using 100 pM random primer (Takara Bio., Japan) in 20  $\mu$ l of reverse-transcription (RT) reactions and then used for polymerase chain reaction. Oligonucleotide PCR primers (shown with final product size) were as follows: Gapdh (glyceraldehyde-3-phosphate dehydrogenase) (308 bp), forward: 5'-TCC CTC AAG ATT GTC AGC AA-3', reverse: 5'-AGA TCC ACA ACG GAT ACA TT-3'; Card15/Nod2 (219 bp), forward: 5'-AGT GAA GGC AAA TGG ACT GG-3', reverse: 5'-TCT GTC CGC AGC TCT AAG GT-3'; Tlr4 (272 bp), forward: 5'-ATG CCA GGA TGA TGC CTC TCT TGC A-3', reverse: 5'-TTC ACA CCT GGA TAA ATC CAG CCA C-3'; Tlr2 (202bp), forward: 5'-CCA GCA GCT GGA

GAA CTC TGA CCC A-3', reverse: 5'-CTC GTC AAA GAG CCT GAA GTG GGA G-3'; Myeloid differentiation factor 88 (Myd88, 399 bp), forward: 5'-AGT TGC TAG CCT TGT TAG ACC GTG AGG-3', reverse: 5'-AAA CAA CCA CCA CCA TGC GAC GAC ACC-3'. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. Expression of each of the transcripts was normalized to Gapdh expression and quantitated densitometrically with Scion Image beta 4.0.2 analysis software (Frederick, MD, U.S.A.).

**Generation of cRNA probes and *in situ* hybridization:** The 687 bp DNA fragments (Genbank XM\_226330) were isolated from rat peritoneal exudative cDNAs by PCR, and using the specific primers 5'-AGC TGC TAT GTG TTC TCA GC-3' (forward; 2032–2051) and 5'-TTG TGC TGC AGC ATC TGC AG-3' (reverse; 2699–2718) were subcloned into the pCR<sup>II</sup> plasmid (Invitrogen). The clone was linearized with the appropriate restriction enzyme, and antisense RNA probes were generated by *in vitro* transcription using DIG-labeled UTP with Sp6 or T7 RNA polymerase (Roche Diagnostics Inc., Mannheim, Germany). To identify the distribution of rat Card15/Nod2 transcripts, *in situ* hybridization was performed as previously described [5]. In short, deparaffinized sections were pretreated with 0.3% Triton X-100 in 10 mM phosphate buffer saline and 20 mg/ml proteinase K in Tris-HCl buffer (pH 7.5) containing CaCl<sub>2</sub> and then hybridized with DIG-labeled RNA probes in a solution containing 50% formamide, 10% dextran sulfate, 5  $\times$  SSC, 1  $\times$  Denharts' solution, 1% SDS, 100  $\mu$ g/ml heparin, 10 mM DTT, and 1 mg/ml denatured tRNA and ssDNA at 50°C for 12–16 hr. After treatment with RNase A (20  $\mu$ g/ml; Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37°C for 1 hr, the specimens were finally washed twice with 0.1  $\times$  SSC at 65°C for 1 hr. The signals were detected by immunohistological method using alkaline phosphatase-conjugated anti-DIG antibody and nitroblue tetrazolium as the chromogen (Roche Diagnostics).

**Statistical analysis:** All results are expressed as the mean  $\pm$  SEM. A student's *t*-test or nonparametric repeated-measures analysis of variance (ANOVA) was used for statistical analysis. P values were considered significant when  $<0.05$ .

## RESULTS

**Assessment of colonic inflammation:** Histological assessment of TNBS colonic samples showed disruption of the mucosal structure of the colon, with extensive ulceration and inflammation in the TNBS-treated rat (Fig. 1). At 2 days after TNBS treatment (Fig. 1C, D), destruction of the epithelial cell layer and lamina propria was observed, along with the infiltration of inflammatory cells that were seen surrounding the remains of the necrotic epithelial cell layer (Fig. 1C). A remarkable cellular infiltration was also observed in the submucosa. Although both the boundary portions including the submucosa-muscle layer and the circular-longitudinal muscle layer were still distinct, dropsy and macrophage-like cell infiltration were seen in the circu-

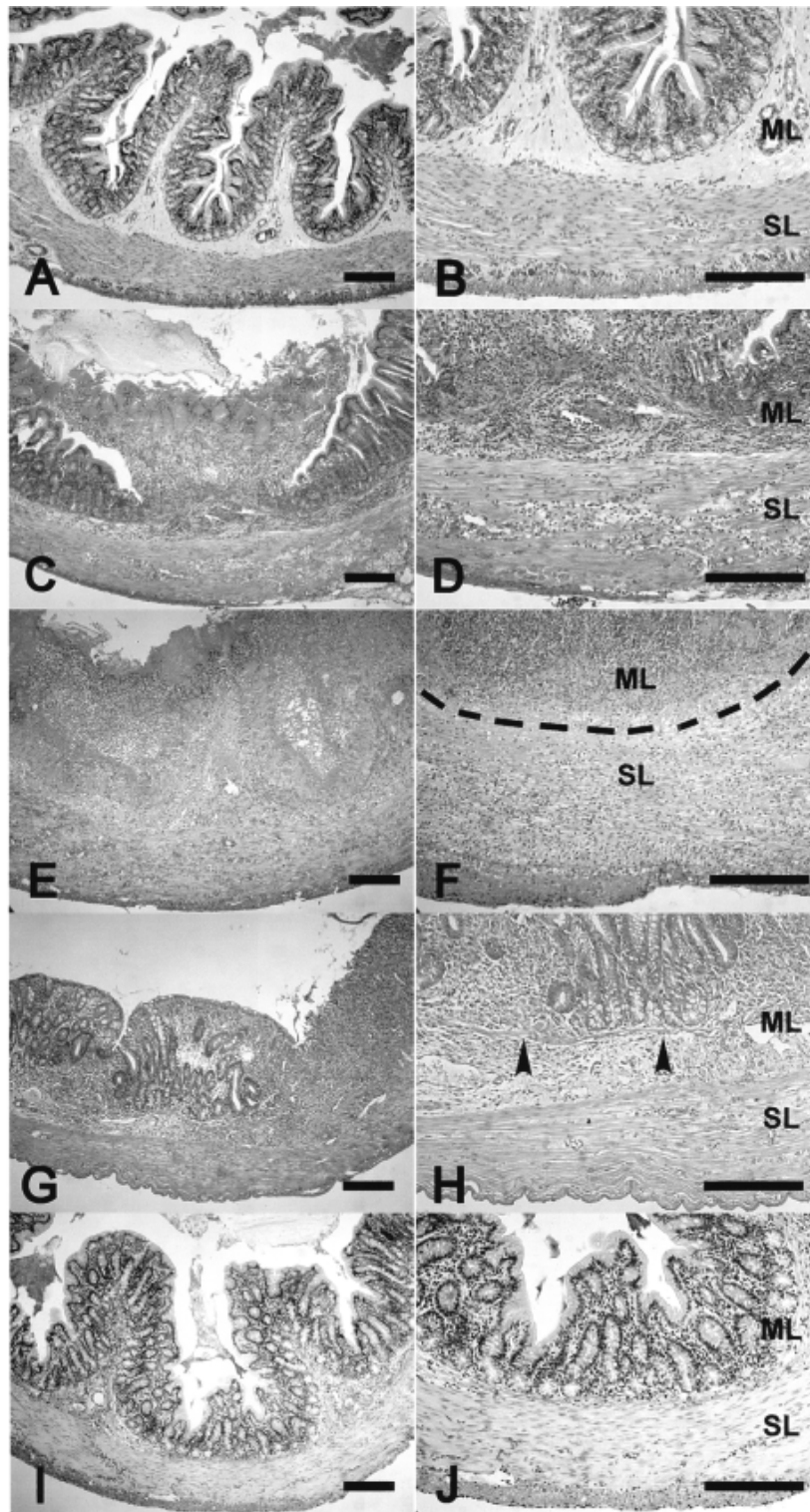


Fig. 1. A typical microscopic appearance of the colon in a TNBS-treated rat. Serial paraffin embedded sections ( $6\ \mu\text{m}$ ) were stained with H&E to assess the degree of inflammation and smooth muscle wall thickness. Proximal colon microscopic image of a nontreatment control rat with intact epithelial, mucosal and smooth muscle layers (A, B). A TNBS-induced colitis rat at 2 days (C, D) shows destruction of the mucosal layer (ML) including inflammatory cell infiltration and smooth muscle layer (SL) architecture. TNBS-treated colons at 4 days (E, F), 7 days (G, H) and 14 days (I, J) are also shown. The uncertain border between the submucosal and circular smooth muscle layers (dotted line, F) gradually changes and becomes clear at 7 days after the TNBS treatment (H). Arrowheads indicate the layer of muscularis mucosae (H). Scale bar= $250\ \mu\text{m}$ .

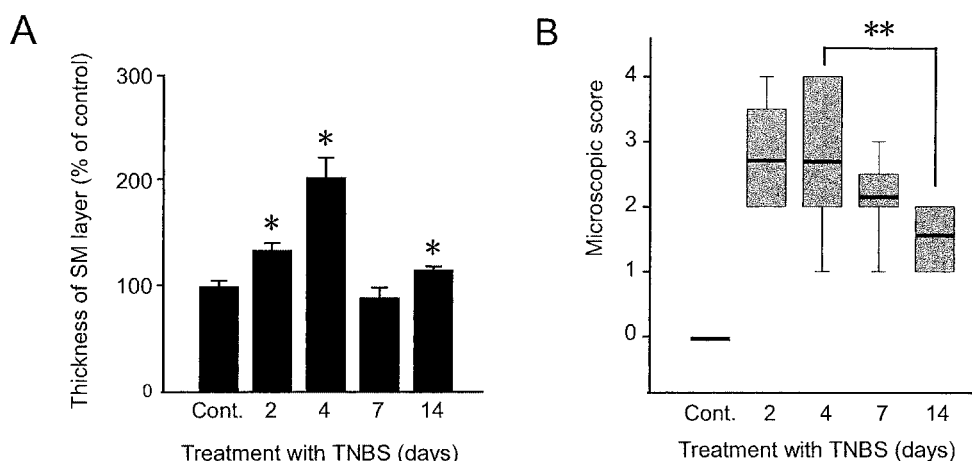


Fig. 2. The thickness of smooth muscle (A) and microscopic damage score (B) of TNBS-treated rat colon are shown. Results are expressed as boxplots of the median (B). (\*;  $p < 0.05$  vs. non-treatment control, \*\*;  $p < 0.05$  vs. TNBS-treated colon at 4 days).

lar muscle layer (Fig. 1D). At 4 days after TNBS treatment (Fig. 1E, F), the boundary line between the mucosal layer and tunica muscularis became much more obscure (anticipated border is shown by the dotted line, F).

TNBS treatment increased the inflammation score in a time-dependent manner, which reached a maximum at 4 days and then gradually decreased toward resting levels (Fig. 2A, B). In response to the amelioration in the inflammation process, many crypts and goblet cells recovered and existed in the epithelial layer (Figs. 1G, I); however, neither the epithelial cells nor the mucoderma were completely restored (Fig. 1G). While the boundary part of the submucosa and muscle layer became clear, there was still aggressive cellular infiltration in the submucosa at 7 days after TNBS treatment (Fig. 1H). Most of the samples showed almost complete restoration of the epithelial cell layer and smooth muscle layer at 14 days (Fig. 1I, J). On the other hand, the infiltration of mononuclear cells in the lamina propria and crypt distortion were still present at 14 days. The variation of thickness of the smooth muscle layer showed a maximum score at 4 days after TNBS treatment and returned to control levels at 14 days (Fig. 2A).

**mRNA expressions of *Card15/Nod2*, *Tlr4*, *Tlr2* and *Myd88*:** Expressions of *Card15/Nod2*, *Tlr4*, *Tlr2* and *Myd88* mRNA were assessed by semi-quantitative RT-PCR in the TNBS-treated rat colon. In this series of experiments, we separated the affected colonic tissue into mucosal and muscular regions to determine whether there was a difference in mRNA expressions between them. The level of *Card15/Nod2* mRNA increased and peaked at 4 days after the TNBS treatment. The level at 4 days was about four times higher than that observed in the control group, after which the level gradually reduced to that found for the control (Fig. 4A). There was no clear difference in the quantity of expression of *Card15/Nod2* (relative to *Gapdh* mRNA) in the muscle and the mucosal layers. *Myd88* is a shared adaptor mole-

cule in IL-1, IL-18, and TLR signaling [1, 13]. The level of *Myd88* mRNA also increased and peaked at 2 days after the TNBS treatment (Fig. 4B), after which it gradually reduced to the level found for the control during the amelioration of colitis. On the other hand, in control rats, *Tlr4* and *Tlr2*, which are the representative PAMP receptors involved in mediating inflammatory responses, showed a higher expression in the mucosal layer compared to that in the muscle layer (Fig. 4C, D). After TNBS treatment, the level of *Tlr4* and *Tlr2* mRNAs increased and reached maximums at 2 and 4 days, respectively. Although the expressional change in *Tlr4* mRNA was transient, similar to *Nod2* and *Myd88*, *Tlr2* mRNA continued to be elevated with high levels seen even at 7 and 14 days.

Since the GenBank record of rat *Card15/Nod2* mRNA sequence from XM\_226330 has been predicted by an automated computational analysis, it was necessary to determine whether the PCR product in this study was really *Card15/Nod2*. Although the alignment of a partial sequence of *Card15/Nod2* mRNA showed that the rat *Card15/Nod2* cDNA amplification products might be 285 bp, we detected a shorter amplicon from intraperitoneal macrophages, mesenteric lymph node, spleen (Fig. 3B) and colonic smooth muscle tissue (Fig. 3C). When using a sequence analysis, we confirmed that the amplified cDNA product of the mouse *Card15/Nod2* gene was the same as that found in humans. The 66 bp region, which is absent in mouse and human, was also absent in the rat *Card15/Nod2* transcript.

**Distribution of rat *Card15* transcripts in TNBS-treated rat colon:** *In situ* hybridization analysis showed that a strong signal for the *Card15/Nod2* transcripts was detected in the mucosal layer, especially in the basal epithelial cell layer of the crypt region. This region contained many goblet cells at 2 days after TNBS administration (Fig. 5A, B, indicated with arrows). A strong signal was also observed for monocytes in the lamina propria and mononuclear cells, and in the

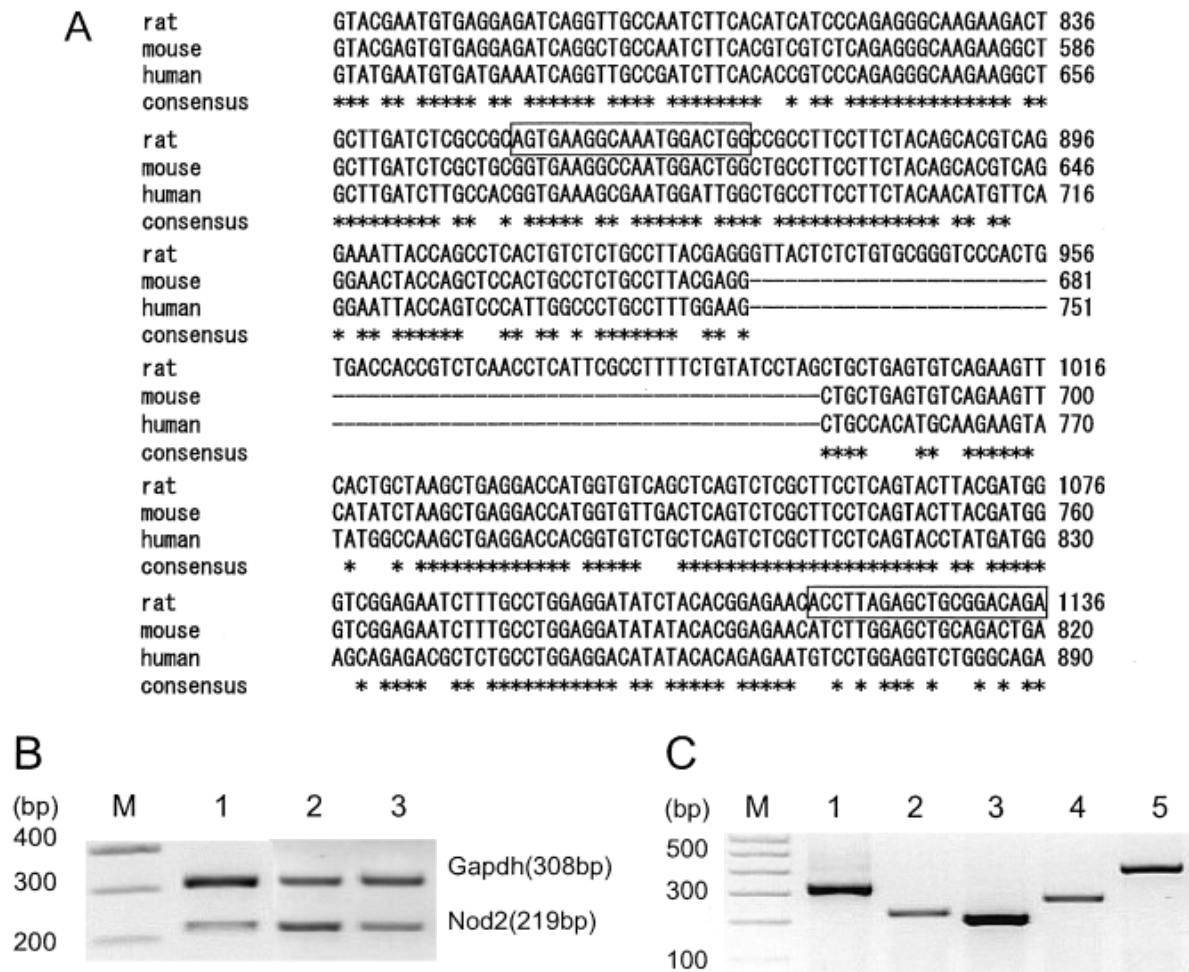


Fig. 3. A. Alignment of a partial sequence of Card15/Nod2 mRNA. Rat (XM\_226330), mouse (NM\_145857), and human (NM\_022162) Card15/Card15/Nod2 mRNAs were aligned using ClustalW (1.82). The portion surrounded by the square is the primer sequence used for Card15/Nod2 cDNA amplification. B. Card15/Nod2 mRNA expressions in the rat primary intraperitoneal macrophages (1), mesenteric lymph node (2) and spleen (3) are shown. C. The predicted, typical PCR products are shown (1: GAPDH, 308 bp, 2: Card15/Nod2, 219 bp, 3: Tlr2, 202 bp, 4: Tlr4, 287 bp, 5: Myd88, 399 bp).

macrophages in the submucosa (Fig. 3B, arrowheads). At 7 days after TNBS administration, a strong signal was also detected in the basal crypt of the epithelia, which showed hyperplasia and distortion (Fig. 5C, D). A weak but significant signal was detected in the myenteric plexus region of the smooth muscle layer, including macrophages (Fig. 5F, arrows). No signal was detected in the smooth muscle cells (Fig. 5G), but a weak signal for the mononuclear cells was observed inside the muscle layer (arrowheads). The region of colonic GALT (gut-associated lymphoid tissue) showed a slight signal around the germinal centers (Fig. 5E, indicated by GC).

## DISCUSSION

In this study, we found an increase of Card15/Nod2 transcripts both in the mucosal layer and in the tunica muscu-

laris of the colon after the TNBS-induced inflammation. Analysis of the expression pattern of the Card15/Nod2 gene during colonic inflammation, found that the level of mRNA transcripts at 4 days was about 4 times higher as compared to controls (Fig. 4A). This change is roughly correlated with the infiltration of inflammatory cells into the submucosal and myenteric regions, which was induced by TNBS treatment.

Previous studies have shown that Card15/Nod2 expression is enhanced by proinflammatory cytokines and bacterial components in various cell populations via a NF $\kappa$ B signal cascade [7, 25]. By destroying the epithelial cell barrier after TNBS administration, there is a great likelihood that the submucosal layer will be exposed to the scattering PAMPs (pathogen-associated molecular pattern), such as LPS and peptidoglycan. The *in situ* hybridization analysis of the present study indicated that there was a strong

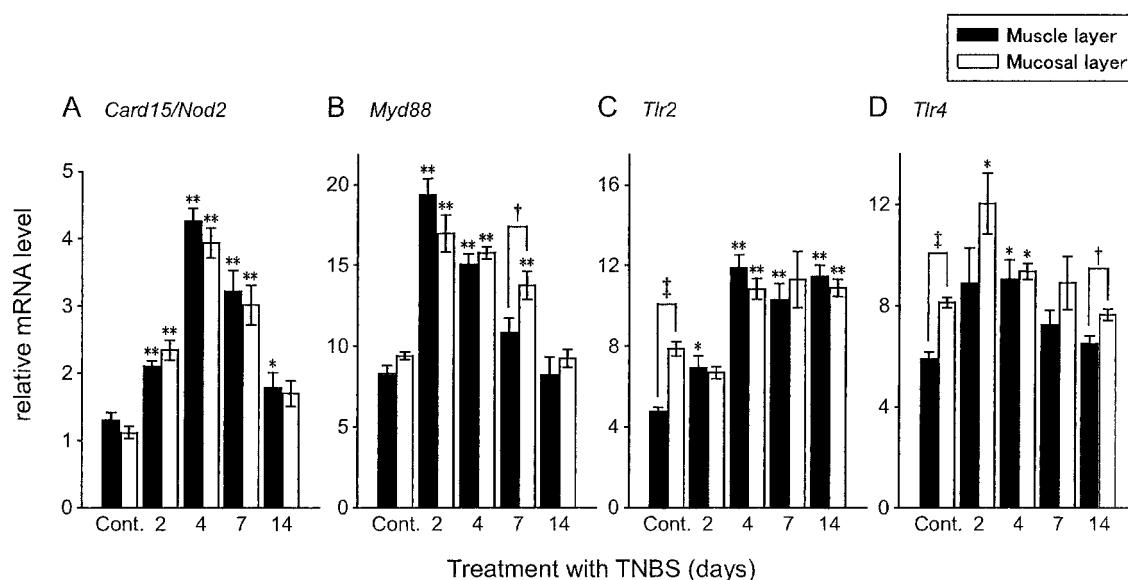


Fig. 4. Alterations in Card15/Nod2, Myd88, Tlr2 and Tlr4 mRNA expressions in TNBS-induced inflamed colon. Each stage of the colonic sample was separated into the smooth muscle layer and mucosal layer. Card15/Nod2: nucleotide oligomerization domain 2 (A), Myd88: myeloid differentiation primary response gene 88 (B), Tlr2: toll-like receptor 2 (C), Tlr4: toll-like receptor 4 (D) mRNAs were normalized to GAPDH mRNA and quantitated densitometrically and expressed as mean  $\pm$  SEM (n=5–7 rats per group; \*, p<0.05, \*\*, p<0.01 vs. non-treatment control. †, p<0.05, ‡, p<0.01).

Card15/Nod2 signal detected in the epithelial cell layer, especially in the crypt region and in the monocytes or fibroblasts in the lamina propria (Fig. 5). In general, there are no or only a few Paneth cell-like cells in the colonic region of rodents, and therefore in this study, the majority of the positive cells in the basal epithelial cell layer were most likely goblet cells (Fig. 5D). Previous studies using samples of colon from patients with Crohn's disease have shown that there was an increase in Card15/Nod2 in both macrophages of the lamina propria and the intestinal epithelial cells, either as goblet cells [2] or metaplastic Paneth cells [22]. In the present study, Tlr2 and 4, Myd88 and Card15/Nod2 were upregulated at the mRNA level in the mucosal region of the TNBS-treated colon. These findings suggest that, in addition to the TLRs system, Card15/Nod2, which is a cytosolic protein involved in intracellular recognition of microbes, might play an important role as a critical regulator of bacterial immunity within the mucosal region.

It has been reported that there is a population of macrophages, which are distributed in the muscle layer in mammalian intestine, that are distinct from the mucosa macrophages [17, 18]. It has also been reported that LPS activates these muscularis macrophages, which were observed to secondarily suppress circular muscle contraction *in vivo* [4] or *in vitro* [24, 27]. Hori *et al.* have shown that through the excess production of prostaglandins and nitric oxide, the muscularis resident macrophages play a critical role in the pathogenesis of gastrointestinal dysfunction during inflammation [8]. In the endothelin B receptor null rat, which congenitally induces severe obstruction [26], and also in the experimental obstruction model rat [28], the

number of muscularis macrophages was increased, and mRNA of Tlr4 and proinflammatory cytokines were upregulated in the muscle layer, resulting in induction of gastrointestinal dysmotility. In the present study, we observed mRNA upregulation of Tlr2, 4 and Myd88 in addition to Card15/Nod2 in the muscle layer. These results suggest that both the TLR and Card15/Nod2 systems may play some pathophysiological roles in motor dysfunction during the development of muscularis inflammation.

In summary, this study demonstrates for the first time that the expression of Card15/Nod2 transcripts increased in the experimental gut inflammation model. Card15/Nod2 mRNA was expressed not only in the basal epithelial cell layer of the crypt region but also in the myenteric plexus region of the smooth muscle layer, which indicates the possible importance of this molecule in the gut dysmotility that is frequently observed in IBD patients.

**ACKNOWLEDGEMENT.** This work was supported by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), and a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture and Science.

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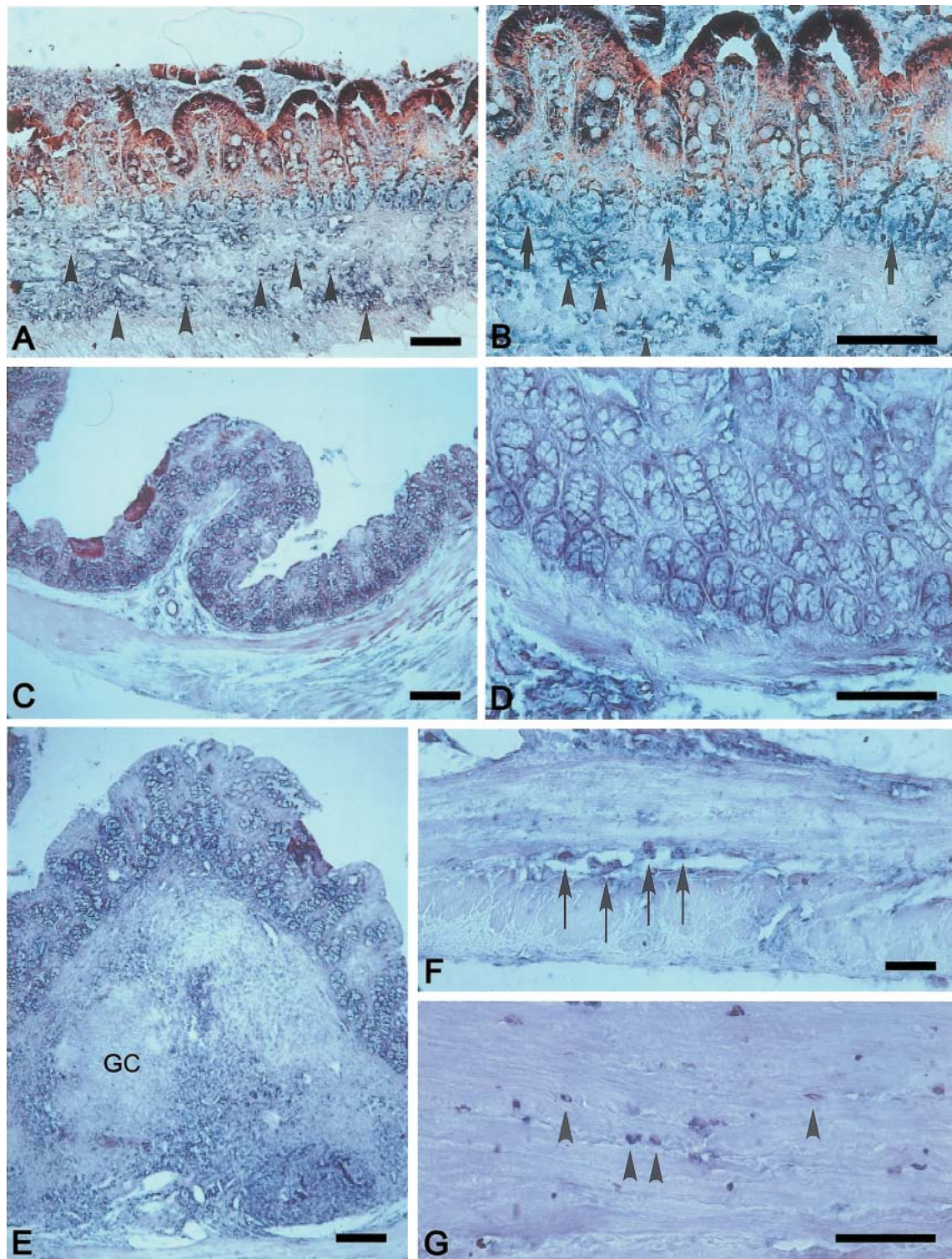


Fig. 5. *In situ* hybridization analysis of Card15/Nod2 expression in TNBS-treated rat colon. Card15/Nod2 transcripts were hybridized with a 687 bp DIG-labeled cRNA probe. The strong signals of Card15/Nod2 mRNA were present in the submucosal layer which contained mononuclear cells in the form of macrophages (indicated with arrowheads) and in the crypt region of the epithelial cells (arrows) at 2 days after TNBS treatment (A, B, blue signal). A slight signal was also observed in the myenteric plexus region containing resident macrophages (F, arrows) and in the resident macrophages in the circular muscle layer (G, arrowheads) at 2 days after TNBS treatment. At 7 days after TNBS treatment, a strong signal was also detected in the crypt region and submucosal part (C, D). The region of colonic follicles (patches) is indicated in E. A slight signal was detected around the germinal center (GC). Scale bar: 250  $\mu$ m.

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