Epigenetic Regulation of TLR4 Gene Expression in Intestinal Epithelial Cells for the Maintenance of Intestinal Homeostasis

Kyoko Takahashi, Yutaka Sugi, Akira Hosono and Shuichi Kaminogawa

*J Immunol* published online 21 October 2009
http://www.jimmunol.org/content/early/2009/10/21/jimmunol.0901271
Epigenetic Regulation of TLR4 Gene Expression in Intestinal Epithelial Cells for the Maintenance of Intestinal Homeostasis

Kyoko Takahashi, Yutaka Sugi, Akira Hosono, and Shuichi Kaminogawa

Intestinal epithelial cells (IECs) are continuously exposed to large numbers of commensal bacteria but are relatively insensitive to them, thereby averting an excessive inflammatory reaction. In this study, we show that the low responsiveness of human IEC lines to LPS was mainly brought about by a down-regulation of TLR4 gene transcription. Additionally, the presence of an IEC-specific repressor element in the 5′ region of the TLR4 gene and binding of a NF to the element was shown. The transcription factor ZNF160, which was expressed more abundantly in a LPS-low responder IEC line than in a LPS-high responder IEC line, repressed TLR4 gene transcription. ZNF160 is known to interact with the scaffold protein KAP1 via its N terminus to recruit histone deacetylase. Histone deacetylation, as well as DNA methylation, at the 5′ region of the TLR4 gene was significantly higher in LPS-low responder IEC lines than in a monocyte line or a LPS-high responder IEC line. It was demonstrated that TLR4 gene transcription was repressed by these epigenetic regulations, which were, at least in part, dependent on ZNF160. Down-regulation of TLR4 gene expression by these mechanisms in IECs possibly contributes to the maintenance of homeostasis in the intestinal commensal system. The Journal of Immunology, 2009, 183: 0000 – 0000.

The intestinal immune system, which is the largest immune system in the body, plays an essential role in the maintenance of health. In addition to food, pathogenic bacteria and viruses enter the intestinal tract through the oral cavity. On the other hand, an enormous number of commensal bacteria inhabit the intestinal tract. The intestinal immune system accurately recognizes these different organisms, discriminates between safe/beneficial and dangerous components, and attacks only those that are hazardous to the host.

Lately, the crucial role of the symbiosis between the commensal bacteria and the intestinal immune system in maintaining good health has attracted considerable attention. For instance, the development of GALTs such as Peyer’s patches and the induction of oral tolerance are known to be impaired or delayed in germ-free animals (1–5). Moreover, a correlation between the incidence of atopic eczema in children and the composition of their intestinal microbiota has been reported (6–11). The following phenomena have been shown to receive stimulation from intestinal commensal bacteria through TLRs, the pattern recognition receptors for microbial components, to maintain their homeostasis (17). However, to prevent triggering excessive inflammatory reactions, they do not respond in a sensitive manner to the commensals. Specifically, IECs contribute to the maintenance of intestinal homeostasis by partially tolerating the commensals and regulating mucosal inflammation. Decreased expression of specific TLRs and accessory molecules such as MD2 in IECs has been reported as one of the mechanisms controlling the hyporesponsiveness (18, 19). Additionally, the expression of Toll-interacting protein, a negative regulator of intracellular signals from TLRs, is known to be increased in IECs (18). In contrast, excessive responses to the commensals and increased expression of specific TLRs, including TLR4, are often observed in patients with inflammatory bowel disease (20). Therefore, expression of these molecules is thought to be regulated by cell type-specific mechanisms in IECs to maintain the intestinal symbiosis. Here we have analyzed the regulatory mechanisms of TLR4 gene expression in IECs to elucidate one of the mechanisms for maintaining the intestinal homeostasis.

1 This work was supported in part by a Grant-in Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K.T.) and a Grant-in Aid for Scientific Research from Japan Society for the Promotion of Science (to S.K.).

2 Address correspondence and reprint requests to Dr. Kyoko Takahashi, Food and Physiological Functions Laboratory, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa-shi, Kanagawa 252-8510, Japan. E-mail address: ktaka@brs.nihon-u.ac.jp

3 Abbreviations used in this paper: IEC, intestinal epithelial cell; DN, dominant negative; qPCR, quantitative PCR; ChIP, chromatin immunoprecipitation; 5-aza-dC, 5-aza-2′-deoxycytidine; TSA, trichostatin A; siRNA, small interfering RNA; KRAB, Kruppel-associated box; KAP, KRAB-associated protein; ICSBP, interferon consensus sequence-binding protein.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00
REGULATION OF TLR4 GENE EXPRESSION IN IECs

Materials and Methods

Cell culture

The human epithelial colon adenocarcinoma cell lines Caco-2 and HT-29 and the human epithelial colon carcinoma cell lines HCT 116 and T84, as well as the human macrocyte line THP-1, were purchased from DS Pharma Biomedical. The human epithelial colon adenocarcinoma cell line SW480 was provided by the Cell Resource Center for Biomedical Research, Development, Aging, and Cancer Center at Tohoku University (Miyagi, Japan). Caco-2 cells were cultured in Eagle’s MEM (Nissui) supplemented with nonessential amino acids (Invitrogen). HCT 116 and HT-29 cells were cultured in McCoy’s 5a medium (MP Biomedicals). T84, SW480, and THP-1 cells were cultured in a 1/1 mixture of Ham’s F12 (Nissui) and DMEM (Nissui), a 1/1 mixture of L15 (MP Biomedicals) and DMEM, and RPMI 1640 (Nissui), respectively. All media were supplemented with 10% (v/v) FBS (Biowest), 100 U/ml of penicillin and 100 U/ml of streptomycin (Meiji), and 5 x 10−3 M 2-ME. Cells were cultured at 37°C in a humidified incubator with 5% CO2.

Measurement of IL-8 production

Cells were stimulated for 18 h with 0.1–1000 ng/ml ultra-pure Escherichia coli K12 LPS (InvivoGen), which is guaranteed to only activate the TLR4 pathway. Concentrations of secreted IL-8 in the culture supernatants were measured with a human IL-8 ELISA kit (BioSource International) or Quantikine human CXCL/LR-8 (R&D Systems) according to the manufacturer’s instructions.

Measurement of NF-kB activation by a reporter gene assay

Cells were transfected with 1.5 µg of NF-kB reporter plasmid carrying NF-kB binding sites upstream of the luciferase gene using FuGene HD transfection reagent (Roche). Alternatively, cells were cotransfected with 1.5 µg of NF-kB reporter plasmid and 1.5 µg of an expression plasmid encoding the dominant-negative (DN) form of MyD88. The plasmid phRL-TK (1.25 ng/well; Promega) carrying the Renilla luciferase gene under the control of the CMV promoter was introduced to normalize the transfection and cell lysis efficiencies in every experiment. Four hours after transfection, ultra-pure E. coli K12 LPS (InvivoGen) was added to the cells. After culturing the cells for an additional 20 h, the cells were harvested. Cell lysis and determination of luciferase activity were conducted using a dual-luciferase assay kit (Promega) according to the manufacturer’s instructions. Luminescence was measured with a Tristar LB941 luminometer (Berthold).

Quantitative RT-PCR (qRT-PCR)

Total RNA was prepared from each cell line using an RNeasy Mini Kit (Qiagen) or a High Pure RNA isolation kit (Roche), and the first-strand cDNA synthesis was then conducted using SuperScript II reverse transcriptase (Invitrogen). Messenger RNA expression was quantified by real-time PCR with a LightCycler 480 (Roche) using SYBR Green I Master reagent (Roche). The sequences of the synthetic oligonucleotides used as primers are shown below for detection of TLR4 gene expression, forward, 5’-AAGC GAAAGGTGATTGTTG-3’; reverse, 5’-ACACCACTTTACACAACTGG-3’. qPCR was performed with the ABI Prism 7300 system (Applied Biosystems). The reaction mixture was subjected to an initial step at 50°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Relative quantification was performed using the comparative method (2ΔΔCt).

Plasmid construction

A DNA fragment corresponding to the nt −1013/+188 region of the human TLR4 gene (nucleotide numbers are counted from the major transcription start site as +1) was amplified by PCR from a human genomic DNA library (Clontech). The following synthetic oligonucleotides were used as primers: forward, 5’-ACATCGAGTTATGACCTTCTGAA GCTGCTT-3’; reverse, 5’-CTAGAGCGGTCCCTTCTCC AC-3’; for detection of GAPDH gene expression as a control, forward, 5’-TTCAGTATATTCCATTCTGAAA-3’; reverse, 5’-TTCACACCCCTGTTGC TGTA-3’. The amplified product was cloned into the pcDNA3.1 vector (Invitrogen) to confirm the nucleotide sequence and then inserted into the pCR2.1 vector (Invitrogen). The double-stranded DNA was inserted into pBAsi-hU6 Pur vector (Takara Bio) at the BamHI site. After confirming the sequence, the resulting plasmid was named pBAsi-TLR4. The control plasmid, pBAsi-cont, carrying the insert with the same A, T, C, and G composition as seen in pBAsi-TLR4 was similarly constructed using the following synthetic oligonucleotides: top strand, 5’-GGAGCACTATATCCACACCTT CTGACACTCTGGTTGATATGGTTGAGCTGTTTAC-3’; bottom strand, 5’-AGTTTAAAAGCTCAGTTTCTGCTGATGACACTCTG AGTGGTCGTTCACTCCCTGACACTCTGGTTGATATGGTTGAGCTGTTTAC-3’. The amplified product was cloned into pcDNA3.1 vector (Invitrogen) in the correct and reverse directions to yield pcDNA3.1-ZNF160 sense and pcDNA3.1-ZNF160 antisense, respectively. The control empty vector pcDNA3.1-empty was generated by self-ligation of BstXI-digested pcDNA3.1.

Knockdown of TLR4 expression

Cells were transfected with pBAsi-TLR4 or pBAsi-cont, both of which carry a puromycin resistance gene as a selectable marker, using FuGene HD transfection reagent (Roche) and cultured for 24 h. After adding 5 µg/ml puromycin (Sigma-Aldrich) to select the transfected cells, cells were cultured for an additional 48 h. Cells were then employed for measurement of IL-8 production upon LPS stimulation as described above.

FACS

After treatment with Fc block (anti-mouse CD16/CD32; BD Biosciences), cells were incubated with anti-human TLR4 mAb (clone HTA125; eBioscience) or mouse IgG2a isotype control (eBioscience) in FACS buffer (DMEM (pH 7.2) containing 0.5% BSA, 1 mMEDTA, 10 mM HEPES, 2 mM sodium pyruvate, and 0.05% sodium azide) on ice for 30 min. After washing, the cells were incubated with biotinylated anti-mouse IgG (H+L) (eBioscience) on ice for 30 min followed by incubation with streptavidin-labeled PE (Invitrogen) and then analyzed on a FACS Canto (BD Biosciences).

Measurement of transcriptional promoter activity by a reporter gene assay

The reporter plasmid pGL-TLR4−1013/+118 (0.7 µg/well) was introduced into cells using FuGene HD transfection reagent (Roche). For overexpression experiments, cells were cotransfected with 0.5 µg of the reporter plasmid pGL-TLR4−1013/+118 and 0.5 µg of pcDNA3.1-ZNF160 sense, pcDNA3.1-ZNF160 antisense, or pcDNA3.1-empty. The plasmid phRL-CMV (1.25 ng/well) was introduced to normalize the transfection and cell lysis efficiencies in every experiment. After 20–24 h of culture, cells were harvested and washed with PBS. Cell lysis and determination of luciferase activity were performed as described above.

Immunoprecipitation

Cells were washed with ice-cold PBS and incubated on ice for 30 min in lysis buffer (20 mM Tris (pH 7.6), 1% Nonidet P-40, 60 mM octyl β-D-glucoside, 2 mM PMFS, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin). Cell lysates were immunoprecipitated with anti-TLR4 mAb (clone 2R2) or mouse IgG (negative control) and protein A-Sepharose (GE Healthcare). Immunoprecipitated proteins were detected by immunoblotting with anti-TLR4 or anti-TLR2 mAb. Part of each immunoprecipitated cell lysate was subjected to immunoblotting with
expressed as means ± SD of two independent experiments. 

B. Activation of NF-kB by stimulation with LPS was measured by reporter assays. Each cell line was cotransfected with a NF-kB reporter plasmid and an expression plasmid carrying MyD88 DN or an empty vector control and stimulated with the indicated concentrations of LPS. The fold increase in luciferase activity relative to that of unstimulated cells transfected with the empty vector control is shown. Results are represented as means ± SD of three independent experiments. 

C. TLR4 mRNA expression in each cell line was determined by qRT-PCR. Relative values normalized using GAPDH mRNA levels are given. Results are expressed as means ± SD of three independent experiments. 

D. A TLR4 siRNA expression plasmid or a control siRNA expression plasmid was introduced into SW480 cells. After selection of the transfected cells with puromycin, TLR4 mRNA expression was determined by qRT-PCR. Relative values normalized using GAPDH mRNA levels are given. Results are expressed as means ± SD of four independent experiments: - - -, no transfection; TLR4, transfected with the TLR4 siRNA expression plasmid; control, transfected with the control siRNA expression plasmid; *, p < 0.001. E. SW480 cells were transfected with the TLR4 siRNA expression plasmid (filled bars) or the control siRNA expression plasmid (open bars). After selection with puromycin, the transfected cells were stimulated with 0.1–1000 ng/ml LPS for 18 h to measure IL-8 secretion into the culture supernatant by ELISA. IL-8 production was calculated by subtracting the concentrations in the culture supernatant of unstimulated cells from those of stimulated cells. Results are represented as means ± SD of two independent experiments. 


tivity less than half of that of the empty vector generated by self-ligation of reporter plasmid pGL3-TLR4, along with either MyD88 DN or an empty vector control and stimulated with the indicated concentrations of LPS. Expression plasmids, contained in mixtures that gave luciferase activity above the background, were subjected to ChIP assays employing anti-acetyl histone H3 Ab. The 5′ region of the TLR4 gene corresponding to nt 5290–539 was amplified by PCR from the recovered DNA using the synthetic oligonucleotides 5′-ACATATCGAAGTCCTAACCCCTCTAC-3′ and 5′-GGAGGCTTTCAACTGACACCTTGACTGACTC-3′ as primers. The GAPDH promoter region was amplified as a control using primers with the sequences 5′-TACTAGCGGTTTTACGCG-3′ and 5′-TGAAACGAGAGAAGAAGCGA-3′. For the ZNF160 overexpression experiment, cells were transfected with pcDNA3.1-ZNF160 sense or pcDNA3.1-empty using FuGene HD transfection reagent. After 8 h, G418 (Sigma-Aldrich) was added to select for transfected cells. Cells were cultured for an additional 40 h, harvested, and subjected to ChIP assays employing anti-acetyl histone H3 Ab. The 5′ region of the TLR4 gene and that of the GAPDH gene in the immunoprecipitated DNA were quantified by real-time PCR analyses using the same primers described above.

Nuclear extract preparation

Nuclear extract was prepared from Caco-2 and THP-1 cells as described previously (21).

EMSA

Double-stranded DNAs were prepared as probe A, B, and C by annealing Alexa 647-labeled synthetic oligonucleotides. Nucleotide sequences of the probes were as follows: probe A, 5′-GACTGTATATGGAGAGAGAGCC TTG-3′; probe B, 5′-CCCTGAAGAGGTATGAAGTGA-3′; probe C, 5′-GTAGAAAGGTTCATTATGGTG-3′.

Fifteen micrograms of nuclear extract and 5 pmol of DNA probe were incubated at room temperature in 10 mM HEPES buffer (pH 7.9) containing 400 ng of poly(dI:dC), 1 mM MgCl2, 30 mM KCl, 1 mM DTT, and 5% glycerol for 20 min. The mixtures were separated by electrophoresis on 4% polyacrylamide gels at 120 V for 2.0–2.5 h in 25 mM Tris, 22.5 mM boric acid, 0.5 mM DTT. Fluorescence was detected using a Typhoon 9410 (GE Healthcare).

cDNA subtraction

cDNAs of the genes that were expressed more abundantly in Caco-2 cells than in SW480 cells were enriched with a PCR-Select cDNA subtraction kit (Clontech) according to the manufacturer’s instructions. Obtained cDNA candidates were inserted into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) to yield their expression plasmids. The expression plasmids were introduced into SW480 cells as groups of five clones together with the reporter plasmid pGL3-TLR4−1015/+188 for a transient expression as–

expression TOPO vector. The mixtures were separated by electrophoresis on 4% polyacrylamide gels at 120 V for 2.0–2.5 h in 0.25× TBE buffer (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM DTT). Fluorescence was detected using a Typhoon 9410 (GE Healthcare).

Bisulfite conversion reaction

Genomic DNA was prepared from cells using a PureLink Genomic DNA Mini kit (Invitrogen) according to the manufacturer’s instructions. To analyze methylation of CpG motifs, 400 ng of genomic DNA was denatured

anti-β-actin mAb (Abcam) to quantify the amounts of proteins present in each lysate before immunoprecipitation.
at 98°C for 10 min, modified by conversion reagent at 64°C for 2.5 h, and then purified using a MethylCode bisulfite conversion kit (Invitrogen). The 5′ region of the TLR4 gene was amplified by PCR from the modified genomic DNA. The sequences of the synthetic oligonucleotides used as PCR primers are as follows: forward, 5′-GGTAGAGGTAGATGATTAA TTGGG-3′; reverse, 5′-CCCCAATAACTACCTGTAACCTCCT-3′.

Following purification of PCR products, they were cloned into the pCR2.1 vector for sequencing. Nucleotide sequences of 14–16 clones for each cell line were analyzed.

**Results**

**Responsiveness of human IEC lines to LPS is down-regulated mainly by repressing TLR4 gene transcription**

Various human IEC lines were first analyzed for their ability to respond to stimulation through TLR4. Five human IEC lines (Caco-2, HT-116, T84, HT-29, SW480) and a control monocyte line (THP-1) were stimulated with LPS to measure IL-8 secretion into the culture supernatant (Fig. 1A). Most IEC lines, with the exception of SW480, showed low responsiveness to LPS compared with the monocyte line THP-1. Caco-2 and HT-116 cells barely responded, and HT-29 and T84 cells weakly responded to LPS stimulation. In contrast, production of IL-8 from SW480 cells was similar to, or even higher than, IL-8 production from the monocyte line THP-1. The MyD88 dependency of these responses was further examined by NF-κB reporter assays (Fig. 1B). Consistent with the results for IL-8 production, activation of NF-κB was observed in SW480 and THP-1 cells when stimulated with LPS, while it was hardly detected in Caco-2 cells. Activation of NF-κB was inhibited by exogenous expression of the DN form of MyD88 in SW480 and THP-1 cells, indicating that the responses to LPS were dependent on MyD88. The TLR4 mRNA expression in each cell line was determined by qRT-PCR (Fig. 1C). The amounts of TLR4 mRNA almost exactly correlated with the LPS responsiveness of each cell line. Furthermore, TLR4 mRNA expression was reduced to about one-third in SW480 cells transfected with a TLR4 siRNA expression plasmid when compared with the cells transfected with a control siRNA expression plasmid. These results show that LPS sensitivity is regulated by TLR4 gene expression in IECs. Next, the cell surface expression of TLR4 and the transcriptional enhancing activity of the 5′ region of the TLR4 gene were compared between Caco-2 and THP-1 cells. The shifted band appeared when the nuclear extract stained with isotype control Ab; bold lines, stained with anti-TLR4 Ab. Results are representative of three independent experiments. Shadowed areas, unstained; thin lines, stained with isotype control Ab; bold lines, stained with anti-TLR4 Ab. B, Transcriptional enhancing activity of the 5′ region of the human TLR4 gene (nt -1013/+188) in each cell line was measured by a reporter gene assay. Luciferase activities relative to that from a SV40 promoter control are shown. Results are represented as means ± SD of three independent experiments. C, Protein expression of TLR4 and TLR2 in each cell line was determined by immunoprecipitation. Amounts of protein included in the cell lysates before immunoprecipitation were determined by immunoblotting with anti-β-actin Ab. Data are representative of two independent experiments.

**Inhibition of histone deacetylase and DNA methyltransferase**

Caco-2 cells were treated with 10 μM 5-aza-2′-deoxycytidine (5-Aza-dC; Calbiochem) for 4 days, with 80 nM trichostatin A (TSA; Cayman Chemical) for 24 h, or with TSA and 5-Aza-dC for 24 h after treatment with 5-aza-dC alone for 3 days. Cells were then harvested to obtain total RNA. TLR4 mRNA expression was quantified by qRT-PCR as described above.

**FIGURE 2.** LPS responsiveness of IEC lines is mainly regulated at the transcriptional level. A, Cell surface expression of TLR4 on each IEC line was determined by FACS using anti-TLR4 Ab. Results are representative of three independent experiments. Shadowed areas, unstained; thin lines, stained with isotype control Ab; bold lines, stained with anti-TLR4 Ab. B, Transcriptional enhancing activity of the 5′ region of the human TLR4 gene (nt -1013/+188) in each cell line was measured by a reporter gene assay. Luciferase activities relative to that from a SV40 promoter control are shown. Results are represented as means ± SD of three independent experiments. C, Protein expression of TLR4 and TLR2 in each cell line was determined by immunoprecipitation. Amounts of protein included in the cell lysates before immunoprecipitation were determined by immunoblotting with anti-β-actin Ab. Data are representative of two independent experiments.

**Nucleotide –489/–428 region of the TLR4 gene contains an IEC-specific repressor element and is recognized by an IEC-specific NF**

To further examine the mechanisms of transcriptional repression of the TLR4 gene in IECs, cis-acting elements in the 5′ region of the TLR4 gene were compared between Caco-2 and THP-1 cells. For this purpose, a series of deletion constructs were employed for luciferase assays. Deletion of the nt –675/–428 region increased the luciferase activity in Caco-2 cells, while it hardly affected the activity in THP-1 cells, suggesting that this region contains an IEC-specific repressor element (Fig. 3A). Moreover, this region was suggested to contain a responsive element that is repressed by some microbe, because the transcriptional enhancing activity of nt –675/+188 but not of nt –427/+188 was decreased by the synthetic TLR2 ligand, Pam3CSK4 (Fig. 3B). Further mapping of the repressor element in the nt –675/–428 region by luciferase assays using deletion constructs in Caco-2 cells revealed that the nt –489/–428 region acts as a repressor element (Fig. 3C). We next examined the presence of a NF binding to this region by EMSA using three oligonucleotide probes covering this region and nuclear extracts prepared from Caco-2 and THP-1 cells. The shifted band indicated by an arrow in Fig. 3D appeared when the nuclear extract...
from Caco-2 cells but not from THP-1 cells was added, indicating that a NF that was specifically expressed in Caco-2 cells bound to a NT that a NF that was specifically expressed in Caco-2 cells bound to that of a NT that a NF that was specifically expressed in Caco-2 cells bound to THP-1 cells but not from THP-1 cells was added, indicating that ZNF160 repressed the transcriptional enhancing activity of the TLR4 gene in IECs, indicating that ZNF160 acts through the nt region of the TLR4 gene and nuclear extracts prepared from Caco-2 and THP-1 cells. Results are representative of three independent experiments.

**TLR4 gene transcription is down-regulated by epigenetic modification including histone deacetylation and DNA methylation in IECs**

KRAB domains have been reported to recruit KRAB-associated protein (KAP) 1 when tethered to DNA via their zinc finger motifs (24, 25). KAP1 directly binds to KRAB and functions as a scaffold for the formation of a multimolecular complex comprising histone deacetylases, which induces transcriptional repression through the formation of heterochromatin (26–28). In addition to histone deacetylation, it was recently reported that the KRAB domain can trigger de novo DNA methylation (29). Histone acetylation and DNA methylation are known to mediate epigenetic regulation of gene expression. To investigate if TLR4 gene expression is controlled by these epigenetic mechanisms in IECs, acetylation of histones interacting with the 5′ region of the TLR4 gene was analyzed by ChiP assays using anti-acetyl histone H3 Ab. As shown in Fig. 5, acetylated histones interacting with the 5′ region of the TLR4 gene were significantly lower in Caco-2 and HCT 116 cells, both of which barely express TLR4, than in SW480 and THP-1 cells, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were significantly lower in Caco-2 and HCT 116 cells, both of which barely express TLR4, than in SW480 and THP-1 cells, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs, indicating that the histones interacting with the 5′ region of the TLR4 gene were highly deacetylated in LPS-low responder IECs.
that DNA methylation and histone deacetylation are cooperatively involved in the repression of TLR4 gene transcription (Fig. 7A). Collectively, these results show that TLR4 gene transcription is down-regulated by epigenetic modification including histone deacetylation and DNA methylation in LPS-low responder IEC lines.

The epigenetic modification is, at least in part, dependent on ZNF160

To analyze the involvement of ZNF160 in epigenetic regulation, ChIP assays of the endogenous TLR4 gene were performed employing SW480 cells transfected with a ZNF160 expression plasmid or an empty vector control. Acetylation of histones that interacted with the 5′ region of the TLR4 gene was significantly reduced by the overexpression of ZNF160, while acetylation of histones that interacted with the 5′ region of the GAPDH gene was not affected (Fig. 7, B and C). The results indicate that epigenetic

Furthermore, the effects of the DNA methyltransferase inhibitor 5-aza-dC and/or the histone deacetylase inhibitor TSA were analyzed in Caco-2 cells. The expression of TLR4 increased ~10-fold as a result of treatment with both 5-aza-dC and TSA, indicating

that DNA methylation and histone deacetylation are cooperatively involved in the repression of TLR4 gene transcription (Fig. 7A). Collectively, these results show that TLR4 gene transcription is down-regulated by epigenetic modification including histone deacetylation and DNA methylation in LPS-low responder IEC lines.

The epigenetic modification is, at least in part, dependent on ZNF160

To analyze the involvement of ZNF160 in epigenetic regulation, ChIP assays of the endogenous TLR4 gene were performed employing SW480 cells transfected with a ZNF160 expression plasmid or an empty vector control. Acetylation of histones that interacted with the 5′ region of the TLR4 gene was significantly reduced by the overexpression of ZNF160, while acetylation of histones that interacted with the 5′ region of the GAPDH gene was not affected (Fig. 7, B and C). The results indicate that epigenetic
modification at the 5’ region of the TLR4 gene is, at least in part, dependent on ZNF160.

Discussion
In this report, we show that TLR4 gene transcription is epigenetically suppressed in IECs to prevent excessive inflammatory responses. This means that epigenetic regulation of TLR4 gene expression in IECs can act as one mechanism for maintaining intestinal homeostasis by suppressing excessive responses to the commensals and regulating mucosal inflammation in the gut. Epigenetic information is encoded by differential methylation of DNA on cytosines and by proteins associating with DNA such as histones, which may be modified covalently by acetylation, methylation, phosphorylation, and/or ubiquitination. Since this epigenetic information is heritable beyond cell division, the importance of epigenetic regulation is established especially in the fields of developmental and cancer biology. This is the first report to describe the involvement of epigenetic regulation of transcription in the maintenance of the intestinal commensal system. Although the intestinal epithelium is known to be continuously renewed by rapid turnover of IECs, there are many reports supporting that the characteristics of IECs including their tolerated responses to the commensals are inherited by renewed cells, raising the possibility that epigenetic regulation as seen in our study is involved in such inheritance. These basic mechanisms may work commonly in other commensal systems in specific tissues in our body such as the skin epidermis or the mucosa of the oral cavity, in addition to the intestine, all of which are continuously exposed to nonpathogenic microbes. Zampetaki et al. recently reported that murine TLR4 gene expression is epigenetically repressed in embryonic stem cells but not in embryonic stem cell-derived differentiated smooth muscle cells (30). Epigenetic repression may be released in differentiated cells except specific types of cells including IECs.

Rehli et al. reported that transcription factors of PU.1 and IFN consensus sequence-binding protein (ICSBP) regulate human TLR4 gene expression in myeloid cells through elements just upstream of the transcription start site (31). As shown in Fig. 3A, additional enhancer elements seem to be present further upstream of the PU.1 and ICSBP binding sites identified by Rehli et al. Moreover, the presence of an IEC-specific repressor element in the 5’ region of TLR4 gene and an IEC-specific NF binding to the element has been suggested. We tried to identify the transcription factor forming the Caco-2-specific band shown in Fig. 3D but were unsuccessful. Since it was found that ZNF160 acts through the nt −675/−428 region (Fig. 4, D and E), it was thought that ZNF160 binds to this region. However, in vitro translation products of ZNF160 did not bind to the double-stranded DNA probe containing this region in EMSA (data not shown). Some modification of the ZNF-160 protein may be required for DNA binding, or, alternatively, ZNF-160 may indirectly bind to this region through another DNA binding factor. Stimulation with Pam3CSK4 repressed the transcriptional enhancing activity of the 5’ region of the TLR4 gene through the nt −675/−428 region (Fig. 3B). It is unclear at present whether specific microbial components modify transcription of the TLR4 gene, although LPS may not affect transcriptional activation because expression of TLR4 recognizing LPS was undetectable on Caco-2 cells (Fig. 2A).

The epigenetic regulation of TLR4 gene expression has been found to be partly dependent on ZNF160, a repressive transcription factor possessing the KRAB domain, which has been reported to recruit histone deacetylase through the scaffold protein KAP1. KAP1 is characterized by the presence of a RING finger, B boxes, a coiled-coil region, a PHD finger, and a bromodomain; the first three of these motifs are both necessary and sufficient for homooligomerization and direct binding to KRAB (24, 25). A reduction in acetylation of histones that interact with the 5’ region of the TLR4 gene in IECs is thought to be dependent on histone deacetylase, which is recruited by ZNF160 through KAP1. Similarly, an increase in methylation of the 5’ region of the TLR4 gene in IECs is thought to be dependent on ZNF160. However, the contribution of ZNF160 to the regulation of TLR4 gene expression seems to be rather small compared with the large contribution of epigenetic modifications to the repression of TLR4 gene expression. Actually, overexpression of ZNF160 only suppressed transcriptional promoter activity of the TLR4 gene to about a half in SW480 cells (Fig. 4B), while inhibitors of histone deacetylase and DNA methyltransferase together increased TLR4 gene expression by ∼10-fold in Caco-2 cells (Fig. 7A). Additionally, overexpression of ZNF160 in the monocyte line increased transcriptional promoter activity, while overexpression in the IEC line suppressed transcription (Fig. 4, B and C). These results suggest that ZNF160 and an additional cell type-specific regulatory factor cooperatively repress transcription of the TLR4 gene in IECs. Recently, a single nucleotide polymorphism with strong association to ileal Crohn’s disease was mapped to an intergenic region that is flanked on the centromeric side by a gene encoding another zinc finger protein, ZNF365 (32). Although the functions of ZNF365 are little known, specific members of the C2H2 zinc finger proteins, which constitute the largest class of transcription factors in humans, may play a role in the maintenance of intestinal homeostasis.

LPS responsiveness of IECs depends on TLR4 expression levels as shown in Fig. 1E. Additionally, expression of MD-2 is thought to be another important factor that determines the LPS responsiveness because optimal LPS recognition by TLR4 requires MD-2 as a coreceptor. It has been reported that MD-2 expression in healthy, normal intestinal mucosa is minimal, while it is increased in active inflammatory bowel disease colitis (33, 34). Moreover, inhibition of CpG methylation and histone deacetylation was shown to result in increased mRNA expression of MD-2 gene in IECs just recently (35). TLR4 and MD-2 expression may be down-regulated in part by common or related mechanisms in IECs. On the other hand, expression of TLR4 and TLR2 is thought to be regulated at different stages. TLR4 expression has been found to be regulated mainly at the transcriptional level because its cell surface expression correlates relatively well with mRNA expression levels in each cell line. However, regulation at the posttranslational level is also thought to be present, as a difference in the cell surface TLR4 expression was seen between SW480 and THP1 cells despite these cells having almost the same mRNA expression levels (Figs. 1 and 2). Since IL-8 production in response to LPS stimulation was rather higher in SW480 cells than in THP-1 cells, a certain extent of cell surface TLR4 expression seems to be sufficient to respond to LPS. On the other hand, expression of TLR2 seemed to be regulated mainly at the posttranslational level because its mRNA and intracellular protein expression was not apparently different between the IEC lines and the monocyte line, but its cell surface expression was much lower in the IEC lines than in the monocyte line (Fig. 2C and our unpublished data). Intracellular transport of TLR2 protein to the cell surface is thought to be inhibited by a specific mechanism in IECs.

Further study should allow elucidation of the entire regulatory mechanism underlying transcription of symbiosis-associated genes, including TLR4 in IECs and its contribution to regulation of mucosal inflammation triggered by IECs, and, as a result, to maintenance of the intestinal commensal system.

Disclosures
The authors have no financial conflicts of interest.

The Journal of Immunology
References


