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Expression and Function of TLR2, TLR4, and Nod2 in Primary Canine Colonic Epithelial Cells

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Abstract

The gut maintains a delicate balance between the downregulation of inflammatory reactions to commensal bacteria and the capacity to respond to pathogens with vigorous cellular and humoral immune responses. Intestinal epithelial cells, including colonic epithelial cells (CECs) possess many properties of cells of the innate immune system, in particular the ability to recognize and respond to microbial antigens. Recognition of microorganisms by CECs is based upon their recognition of signature molecules, called microbe-associated molecular patterns (MAMP), by pattern recognition receptors (PRR) including membrane Toll-like receptors (TLR) and cytosolic Nod2, an intracellular counterpart of TLRs.

The purpose of this study was to determine whether primary CECs from normal dogs express a functional TLR2, TLR4 and Nod2 and whether they are regulated by inflammatory mediators. We show that canine primary CECs express TLR2, TLR4, and Nod2 that can be modulated in response to their respective MAMPs, lipopolysaccharides (LPS) or peptidoglycans (PGN). Furthermore, we demonstrate that these receptors are functional as evidenced by the induction of cytokine gene expression in response to LPS or PGN.

Keywords

Dog; toll-like receptor; intestine; epithelial cells; innate immunity

INTRODUCTION

Diseases involving the gastrointestinal system are a common problem in veterinary clinical medicine. Intestinal inflammation is a consequence of many of these diseases, including infectious diseases and inflammatory bowel disease (IBD). The gut maintains a delicate balance between the downregulation of inflammatory reactions to the commensal bacteria and food antigens, and the capacity to respond to pathogens with vigorous cellular and humoral

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immune responses. The underlying mechanisms that maintain this balance are poorly understood. The barrier function of intestinal epithelial cells has historically been considered to be their most important contribution in preventing contact and subsequent activation of the mucosal immune system by the commensal bacteria and protein antigens.

Over the past several years it is becoming clear that human and murine intestinal epithelial cells possess many properties of cells of the innate immune system, especially their ability to recognize and respond to microbial antigens. The recognition of microorganisms by innate immune cells is based upon recognition of signature molecules on microorganisms called microbe (or pathogen)-associated molecular patterns (MAMPs) (Akira, 2003a, b; Inohara et al., 2002; Medzhitov, 2001). MAMPs are shared by large groups of microorganisms, including peptidoglycans (PGN) and lipoteichoic acid (LTA) found in most gram-positive bacteria, and lipopolysaccharides (LPS) of Gram-negative bacteria. Since different pathogen classes express distinct MAMPs, recognition of the motifs informs the host about the nature of the potentially threatening microbe. The response to microbial MAMPs is mediated by pattern recognition receptor (PRR) families of which two types have been identified to date (Akira, 2003a, b; Inohara et al., 2002; Medzhitov, 2001). The membrane Toll family of proteins, (i.e. Toll-like receptors; TLR) and the cytosolic “nucleotide binding site plus leucine rich repeats” (NBS-LRR) plant disease resistance-like protein family which in mammalian cells includes Nod1/CARD4 and Nod2/CARD15 that represent intracellular counterparts of TLRs. Although it was originally thought that these PRRs were restricted to cells of the myeloid lineage, intestinal epithelial cells, in particular colonic epithelial cells (CECs), have recently been shown to express TLR2, TLR4 and Nod2 (Cario et al., 2000; Gutierrez et al., 2002). Activation of CECs through TLRs or Nod 2 results in the production of similar pro-inflammatory cytokines, such as interleukin 8 (IL-8), as other cells of the innate immune system. In addition, stimulation of human CECs with bacterial antigens has been shown to up-regulate the expression of interleukin 7 (IL-7), a growth and survival cytokine (Yamada et al., 1997).

The purpose of this study was to determine whether primary CECs from normal dogs express functional TLR2, TLR4 and Nod2 that are regulated by inflammatory mediators.

MATERIALS AND METHODS

Cell lines

DH82 cells, a canine macrophage-monocyte cell line, were kindly supplied by Dr. Maxie Wellman, College of Veterinary Medicine, The Ohio State University (Wellman et al., 1988). The DH82 cells were cultured in Dulbecco's MEM (DMEM) supplemented with 2 mM L-glutamine and 10% FCS, and incubated at 37°C in a 5% CO₂ atmosphere. The cells were subcultured twice a week. DH82 cells were used to determine the optimum concentration of MAMPs for stimulation of canine cells through their respective receptors, and to determine the time period at which up-regulation of the gene of interest could be documented following stimulation their respective MAMPs. Confluent monolayers of DH82 cells were stimulated with varying concentrations, ranging from 1 ng/ml through 100 µg/ml, of LPS (Sigma) or PGN (Sigma) for 24 hours. Samples were harvested at 0, 2, 4, 8 and 24 hrs and analyzed for TLR2, TLR4 and Nod2 expression. The results of these pilot studies showed the optimal concentrations of LPS and PGN were 100 ng/ml and 1 µg/ml, respectively with the maximal expression observed 4 hours following stimulation.

CEC isolation and culture

The protocol for isolating and culturing canine primary CECs was adapted from our protocol for isolating murine primary CECs (Baumgart et al., 1998a; Telega et al., 2000). Segments of colon were collected from normal 6 to 8 week old puppies following euthanasia. The colon

segments were opened longitudinally to expose the mucosal surface and washed 4 times with calcium- and magnesium-free Hanks balanced salt solution (HBSS) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 µg/ml fungizone to remove mucus and debris. Five millimeter segments of tissue were then incubated at 37°C for 90 min in 10 ml of DMEM supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin containing 10 mg Dispase I (Boehringer Mannheim Biochemicals) in a 50 ml centrifuge tube with constant agitation (Baumgart et al., 1998b; Telega et al., 2000). The resultant epithelial sheets were dispersed into single cell suspensions by pipetting and the purity of the CECs analyzed by flow cytometry as described below. CEC isolations containing >1% contamination with CD45⁺ cells were not used in these studies. Primary canine CECs were dispensed into 6-well culture plates and cultured in DMEM supplemented with 10% FCS and used for these studies when they reached approximately 75% confluency. Duplicate cultures were evaluated for mRNA expression pre-stimulation and at 4 hours following stimulation with either LPS (100 ng/ml) or PGN (1 µg/ml).

Flow cytometry

The purity of the CECs was analyzed by flow cytometry. Isolated CECs were stained with either canine phycoerythrin (PE)-labeled anti-CD45, a pan leukocyte marker, or FITC-labeled anti-pan cytokeratin (Dako), an epithelial marker, monoclonal antibodies. PE- or FITC-labeled isotype controls were used to control for nonspecific staining. Following incubation with the respective antibodies, the cells were washed, and fixed in PBS containing 2% paraformaldehyde and analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

RNA isolation

Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. To prevent DNA contamination, on-column DNase digestion was performed with the RNase-Free DNase Set (Qiagen). After completion of the protocol, RNA was treated with Amplification Grade DNase I (LTI) and followed by the RNA cleanup protocol according to the manufacturer's protocol. RNA was stored at -80°C until analyzed.

Primer design

Primers for PCR amplification of β actin, TLR4, and IL-8 were designed from published canine specific sequences (β actin, GenBank accession number Z70044; TLR4, DDBJ accession number AB080363; IL-8, GenBank accession number Z70048). Primers for TLR2, Nod2 and IL-7 were retrieved from canine whole sequence from the TRACE archive (<http://www.ncbi.nlm.nih.gov/Traces/trace.cgi>) using default settings of the MEGABLAST algorithm. Human RefSeq mRNA sequences were used as queries. Orthology of the retrieved canine sequences was confirmed by reciprocal searches of the human genome sequence. Appropriate primers were designed using MacVector. The amplified products were gel purified and their identity confirmed by direct sequencing. The primers for IL-7 and IL-8 cross an intron.

RT-PCR

cDNA was synthesized by a reverse transcription PCR from 5µg of total RNA using 200 units of Superscript II Reverse Transcriptase (Invitrogen) and random hexamer primers. Sterile, distilled water, dNTP, 5X first Strand Buffer, 0.1M DTT and 40 U RNasin were added for a total reaction volume of 20µl using the protocol for Superscript II. The cDNA samples were amplified by PCR in total reaction volume of 50µl containing 0.25µl Platinum Taq polymerase (Invitrogen), 5µl 10X buffer, 1.5 µl magnesium chloride, 1µl assorted dNTP, and 0.5 µl of each primer (50mM). Conditions of the PCR were an initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, annealing temperature for 1 minute (Table 1), elongation at 72°

C for 1 minute, and followed by a final 7 minute elongation at 72°C. Products were visualized by electrophoresis through a 1.5% agarose gel followed by gel staining in ethidium bromide. Parallel reactions lacking reverse transcriptase were included as controls for genomic DNA contamination, and reactions lacking RNA served as a negative control for DNA contamination in reagents.

RESULTS

Characterization of canine primary CECs

The isolated canine CECs consisted of crypts, partial crypts, and individual CECs (Fig. 1A). Flow cytometric analysis of the isolated cells revealed that the majority of the cells (>95%) were positive for cytokeratin characteristic of epithelial cells (Fig. 1B) with less than 1% contaminating hematopoietic cells as determined by staining for CD45, a pan leukocyte marker (Fig. 1C).

Canine primary CECs express TLR2, TLR4 and Nod2

The expression of TLR2, TLR4 and Nod2 was examined in freshly isolated CECs and compared with DH82 cells, a canine macrophage cell line, as a positive control. Canine primary CECs, like murine CECs, constitutively express TLR2, TLR4, and Nod2 (Fig. 2). The constitutive expression in CECs appeared to be less than that observed in myeloid cells as has been reported for both primary murine CECs and human IEC cell lines.

Modulation of TLR2, TLR4 and Nod2 expression by their MAMPs

Although canine primary CECs constitutively express TLR2, TLR4 and Nod2 (Fig. 2), it was important to determine whether their expression was modulated following stimulation with their respective MAMPs. Fig. 3A shows that TLR4 is up-regulated in canine primary CECs following stimulation with LPS, and Fig. 3B illustrates that both TLR2 and Nod2 are up-regulated following stimulation with PGN.

Canine primary CECs express a functional TLR2, TLR4 and Nod2

To determine whether the stimulation of TLR2, TLR4 and/ or Nod2 on canine primary CECs results in a functional response, we evaluated the level of IL-8 and IL-7 expression following stimulation with either LPS or PGN. Activation of primary canine CECs results in the up-regulation of both IL-8 (Fig. 3C) and IL-7 (Fig. 3D).

DISCUSSION

This study demonstrates for the first time the expression of functionally active TLR2, TLR4 and Nod2 on primary canine intestinal epithelial cells, in this case CECs. We have shown that TLR2, TLR4 and Nod2 are expressed at low levels in “non-stimulated” canine primary CECs, however they are rapidly up-regulated in response to challenge with various infectious inflammatory stimuli. In addition, we have shown that stimulation of canine primary CECs with MAMPs specific for TLR2, TLR4 and Nod2 results in the up-regulation of the expression of the pro-inflammatory cytokine IL-8 that is one of the downstream consequences of TLR and Nod2 signaling. In the absence of antibodies specific for canine TLR2 and TLR4 it is difficult to say with certainty that TLR2 and TLR4 are expressed on the surface of canine CECs. However, the fact that TLR2 and TLR4 mRNA are up-regulated in response to their respective MAMPs with subsequent up-regulation of downstream events, expression of IL-8, is highly suggestive that both TLR2 and TLR4 are expressed on the cell surface. The up-regulation of TLR2, TLR4 and Nod2 by canine primary CECs in response to MAMPs is

consistent with these cells acting as the first line of defense in the canine intestinal tract and with their involvement in regulating CEC response to microbial antigens.

We have also shown that stimulating canine primary CECs with LPS results in the up-regulation of IL-7 expression. These findings are similar to those reported in the human colonic epithelial cell line, T84, following in vitro exposure to bacterial antigens in which the expression of IL-7 was up-regulated followed by the up-regulation of IL-7R α (Yamada et al., 1997). The importance of these findings is unclear. Although it is well documented that murine and human CECs secrete IL-7 (Madrigal-Estebas et al., 1997; Reinecker and Podolsky, 1995; Watanabe et al., 1995), the primary effect of the IL-7 secreted by CECs has been hypothesized to be on the surrounding intraepithelial lymphocytes. One role of IL-7 is maintaining peripheral lymphocyte homeostasis by acting as both growth and survival (anti-apoptotic) factors (Fry and Mackall, 2002). Alternatively, one could hypothesize that the production of IL-7 by CECs following antigenic challenge may act in an autocrine fashion to maintain the integrity of the intestinal epithelium following infection or inflammation either acting as a growth and/or survival factor for CECs. In support of this hypothesis is the finding that IL-7R $\alpha^{-/-}$ mice have been shown to have abnormal repair of intestinal epithelial cells following irradiation compared to wild-type mice resulting in death that was attributed to increased apoptosis of intestinal epithelial cells (Welniak et al., 2001).

Although there seems little doubt that commensal bacteria play a direct role in the development of intestinal inflammation and inflammatory bowel disease (IBD), how they bring this about is not at all clear. Evidence is starting to accumulate that breakdown of immune tolerance toward the normal bacterial flora involves dysregulated and/or constant activation of the innate immune system, including intestinal epithelial cells (Berrebi et al., 2003; Cario and Podolsky, 2000; Hausmann et al., 2002; Hugot et al., 2001; Ogura et al., 2001). Dysregulation of TLR2, TLR4, and/or Nod 2 expression by intestinal epithelial cells may be involved in the breakdown of immune tolerance toward the normal bacterial flora of the gut thereby contributing to the pathogenesis of IBD. There are several reports of TLR2, TLR4 and/or Nod2 expression being strongly up-regulated in CECs from human IBD patients (Berrebi et al., 2003; Cario and Podolsky, 2000; Hausmann et al., 2002) although it is unclear whether this is a cause or consequence of inflammation. It could be argued that these abnormalities could be associated with aberrant cytokine production by activated T cells that are associated with clinical disease. However, we have recently shown that in a well-documented murine model of IBD that dysfunction of TLR2, TLR4 and Nod2 in primary CECs is associated with the development of IBD and, in fact, precedes the development of clinical and histologic signs of IBD suggesting that the CECs themselves may be defective (Lan et al., 2005; Singh et al., 2005).

The results of this study provide the basis for determining whether there is increased expression of TLR2, TLR4 and Nod2 in primary CECs from dogs with IBD as has been reported in human IBD and a murine model of IBD. Since these studies can be performed on CECs freshly isolated from intestinal biopsies, comparison of expression in cells isolated from non-inflamed (normal) versus inflamed (diseased) gut tissue from the same dog can may provide evidence whether any dysregulation may be causal or secondary to inflammation. Lastly, they provide the basis to examine differences in TLR2, TLR4 and/or Nod2 expression between intestinal epithelial cells isolated from the small intestine and large intestine.

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Figure 1.

Morphology and purity of canine CECs. The morphology of freshly isolated Canine CECs was evaluated by Wright-Giemsa staining (A). The purity of the freshly isolated canine CECs was evaluated by flow cytometry using monoclonal antibodies reactive with cytokeratin (B) and canine CD45 (C).

Figure 2.

Constitutive expression of TLR2, TLR4 and Nod2 in DH82 cells and primary canine CECs.

Figure 3.

(A.) TLR4 expression in canine primary CECs before and 4 hours following stimulation with LPS. (B.) TLR2 and Nod2 expression in canine primary CECs before and 4 hours following stimulation with PGN. C.) Expression of IL-8 in canine primary CECs before and 4 hours following stimulation with LPS or PGN. (D). Expression of IL-7 in canine primary CECs before and 4 hours following stimulation with LPS.

Table 1

Primer sequences, annealing temperatures, and size of amplicon.

Primer	Forward Primer	Reverse Primer	Annealing Temperature	Product Length
Beta Actin	TGACC CAGA TCA TGT TTGA GACC	TCC TGC TTG CTG A TCCA CAT CT	60	700bp
TLR2	TGT TGTG AAAGT GAG CAAGGAA C	AAG CAGC CTG TTT TAC TGGTG G	53	440bp
TLR4	AGAG TT TC CTG C AG TGGG TCAA	GTCTGGGCAA TCT CA TAC TCA A	55	518bp
Nod2	CC TGA ACT CAT C AAAG CCA TCG	TGC TCAC CAT C CT AC C TCAT T	58.6	559bp
IL-7	TGT TCTGT TGC C AG TAGCA TCTTC	GC C CTGTG AAAC T CTTGATAA GTGG	52.4	280bp
IL-8	GC TGC AGTTCTG TCAAGAG T	CG GAT CTTGTTTC TCAGC	53	240bp