New Role of Nod Proteins in Regulation of Intestinal Goblet Cell Response in the Context of Innate Host Defense in an Enteric Parasite Infection

Huaqing Wang,a,b Janice J. Kim,a,b Emmanuel Denou,a,c Amanda Gallagher,d David J. Thornton,d M. Sharif Shajib,a,b Lijun Xia,e Jonathan D. Schertzer,e Richard K. Grencis,d Dana J. Philpott,f Walilu I. Khan,a,b

Farncombe Family Digestive Health Research Institute,a Department of Pathology & Molecular Medicine,a,b; and Department of Biochemistry and Biomedical Sciences,c McMaster University, Hamilton, Ontario, Canada; Faculty of Life Sciences, University of Manchester, Manchester, United Kingdomf; Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USAe; Department of Immunology, University of Toronto, Toronto, Ontario, Canada

Mucins secreted by intestinal goblet cells are considered an important component of innate defense in a number of enteric infections, including many parasitic infections, but also likely provide protection against the gut microbiota. Nod proteins are intracellular receptors that play key roles in innate immune response and inflammation. Here, we investigated the role of Nod proteins in regulation of intestinal goblet cell response in naive mice and mice infected with the enteric parasite Trichuris muris. We observed significantly more peroxidase-antiperoxidase (PAS)-stained intestinal goblet cells and less mucin (Muc2) in Nod1 and Nod2 double-knockout (Nod DKO) mice after T. muris infection than in wild-type (WT) mice. Expulsion of parasites from the intestine was significantly delayed in Nod DKO mice. Treatment of naive WT mice with Nod1 and Nod2 agonists simultaneously increased numbers of PAS-stained goblet cells and Muc2-expressing cells, whereas treatment with Nod1 or Nod2 separately had no significant effect. Stimulation of mucin-secreting LS174T cells with Nod1 and Nod2 agonists upregulated core 3 glycoprotein (C3GnT; an important enzyme in mucin synthesis) and MUC2. We also observed lower numbers of PAS-stained goblet cells and less Muc2 in germfree mice. Treatment with Nod1 and Nod2 agonists enhanced the production of PAS-stained goblet cells and Muc2 in germfree mice. These data provide novel information on the role of Nod proteins in goblet cell response and Muc2 production in relation to intestinal innate defense.

Goblet cells are the main source of mucins, and the mucus layer coating the gastrointestinal (GI) tract and containing mucins represents the front line of innate defense in the GI tract (1–3). Mucins act as the main structural component of the mucus, giving rise to its polymeric, viscoelastic, and protective properties. Up to 21 different mucin genes have been identified, cloned, and partially sequenced in humans, and the majority of their homologues have been identified in mice (4). The mucin genes MUC2/Muc2 and MUC3/Muc3 are found in large amounts in the GI tract of mice and play the key role in mucin production (1, 5). (MUC and MUC refer to the human gene and protein, whereas Muc and Muc refer to the mouse counterparts [6, 7].) Core 3 and Muc2 protein (8). Hyperplasia of goblet cells has been observed in a number of parasitic infections, including infection with Nippostrongylus brasiliensis, Trichinella spiralis, Hymenolepis diminuta, and Trichuris muris (5, 9–11). Putative mechanisms underlying the protective role of mucins against parasites include the trapping of worms in the mucus and inhibition of parasite motility and feeding capacity (5, 9, 11). Recently, we showed that in T. muris infection, worm expulsion in the initial stage of infection is Muc2 dependent, whereas worm expulsion in the late stage is Muc2 independent (12). Goblet cell response in nematode infection is thought to be under the control of the T helper 2 (Th2)-type response and is considered a potential effector mechanism (3, 5). However, a precise mechanism by which goblet cell response is modulated in intestinal parasite infection remains to be elucidated.

The gut is colonized by a complex, dynamic microbial ecosystem. The resident microbiota in the gut constitutes a heterogeneous microbial ecosystem containing up to $1 \times 10^{13}$ CFU of bacteria (13). Pattern recognition receptors (PRRs), such as cell surface Toll-like receptors (TLRs) and cytoplasmic nucleotide-binding oligomerization domain-like receptors (NLRs), have a crucial role in innate defense, that of recognizing pathogen-associated molecular patterns (PAMPs) (14). Studies have identified a role of Nod proteins in recognizing bacterial infection through their detection of peptidoglycan, which can enter the cells through specific transporters or as a consequence of infection with certain pathogenic bacteria (15). Once triggered, Nod proteins commence a pattern of gene expression in cells that help to clear infection. There are two Nod proteins, Nod1 and Nod2, and while Nod1 senses diaminopimelic acid (DAP)-containing peptidoglycan, which is found mainly in Gram-negative bacteria, Nod2 senses muramyl dipeptide (MDP), which is present in both Gram-
positive and Gram-negative bacteria (16). Despite a significant increase in knowledge of Nod proteins in bacterial infections in recent years, the precise role of these innate receptors in intestinal cellular and immune responses in enteric parasitic infections remains unexplored.

Gut microbes can regulate mucin production by activating different signaling cascades and secretory elements. Probiotics such as Lactobacillus plantarum were reported to influence MUC2 and MUC3 and inhibit the adherence of enteropathogenic Escherichia coli, indicating that the enhanced mucus layer and glycocalyx overlaying the intestinal epithelium and the occupancy of the microbial binding sites by Lactobacillus spp. provide protection against invasion by the pathogens (17). Bacterial products such as lipopolysaccharides (LPS) and flagellin A from Gram-negative bacteria and lipoteichoic acids (LTA) from Gram-positive bacteria are the most common modulators of mucin production, affecting mainly Muc2 and Muc5AC. Altered goblet cell response is important role in regulation of intestinal goblet cell hyperplasia and mucin production in the context of innate defense in T. muris infection.

**MATERIALS AND METHODS**

**Animals.** Breeding pairs of Nod1 and Nod2 double-knockout (Nod DKO) mice on a C57BL/6 background were provided by D. Philpott (University of Toronto). Nod DKO mice were originally generated by crossing Nod1 single-knockout mice and Nod2 single-knockout mouse (20). Breeding pairs of C3GnT1-deficient (C3GnT1−/−) mice on a C57BL6/6 background were provided by L. Xia (University of Oklahoma). C3GnT1−/− mice were generated by targeted homologous recombination in mouse embryonic stem cells (8). Nod DKO and C3GnT1−/− mice were kept in sterilized, filter-topped cages and fed autoclaved food under specific-pathogen-free (SPF) conditions in the animal facility at McMaster University, Hamilton, Ontario, Canada. Germfree C57BL/6 mice were acquired from the Farcombe Axenic Gnotobiotic Unit (AGU) at McMaster University. In some experiments, C57BL/6 mice received nonabsorbable antimicrobials (neomycin [5 mg/ml] or rifampin [0.2 mg/ml]) in drinking water for 7 days. Control mice received sterile water. All animal experiments were approved by the McMaster University Animal Care Committee and conducted in accordance with guidelines set by the Canadian Council on the Use of Laboratory Animals.

**Parasite infection.** Trichuris muris parasites were harvested and ova were collected and maintained as previously described (21). All infected mice received approximately 300 T. muris ova in 200 μl distilled water by oral gavage. Mice were sacrificed at various time points postinfection, and worm burden was assessed by counting the number of worms present in the cecum.

**Histological analysis and immunohistochemistry.** Formalin-fixed, paraffin-embedded sections of intestines were stained with periodic acid-Schiff (PAS) stain to detect intestinal goblet cells. The number of PAS+ goblet cells was expressed per 10 crypts. For immunohistochemistry, formalin-fixed, paraffin-embedded colonic segments were sectioned to 5 μm in thickness, deparaffinized by heating at 60°C for 30 min, cleared with CitriSolv (Fisher Scientific, ON, Canada), and rehydrated in a graded ethanol series of decreasing ethanol concentrations. Sections were subjected to heat-induced epitope retrieval (10 mM sodium citrate buffer–0.05% Tween 20, pH 6.0), blocked, and incubated with a polyclonal antibody against Muc2 (1:75 dilution; sc-15334; Santa Cruz Biotech) overnight at 4°C. Sections were washed with phosphate-buffered saline (PBS)–0.5% Tween 20 and incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1,000) (Molecular Probes/Invitrogen). Sections were mounted using ProLong Gold antifade reagent containing 4’,6-diamidino-2-phenylindole (DAPI) (Molecular Probes/Invitrogen). Images were captured using a Nikon Eclipse 80i microscope and NIS-Elements Basic Research imaging software. The number of Muc2+ cells was expressed per crypt. Investigators were blinded to the study groups.

**Treatment with Nod agonists both in vitro and in vivo.** Naïve C57BL/6, C3GnT−/−, and germfree mice (n = 5 per group) were intrarctally administered Nod1 ligand (C12-iE-DAP), Nod2 ligand (L18-MDP), both Nod1 and Nod2, or vehicle (PBS) at a dose of 50 μg per mouse. All mice were sacrificed 72 h later to assess colonic goblet cell numbers and expression levels of Muc2 and C3GnT in colonic tissue via real-time PCR.

**Cell culture.** LS174T cells were obtained from Kris Chadee (Gastrointestinal Research Group, University of Calgary, Canada). Cells were cultured in T75 tissue culture flasks (Costar, Cambridge, MA, USA) in Dulbecco’s modified Eagle medium–nutrient mixture F-12 (DMEM–F-12) medium (Gibco BRL Life Technologies, Burlington, Canada) containing 10% fetal bovine serum, 100 units ml−1 penicillin-streptomycin, 100 μg ml−1 streptomycin, and 20 mM HEPES (all purchased from Invitrogen Life Technologies). Cells were maintained at 37°C in a humidified incubator at 5% CO2. Culture medium was replaced with prewarmed medium every 2 days. Confluent cultures (80%) were harvested by trypsin–EDTA digest. Cells from passages 3 to 5 were used in this study.

**PCR.** Colon tissue samples were stored in RNAlater RNA stabilization reagent (Qiagen) and stored at −80°C. Total RNA was isolated from tissue or cultured cells using an RNeasy midikit (Qiagen) and reverse transcribed into cDNA using a Omniscript reverse transcription (RT) kit or QuantiTect RT-PCR kit (Qiagen), as per the manufacturer’s instructions. Total RNA was quantified using a NanoDrop 1000 spectrophotometer. Real-time PCR studies were performed with SsoFast EvaGreen Supermix (Bio-Rad) using a CFX96 real-time system (Bio-Rad). By using multiple classical internal control genes, the variability of each gene using a random subset of cDNA samples was assessed and the stability of the reference gene was assessed using geNorm (https://genorm.cmgg.be/) and as previously described by Vandesompele et al (22). 18S rRNA was used as an internal standard. Primer sequences for Muc2, C3GnT, and 18S rRNA are shown in Table 1. A commercially available premultril used was to determine the expression of MUC2 following the manufacturer’s instructions (Bio-Rad assay ID qHsaCIP0011696; NCBI RefSeq NC_000011.9). Each sample was run in triplicate. The cycling conditions were as follows: for Muc2, initial denaturation at 95°C for 5 min followed by 30 amplification cycles (94°C for 30 s, 55°C for 30 s,
72°C for 45 s) with an extension step of 72°C for 10 min following the final cycle; for C3GnT, initial denaturation at 95°C for 5 min followed by 30 amplification cycles (94°C for 30 s, 53°C for 30 s, 72°C for 30 s).

Data were analyzed by using Gene Expression Macro OM 3.0 software (Bio-Rad).

Colonic cytokines. To assess colonic tissue cytokine levels, colon samples were homogenized in Tris-buffered saline (TBS) containing a protease inhibitor mixture (Sigma). Total protein levels in colon homogenates were quantified using the Bio-Rad DC protein assay kit (Bio-Rad). Cytokine levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine murine; R&D Systems, Minneapolis, MN, USA) or the Bio-Plex Luminex system with a Bio-Rad mouse cytokine multiplex assay kit (Bio-Rad, Canada). In the latter, fluorescence data were acquired and analyzed by Bio-Plex Manager software (version 5.0; Bio-Rad Laboratories).

Statistical analysis. All data are presented as means and standard errors of the means (SEM). An unpaired t test or one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison post hoc test or Mann-Whitney U test was performed using GraphPad Prism version 6.0b for Mac OS X (GraphPad Software, La Jolla, CA). An associated P value of <0.05 was considered statistically significant.

RESULTS

Nod proteins play an important role in the development of T. muris-induced intestinal goblet cell hyperplasia. To investigate the role of Nod proteins in generation of T. muris-induced intestinal goblet cell hyperplasia, we infected Nod DKO mice with T. muris and assessed the numbers of PAS+ goblet cells and Muc2 expression. We observed significantly fewer PAS+ intestinal goblet cells on day 14 postinfection (p.i.) in Nod DKO mice than in wild-type (WT) C57BL/6 mice (Fig. 1A and C). We also observed significant downregulation of Muc2 cells and Muc2 expression in intestinal tissues, as assessed by immunohistochemical staining and RT-PCR analysis, respectively, in Nod DKO mice compared to WT mice after infection (Fig. 1B, C, and D). Goblet cells are considered a key component of host defense in T. muris infection. We observed significantly more worms in Nod DKO mice on day 14 p.i. than in WT mice (Fig. 2A). As T. muris infection induces Th2-type immune response in C57BL/6 mice, we investigated interleukin 4 (IL-4) and IL-13 levels in colonic tissues and observed significantly lower IL-4 levels in Nod DKO mice than WT mice after infection (Fig. 2B). There was no significant difference in
IL-13 levels between the Nod DKO and WT mice with or without infection (Fig. 2C).

Treatment with Nod agonists increases intestinal goblet cell numbers in naive mice. As observed significantly lower numbers of PAS\(^+\) intestinal goblet cells and Muc2\(^+\) cells in Nod DKO mice after *T. muris* infection, next, we investigated whether treatment with a Nod1 (C12-iE-DAP) or Nod2 (L18-MDP) agonist has an effect on intestinal goblet cell response in naive wild-type mice. Naive C57BL/6 mice were treated with either C12-iE-DAP or L18-MDP or both (Nod1 plus Nod2), and colonic PAS-stained goblet cells were investigated 72 h posttreatment. Control mice received vehicle (PBS). Treatment with C12-iE-DAP or L18-MDP alone had no significant effect on the number of PAS-stained goblet cell numbers compared with controls. Treatment with both agonists, however, resulted in a significant increase in numbers of PAS-stained goblet cells (Fig. 3A and B). In addition, by immunohistochemical staining and RT-PCR analysis, we observed a significant upregulation of Muc2 in mice treated with both agonists (C12-iE-DAP and L18-MDP) simultaneously compared to that in mice treated with vehicle or with a single agonist (Fig. 3C, D, and E).

Treatment with Nod agonists upregulates C3GnT expression both in vivo and in vitro. C3GnT is an important enzyme in mucin (Muc2) synthesis. As treatment with Nod1 and Nod2 agonists upregulated intestinal goblet cell numbers, we next investigated whether treatment had any effect on C3GnT gene expression using both in vivo and in vitro systems. In order to investigate whether Nod1 and Nod2 agonists are able to up-regulate C3GnT expression *in vivo*, we investigated C3GnT expression in naive mice treated with C12-iE-DAP and L18-MDP intrarectally. Treatment with both agonists simultaneously induced significant upregulation of colonic C3GnT expression (Fig. 3F), consistent with the results of upregulation in Muc2 production (Fig. 3C, D, and E). In addition, stimulation of mucin-producing LS 174T cells *in vitro* with both C12-iE-DAP and L18-MDP significantly upregulated C3GnT expression compared to that in unstimulated cells or single-agonist-stimulated cells (Fig. 4A). We also observed an increase in MUC2 gene expression in LS 174T cells after simultaneous stimulation with C12-iE-DAP and L18-MDP (Fig. 4B). Together, these observations suggest that Nod proteins play an important role in regulating mucin production by modulating C3GnT gene expression.

Nod agonists fail to enhance goblet cell numbers and Muc2 production in C3GnT deficient mice. To extend our studies and investigate whether Nod1 and Nod2 agonists are able to upregulate intestinal goblet cell numbers and mucin production by influencing C3GnT gene expression, we repeated these experiments using C3GnT gene deficient (*C3GnT\(^{-/-}\)*) mice with or without *T. muris* infection. We observed significantly fewer PAS\(^+\) intestinal goblet cells (Fig. 5A and B) and Muc2\(^+\) cells (Fig. 5D and E) in *C3GnT\(^{-/-}\)* mice than in the WT mice after *T. muris* infection, implying an important role of this enzyme in goblet cell function in this model. There was modest but statistically significant difference between PAS\(^+\) goblet cell numbers in uninfected *C3GnT\(^{-/-}\)* mice and *T. muris*-infected *C3GnT\(^{-/-}\)* mice, suggesting the presence of additional factors in regulation of goblet cell biology during this infection. The reduced numbers of PAS\(^+\) goblet cells and Muc2\(^+\) cells in *C3GnT\(^{-/-}\)* were associated with increased worm burden on day 14 p.i. (Fig. 5C). We also observed that simultaneous treatment of naive *C3GnT\(^{-/-}\)* mice with agonists of Nod1 and Nod2 had no effect on PAS\(^+\) goblet cell numbers, Muc2\(^+\) cell numbers, or Muc2 expression in intestinal tissues (Fig. 6). These findings suggest that C3GnT plays an important role in regulation of Nod protein-stimulated intestinal goblet cell response and mucin production.

Treatment with Nod agonists upregulates intestinal goblet cell numbers and Muc2 in germfree mice. Nod proteins are important innate receptors for bacterial products. As we observed an upregulation of goblet cells in response to Nod agonists, we next tested the hypothesis that the gut microbiota plays an important role in mediating the Nod protein-mediated changes in intestinal goblet cell responses. To understand the role of the gut microbiota in intestinal goblet cell biology, we investigated colonic goblet cell numbers in germfree C57BL/6 mice and in mice with perturbation of the microbiota by oral administration of antimicrobials.
which have been previously shown to alter the bacterial composition of the gut (23). We observed significantly reduced numbers of PAS$^+$ intestinal goblet cells in naive mice treated with the broad-spectrum antibiotic neomycin (Fig. 7A and B). However, there was no significant difference in PAS$^+$ goblet cell numbers after treatment with another antibiotic, rifampin. The role of gut microbiota in intestinal goblet cells response was further investigated in germfree mice. The numbers of PAS$^+$ intestinal goblet cells...
were significantly lower in germfree mice than in SPF mice (Fig. 7C and D). Immunohistochemical studies revealed the presence of less Muc2+ cells in germfree mice (Fig. 7E and F). Importantly, when we treated the germfree mice simultaneously with C12-iE-DAP and L18-MDP, we observed a significant increase in PAS+ intestinal goblet cells and Muc2+ cells (Fig. 7C to F). These observations suggest a potential important role of the gut microbiota-Nod axis in the intestinal goblet cell response.

**DISCUSSION**

Goblet cells are the main source of mucins in the gut and are considered a key component of innate defense mechanisms against various enteric infections (9). Despite a significant increase in knowledge regarding Nod proteins in bacterial infections and mucosal immunity in recent years, the role of these innate receptors in goblet cell responses remains unexplored. In the present study, we demonstrate an important role of Nod proteins in intestinal goblet function and mucin production in the context of an intestinal nematode parasite infection.

The innate immune receptors Nod1 and Nod2 are important components of the first line of host defense for detection of bacterial peptidoglycan (PG) within the cytoplasm of intestinal epi-

---

**FIG 4** Treatment with Nod1 and Nod2 agonists simultaneously in vitro increases C3GnT(A) and MUC2 (B) expression. Mucin-producing LS 174T cells were stimulated with a Nod1 agonist (C12-iE-DAP), a Nod2 agonist (L18-MDP), or both Nod1 and Nod2 agonists for investigation of C3GnT and MUC2. C3GnT and MUC2 expression was assessed by using real-time PCR. Data are represented as means, and error bars represent SEM. Groups were compared using Tukey’s multiple comparison posttest. *, P < 0.05.

**FIG 5** Numbers of PAS+ and Muc2+ cells were significantly lower in C3GnT−/− mice after T. muris infection. C3GnT−/− mice were infected with T. muris and sacrificed on day 14 p.i. (A) PAS+ goblet cell numbers in C3GnT−/− and wild-type mice on day 14 p.i. Groups were compared using Tukey’s multiple comparison posttest. (B) Representative micrograph of PAS-stained goblet cells in colon sections. Magnification, ×200; bars, 100 μm. (C) Worm burden in C3GnT−/− and wild-type (WT) mice on day 14 p.i. The Mann-Whitney U test was used to analyze statistical differences among the groups. (D) Muc2+ cells as assessed by immunohistochemistry. (E) Representative images of Muc2-stained intestinal sections in C3GnT−/− and WT mice with or without T. muris infection. Groups were compared using Tukey’s multiple comparison posttest. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant (5 mice per group).
thelial cells. Recently, it was shown that Nod2 plays an important role in colonic epithelial cells responses to T. muris infection and subsequent recruitment of dendritic cells following infection (24).

Goblet cells are considered a key component of innate defense in many nematode parasite infections, including T. muris infection (12, 25–27), but the role of Nod proteins in the regulation of these cells has not been investigated. In this study, we observed down-regulation of intestinal goblet cell numbers and Muc2 expression following T. muris infection in Nod DKO mice compared to WT mice, suggesting an important role of Nod proteins in generation of intestinal goblet cell hyperplasia and mucin production in this infection. We also observed a significant difference in PAS/β11001 goblet cell numbers in infected Nod DKO mice on day 14 p.i. compared to those in uninfected mice. Previous studies have shown that Th2 cells play an important role in goblet cell response in intestinal nematode infections, including T. muris infection (3, 28, 29). In addition, recent studies have shown a role of TLR4 in goblet cell response in Citrobacter rodentium infection (30) and in differentiation of goblet cells in intestinal organoid and enterocyte cell cultures (31). Therefore, it seems likely that in addition to Nod receptors, other innate receptors, such as TLR, and adaptive immune cells, such as T cells, can also influence the goblet cell response after T. muris infection.

We also observed that simultaneous treatment of naive mice with Nod1 and Nod2 agonists significantly upregulated PAS+ intestinal goblet cell numbers and Muc2 production, whereas treatment with individual agonists had no significant effect. In addition, treatment of naive mice with Nod1 and Nod2 agonists significantly upregulated the expression of C3GnT (the enzyme responsible for synthesizing the major component of Muc2) (32). To further understand the role of Nod proteins in intestinal goblet cell biology, we utilized the mucin-producing cell line LS 174T, and consistent with in vivo findings, we observed that simultaneous stimulation with agonists for Nod1 and Nod2 significantly upregulated the expression of MUC2 and C3GnT in mucin-producing cells. Individual treatment with Nod1 or Nod2 agonists had no significant effect on MUC2 or C3GnT expression. As we observed that the absence of Nod1 and Nod2 in Nod DKO mice

![FIG 6](http://iai.asm.org/ on August 29, 2017 by guest)
inhibited the upregulation of *T. muris*-induced goblet cell hyperplasia, and as C3GnT is a major enzyme in Muc2 synthesis, we next investigated whether C3GnT has any role in goblet cell response or mucin production by utilizing C3GnT−/− mice. C3GnT−/− mice lack the core 3 O-glycans of colonic mucins. These mice have a modest reduction in glycosylation and are susceptible to experimental triggers of colitis (8). We observed significantly fewer PAS+ goblet cells and less Muc2 expression in
C3GnT−/− mice after T. muris infection. The reduced numbers of PAS+ goblet cells and smaller amounts of Muc2 in C3GnT−/− were associated with increased worm burden. We did not observe any significant difference in intestinal IL-4 and IL-13 levels between C3GnT−/− mice and WT mice after T. muris infection (data not shown). Thus, in spite of the delay in worm expulsion, C3GnT deficiency had no significant effect on generation of the Th2-type immune response in T. muris infection. Moreover, simultaneous treatment with Nod1 and Nod2 agonists in naive C3GnT−/− mice failed to upregulate goblet cell numbers and Muc2 production suggesting an important role for C3GnT in Nod protein-mediated goblet cell hyperplasia and mucin production. Together, these findings suggest that Nod proteins play an important role in goblet cell response and mucin production and in generation of T. muris-induced intestinal goblet cell hyperplasia in relation to host defense. The mechanisms underlying the synergistic roles of Nod1 and Nod2 in increasing goblet cell numbers and mucin production in T. muris infection are not known. However, there are studies that report that both Nod1 and Nod2 function in a synergistic fashion to tune the appropriate responses to certain pathogens. For example, Nod1 and Nod2 DKO mice showed a significant reduction in the production of inflammatory cytokines and an increase in the bacterial colonization of the mucosal tissue in a Salmonella model of colitis. These phenotypes were not observed in Nod1 and Nod2 single knockout mice (33). Synergism between Nod1 and Nod2 was also reported in a murine model of Bacillus anthracis. Mice deficient for both Nod1 and Nod2 were more susceptible to lethal challenge with B. anthracis and produced lower levels of proinflammatory molecules than single knockouts (34). Th2 cells play an important role in development of goblet cell hyperplasia in infections with many intestinal nematode parasites, including T. muris (3, 28). Therefore, since stimulation of Nod1 and that of Nod2 drive similar activation pathways, it is likely that synergistic contributions of Nod1 and Nod2 are required for the development of Th2 immune response and subsequent goblet cell hyperplasia during T. muris infection. We also observed that treatment of noninfected mice with Nod1 agonist or Nod2 agonist alone had no significant effect on goblet cell numbers or mucin production. However, simultaneous treatment with both agonists resulted in a significant increase in goblet cell numbers and Muc2 production. Therefore, another possibility is the direct stimulation of Nod proteins by gut microbiota in goblet cell biology. Combined stimulation of both Nod1 and Nod2 is required in influencing goblet cell biology, upregulating goblet cell numbers and Muc2 production.

We recently showed a functional role for the mucus barrier in host protective immunity to T. muris infection, because in the absence of Muc2, worm expulsion is delayed (12). We have shown that around the time of worm expulsion, the mucus barrier is less porous in the resistant strains of mice (BALB/c and C57BL/6 mice, which expel the parasites) than in the susceptible mice (AKR mice, which do not expel the parasites and develop chronic infection), and this alteration in physical properties of the barrier after infection may directly affect the niche of the worms. Another possibility is that the lower levels of mucins result in a network that may compromise host defense because of inappropriate presentation or concentration of other host defense proteins (such as Relm-β and Tff3) in the environment of the worms. As goblet cells are the main source of mucins in the gut, these observations suggest that smaller amounts of mucins, due to reduced numbers of goblet cells, might interfere with worm expulsion.

Immune responses associated with many intestinal nematode infections, including T. muris infection, are characterized by the activation of Th2-type immune response. Previous studies suggested that IL-4 and IL-13 cooperate in the development of Th2 responses, and although their functions overlap, they perform additive roles. Expulsion of the parasites took place in the absence of either cytokine but not in the absence of both cytokines (35). It is also suggested that that blocking of either IL-4 or IL-13 is sufficient to inhibit worm expulsion in T. muris infection (36). In this study, we observed a significant difference in intestinal IL-4 levels but not in IL-13 levels between Nod DKO and WT mice after T. muris infection despite the inhibition in worm expulsion, further supporting the redundancy in the roles of IL-4 and IL-13 in the expulsion of nematode parasites.

The GI tract is colonized by a complex, heterogeneous, and dynamic microbial ecosystem containing up to $10^{13}$ CFU of bacteria (13, 37). This colonization of the mammalian GI tract occurs soon after birth. A mutually beneficial relationship exists between the gut and many of its symbionts: the gut provides nutrients to the resident bacteria, while they aid in the digestion of food and absorption of nutrients, producing biotin and vitamin K, while regulating immune system function and hindering the colonization of pathogenic microorganisms (38). Variations in gut microbial composition can result from genetic and environmental factors such as diet, living conditions, and birthplace (39). The gut microbiota is implicated in various GI disorders, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and enteric infections (40). Gut microbes can regulate mucin production by activating different signaling cascades and secretory elements. Beneficial bacteria, such as Lactobacillus plantarum 299V, were reported to induce MUC2 and MUC3 and inhibit the adherence of enteropathogenic Escherichia coli, indicating that an enhanced mucus layer and glycolcalyx overlying the intestinal epithelium and the occupancy of the microbial binding sites by Lactobacillus spp. provide protection against invasion by pathogens (17). Bacterial products such as LPS and flagellin A from Gram-negative bacteria and lipoteichoic acids (LTA) from Gram-positive bacteria are the most common modulators of mucin production by affecting mainly Muc2 and Muc5AC. In addition to evidence of enhanced mucus secretion in response to intestinal microbes, studies have shown altered goblet cell responses in germfree animals (18, 19, 41). In our recent study, we observed that treatment with beneficial bacteria L. rhamnosus (JB-1) promotes T. muris expulsion in association with upregulation of goblet cells (42). It was also shown that depletion of flora with antibiotics reduced the worm burden in T. muris infection in susceptible mice (43). In this study, we observed significantly fewer PAS+ goblet cells and less Muc2 in germfree mice than in SPF mice. We also observed fewer PAS+ goblet cells in naïve mice treated with a broad-spectrum antibiotic, neomycin, but not those treated with rifampin. These findings demonstrate an important role of gut microbiota in the intestinal goblet cell response. Interestingly, we observed that simultaneous treatment with Nod1 and Nod2 agonists upregulated goblet cell numbers and Muc2 production in germfree mice, indicating an important role of Nod protein-mediated stimulation in the goblet cell response. Taking into consideration the role of gut microbiota in the intestinal goblet cell response and in establishment of T. muris infection (43), the
findings of this study suggest a potential relationship among *T. muris*, gut microbiota, and Nod proteins in goblet cell response and in host defense in this parasitic infection. However, there is extensive complexity in this relationship, and further work is required to explore how the microbiota-Nod protein axis specifically regulates intestinal goblet cell responses that can contribute to parasite expulsion.

The findings of this study provide us with novel information on the role of Nod proteins in intestinal goblet cell response and mucin production and identify Nod proteins as potential new targets in modulating mucin production in the gut. In addition, this study suggests an important contribution of Nod proteins in mediating goblet cell response and Muc2 production in the context of intestinal innate defense in *T. muris* infection.

ACKNOWLEDGMENTS

This work is supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to Waliul Khan and from the Wellcome Trust, UK to David J. Thornton and Richard K. Gencris. Waliul I. Khan is a recipient of a CHF New Investigator Award.

We thank Elena Verdu of the Farncombe Axenic Gnotobiotic Unit (AGU), McMaster University.

FUNDING INFORMATION

Government of Canada | Natural Sciences and Engineering Research Council of Canada (NSERC) provided funding to Waliul Khan under grant number 311764-2013. The Wellcome Trust, UK provided funding to Richard Gencris under grant number WT094757MA. The Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, is supported by core funding from the Wellcome Trust, UK under grant number WT094757MA. The Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, is supported by core funding from the Wellcome Trust, UK under grant number WT094757MA. The Wellcome Trust, UK provided funding to Richard Gencris and David Thornton under grant number WT094757MA.

REFERENCES


