IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Entamoeba histolytica Exacerbates Epithelial Tight Junction Permeability and Proinflammatory Responses in *Muc2*^{-/-} Mice

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Human mucin-2 (MUC-2) is the first line of innate host defense in preventing pathogen-induced epithelial injury. *Entamoeba histolytica* (*Eh*) colonizes the mucus layer by binding of the parasite's surface galactose lectin to galactose and *N*-acetyl-D-galactosamine residues on colonic MUC-2, preventing parasite contact-dependent cytolysis of epithelial cells. We quantified early innate responses to *Eh* in wild-type and MUC-2-deficient mice (*Muc2*^{-/-}) using closed colonic loops. *Eh* infection in wild-type but not *Muc2*^{-/-} mice induced a time-dependent increase in ³H-labeled mucin and nonmucin glycoprotein secretions. Immunohistochemical staining revealed intense MUC-2 secretion, which formed a thick, protective mucus plug overlying the surface epithelium, entrapping *Eh*. In *Muc2*^{-/-} mice, *Eh* induced a pronounced time-dependent secretory exudate with increased gross pathology scores and serum albumin leakage. Colonic pathology, secretory responses, and increased proinflammatory cytokine secretions of TNF- α , IFN- γ , and IL-13 correlated with altered expression of the tight junction proteins claudin-2, occludin, and ZO-1. We identified the putative *Eh* virulence factor that elicits the proinflammatory responses and alters tight junction permeability as *Eh* cysteine protease A5 (EhCP-A5). The present findings demonstrate that colonic mucins confer both luminal and epithelial barrier functions and that, in the absence of MUC-2, mice are more susceptible to *Eh*-induced secretory and proinflammatory responses mediated by EhCP-A5. (*Am J Pathol* 2013; 182: 852–865; <http://dx.doi.org/10.1016/j.ajpath.2012.11.035>)

The surface of the gastrointestinal tract is lined by a viscous mucus layer that acts as a lubricant overlying the surface of intestinal epithelial cells (IECs).¹ The mucus layer is composed of a dense inner portion, impermeable to enteric bacteria, and a more viscous outer part colonized by a variety of commensal species.² Colonic mucin-2 (MUC-2), the main component of this mucus layer, is secreted by intestinal goblet cells, together with intestinal trefoil factor (mITF; encoded by *Tff3*) and resistin-like β (encoded by *Retnlb*), to mediate innate protective functions.^{3–5} The thickness of the colonic mucus layer is reduced in inflammatory bowel disease,⁶ and *Muc2*^{-/-} mice are highly susceptible to dextran sulfate sodium-induced injury and are prone to spontaneously develop colitis and adenomas.⁷ Thus, unraveling the myriad of functions of MUC-2 in innate host defenses is critical for our understanding of host–pathogen interactions

and conditions such as inflammatory bowel disease, for which the etiology is unclear.

One of the best-studied colonic pathogens that interacts with MUC-2 is the enteric human protozoan parasite *Entamoeba histolytica* (*Eh*).^{1,8} *Eh* is the causative agent of amebiasis, resulting in 100,000 deaths worldwide, and is the second leading cause of death due to a parasitic infection after malaria.⁹ In the majority of infections, *Eh* remains harmlessly commensal, but in approximately 10% of cases it invades the colon, causing amebic dysentery and/or liver abscesses.⁹ *Eh* colonizes the mucus layer and can degrade

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MUC-2, allowing the parasite to bind to and destroy the underlying epithelial cells.^{1,5} In genetically resistant mice, *Eh* colonizes the mucus layer but does not invade, and so is a useful model to investigate the early events in host–parasite interactions in the gut. In the present study, we used a closed colonic loop model of infection to investigate innate immune responses in wild-type (WT) and *Muc2*^{−/−} mice and to discern host defenses independent of MUC-2 in epithelial barrier function. The present study revealed that, in the absence of MUC-2, mice are highly susceptible to *Eh*-induced acute proinflammatory responses, leading to aberrant protein secretions and altered tight junction (TJ) permeability. These findings highlight the novel role of MUC-2 in both luminal and epithelial barrier function.

Materials and Methods

Animals and Colonic Loop Study

C57BL/6 and *Muc2*^{−/−} mice on the same genetic background were used in experiments. WT mice were purchased from Charles River Laboratories International (St-Constant, QC, Canada), and *Muc2*^{−/−} mice were obtained from Dr. Velcich⁷ and were bred in-house. All mice were male, 10 to 12 weeks old. Mice were kept in sterilized, filter-top cages and were maintained under specific-pathogen-free conditions with food and water *ad libitum*. All studies were approved by the University of Calgary Animal Care Committee.

Colonic loops were used as a model for short-term infection studies (1 to 6 hours after infection), as described previously.¹⁰ Briefly, WT and *Muc2*^{−/−} mice weighing 23 to 25 g were fasted for 12 hours before experiments began. The mice were anesthetized with 4.25 mg/kg sodium pentobarbital (Ceva Santé Animale, Libourne, France) dissolved in sterile water and injected intraperitoneally. A laparotomy was performed, and the colon was exteriorized and ligated with 3-0 black silk sutures (Ethicon, Somerville, NJ; Peterborough, ON, Canada) at the proximal (distal to the cecum) and distal (proximal to the rectum) ends; one more ligation was made in the middle, thus forming two colonic loops. Care was taken to keep the mesenteries, blood vessels, and nerves intact. Virulent log-phase *Eh* trophozoites (1×10^6) in 100 μ L PBS (pH 7.3) were inoculated into the proximal loop; the distal loop received only PBS. Another group of mice (the sham-treatment group) underwent a similar surgical procedure but without ligation of the colon, to quantify the effects of surgical manipulation alone. Colonic tissues were fixed in Carnoy's solution (3 hours, 4°C) and embedded in paraffin blocks. Microtome sections (7 μ m thick) were rehydrated through an ethanol gradient to water and were stained with H&E (EMD Millipore, Billerica, MA), PAS, Alcian blue (AB), or dual PAS and AB staining (both from Diagnostic BioSystems, Pleasanton, CA).

Assessment of Gross Pathology and Myeloperoxidase Activity

Gross pathology of colonic loops and inflammation was scored on a scale of 1 to 4, as follows: 1, normal colon (uniform thickness, no colon dilation or distension, no blood in luminal contents); 2, minimal damage (visible mucosal thickening and colonic distension, visible mucosal exudates, and expanded loop occupying <50% of the abdominal cavity); 3, extensive damage (thickening of the colonic mucosa, visible dilation of surface blood vessels, colon distension with visible luminal contents, mucosal exudates, and expanded loop occupying 50% of the abdominal cavity); 4, inflamed colon (extensive colon thickening, colon surface with extensive inflamed dilated blood vessels with or without hemorrhage, extensive colon distension with or without visible brown or bloody luminal contents, mucosal exudates under extreme pressure leading to ballooning of the colon, and expanded loop occupying >50% of the abdominal cavity). Myeloperoxidase activity was determined using a standard protocol,¹¹ with some modifications. In brief, colonic tissues were homogenized and sonicated in hexadecyltrimethylammonium bromide buffer. Samples were centrifuged at $9000 \times g$ (10 minutes, 4°C), and the supernatant was collected. 0.53 mmol/L *o*-dianisidine dihydrochloride and 1% hydrogen peroxide were added to 14 μ L of supernatant and the absorbance was determined at 420 nm. Data are expressed as myeloperoxidase units per milligram of tissue.

In Vivo Mucin Secretion Studies

To quantify mucin secretion *in vivo*, mice were fasted overnight and injected intraperitoneally with 20 μ Ci of [³H] glucosamine (PerkinElmer, Waltham, MA) in PBS for 3 hours to metabolically label the newly synthesized mucin pool, as described previously.^{4,5} [³H]glucosamine is converted into sialic acid, *N*-acetyl-glucosamine, and *N*-acetyl-*D*-galactosamine on the mucin monomer and has been used previously to determine the secretion of mucin in response to various agonists.¹² After laparotomy, a single colonic loop was made and injected with 2×10^6 log-phase amebic trophozoites. The mice were sacrificed 3 hours after infection. The colon was excised and opened longitudinally, and mucosal secretions were obtained as described previously.¹³ Mucosal secretions were vortex-mixed (at maximum speed, 15 minutes) and centrifuged ($1000 \times g$, 10 minutes, 4°C) to remove cellular debris. The ³H-labeled secreted glycoproteins were precipitated overnight at 4°C by adding an equal volume of 10% trichloroacetic acid and 1% phosphotungstic acid (Sigma-Aldrich).^{13,14} The trichloroacetic acid/phosphotungstic acid–precipitated proteins were isolated by centrifugation ($400 \times g$, 10 minutes, 4°C), resuspended in PBS, and neutralized to pH 7.0 with 10 mol/L NaOH. The total ³H-labeled glycoproteins were obtained by adding 3 mL Scintiverse I (Fisher Scientific, Pittsburgh, PA;

Ottawa, ON, Canada) scintillation fluid and were quantified as counts per minute, using a scintillation counter (Beckman Coulter, Fullerton, CA).^{13,14} To enumerate the secretion of high molecular weight (V_o) mucin and nonmucin components, secreted ^3H -labeled glycoproteins were fractionated using Sepharose 4B column chromatography, and the counts per minute for each fraction were determined. The column (Bio-Rad Laboratories, Hercules, CA; Mississauga, ON, Canada) was equilibrated with 0.01 mol/L Tris-HCl buffer containing 0.001% sodium azide (pH 8.0; Sigma-Aldrich). Before use, the column was calibrated with blue dextran (molecular weight 2000 kDa), to denote the void volume (V_o) and other gel filtration standards (Bio-Rad Laboratories), including thyroglobulin (TG; 670 kDa), chicken ovalbumin (44 kDa), and vitamin B₁₂ (1.35 kDa).

Eh Cultivation and Harvesting

Eh trophozoites (HM1:IMSS; ATCC, Manassas, VA) were grown axenically in TYI-S-33 medium and were regularly passaged through gerbil livers to maintain high virulence.¹⁴ Trophozoites were harvested during the logarithmic growth phase (48 to 72 hours) by chilling on ice for 10 minutes and were pelleted by centrifugation at $700 \times g$ (5 minutes, 4°C).

Quantification of Proinflammatory Cytokines and TJ Proteins in Colonic Tissues

Gene expression of proinflammatory proteins (IL-1 β , TNF- α , and IFN- γ) and immune-regulatory cytokines (IL-10 and TGF- β), TJ proteins (occludin, ZO-1, and claudins 1 to 5, 8, and 10), and secretory mucins (MUC-6) was examined by quantitative real-time PCR (qPCR). This method was also used to assess the expression of other goblet cell mediators (mITF and resistin-like β). Total RNA was prepared from full-thickness tissue samples with TRIzol reagent (Invitrogen; Life Technologies, Carlsbad, CA), according to the manufacturer's specifications. Spectroscopic analysis was used to determine the purity and yield of the RNA. cDNA was prepared from 1 μg mRNA using random hexamer primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). For qPCR, 1 μL cDNA was used with 10 μL SYBR Green master mix (Invitrogen), and 100 $\mu\text{mol/L}$ primer concentration in a final reaction volume of 20 μL . Primer sequences and conditions were as listed in Table 1. qPCR was performed on a Corbett Rotor Gene 3000 system (Qiagen, Valencia, CA), and results were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method.

Table 1 Mouse Primer Sequences and Annealing Temperatures

Target	Sequence	T_a (°C)	Reference
<i>Il1b</i>	F 5'-GCCTCGTGCTGTCGGACCCA-3' R 5'-CTGCAGGGTGGGTGTGCCGT-3'	60	
<i>Tnfa</i>	F 5'-ATGAGCACAGAAAGCATGATC-3' R 5'-TACAGGCTTGCTACTCGAATT-3'	56	15
<i>Ifng</i>	F 5'-TCAAGTGGCATAGATGTGGAAGAA-3' R 5'-TGGCTCTGCAGGATTTTCATG-3'	54	16
<i>Muc6</i>	F 5'-TGGACCACCCCAAGGAATCCAC-3' R 5'-AGGAGGCCAAGGTGATGTCGGAG-3'	60	
<i>Cldn1</i>	F 5'-TCTACGAGGACTGTGGATG-3' R 5'-TCAGATTACAGCAAGGAGTCG-3'	60	17
<i>Cldn2</i>	F 5'-CCTCGCTGGCTTGTATTATCTCTG-3' R 5'-GAGTAGAAGTCCCGAAGGA-3'	60	18
<i>Cldn3</i>	F 5'-AAGCCGAATGGACAAAGAA-3' R 5'-CTGGCAAGTAGCTGCAGTG-3'	60	17
<i>Cldn4</i>	F 5'-TGGATGAACTGCGTGGTG-3' R 5'-GGTTGTAGAAGTCGCGGATG-3'	60	19
<i>Cldn5</i>	F 5'-GTGGAACGCTCAGATTTCAT-3' R 5'-TGGACATTAAGGCAGCATCT-3'	60	17
<i>Cldn8</i>	F 5'-GCCGGAATCATCTTCTTCAT-3' R 5'-CATCCACCAGTGGGTTGTAG-3'	60	
<i>Cldn10</i>	F 5'-CCCAGAATGGGTACACATA-3' R 5'-CCTTCTCCGCTTGATACTT-3'	60	17
<i>Ocln</i>	F 5'-AGAGGCTATGGACAGGGCTCTTTGG-3' R 5'-CCAACAGGAAGCCTTTGGCTGCTCTTGG-3'	60	18
<i>Tjp1 (Zo1)</i>	F 5'-GGAGCTACGCTTGCCACACT-3' R 5'-GGTCAATCAGGACAGAAACACAGT-3'	60	20
<i>Actb</i>	F 5'-CTACAATGAGCTGCGTGTG-3' R 5'-TGGGGTGTTGAAGGTCTC-3'	54	

F, forward; R, reverse; T_a , annealing temperature.

Enzyme-linked immunosorbent assay (ELISA) was used to quantify the secreted proinflammatory cytokines and serum albumin in the luminal exudates from control and *Eh*-inoculated loops. Briefly, the colon was excised and the two loops separated. The distal suture was removed, and the luminal contents in the loop were collected in an Eppendorf tube, which was stored on a bed of dry ice. Before use, luminal secretions were thawed, protein concentrations were determined using a BCA protein kit (Pierce; Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's specifications, and samples were equilibrated to 100 µg protein per 100 µL reaction. Briefly, 96-well ELISA plates were coated with 1 µL primary antibody against murine serum albumin, secretory IgA, or IgG (Bethyl Laboratories, Montgomery, TX) in 100 µL coating buffer (0.05 mol/L carbonate–bicarbonate solution, pH 9.6) and incubated for 1 hour at room temperature. Plates were rinsed and blocked for 30 minutes at room temperature in blocking solution (50 mmol/L Tris, 0.14 mol/L NaCl, and 1% bovine serum albumin, pH 8.0). To each well, 100 µg of protein sample was added, according to the manufacturer's specifications, and plates were incubated for 60 minutes at room temperature. Horseradish peroxidase detection antibody was added, and the plate was further incubated for 60 minutes. Finally, 3,3',5,5'-tetramethylbenzidine substrate was added, followed by the stop solution, and the protein concentration was determined by colorimetric assay using H₂O₂.

Luminal exudates were also analyzed using a mouse 32-plex cytokine–chemokine panel (Eve Technologies, Calgary, AB, Canada) that allows examination of the levels of IL-8 homologs (KC, LIX, MCP-1, and MIP-2) and proinflammatory (IL-1β, IFN-γ, TNF-α) and immunomodulatory cytokines (IL-10), among others. For Western blotting, whole-thickness colonic tissue preparations were homogenized and sonicated in cell lysis buffer to obtain protein extracts from lysed cells.²¹ Cell lysis buffer contained 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L NaCl, 0.1% SDS, 0.5% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich). Protein samples were analyzed by Western blotting, as described previously.²¹ The protein content of each sample was estimated using a BCA protein assay kit (Pierce; Thermo Fisher Scientific) and was adjusted for a final concentration of 3.33 µg/µL. Protein samples were mixed with 1× sample buffer and boiled for 5 minutes at 100°C. Protein was loaded at 100 µg per well of a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 (PBS-T) for 1 hour at room temperature and incubated with primary antibodies against mouse claudin-2 and occludin (Invitrogen) in 5% bovine serum albumin overnight at 4°C. Blots were washed three times with PBS-T and incubated with secondary antibody in 5% nonfat milk and PBS-T for 1 hour at room temperature. Blots were washed with PBS-T and developed with Immobilon Western chemiluminescent horseradish peroxidase substrate

(Millipore, Billerica, MA), according to the manufacturer's instructions.

Confocal immunofluorescence microscopy was used to determine the localization of TJ proteins in full-thickness tissue preparations. Intestinal tissue was fixed in 1% paraformaldehyde for 15 minutes, after which it was passed through a 10% and a 20% sucrose gradient (30 minutes each), followed by a 30% sucrose solution (overnight at 4°C). Tissue was then embedded in Tissue-Tek OCT optimal cutting temperature compound (Sakura Finetek, Torrance, CA; VWR International, Lutterworth, UK). Tissue sections (5 µm thick) were rinsed in PBS-T and blocked in 3% normal goat serum. Sections were blotted with anti-mouse claudin-2, anti-mouse occludin, and anti-rabbit ZO-1 primary antibodies (Invitrogen 325600, 331500, and 617300, respectively) overnight in a humidified chamber at 4°C. Sections were rinsed with PBS-T and incubated with fluorescently labeled secondary antibodies (anti-mouse DyLight 488, anti-rabbit DyLight 594, both from Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Finally, nuclei were counterstained with DAPI, and sections were mounted with FluorSave reagent (Calbiochem; EMD Millipore, Billerica, MA). Slides were examined using a FluoView FV1000 confocal immunofluorescence microscope (Olympus, Tokyo, Japan). Immunofluorescence signals for the proteins of interest were determined in *en face* planes through the cellular *z* axis at 0.35-µm intervals.

Intestinal Permeability Studies

Intestinal permeability was examined using fluorescein isothiocyanate (FITC)–dextran, as described previously.²² Mice were anesthetized and colonic loops were inoculated with 10 mg FITC–dextran (3 to 5 kDa; Sigma-Aldrich) and 1×10^6 *Eh* trophozoites were suspended in 100 µL PBS; control mice received the same inoculation, but without *Eh*. At 3 hours after inoculation, mice were sacrificed by CO₂ asphyxiation and blood was collected by cardiac puncture. Whole blood was allowed to clot for 3 hours (in the dark) and then was centrifuged at $10,000 \times g$ (10 minutes, room temperature). Serum was collected in a clean Eppendorf tube and was diluted with an equal volume of PBS. Serial dilutions of the stock FITC–dextran solution were used to generate a standard curve, and 100-µL samples were loaded onto a 96-well plate in duplicate. Fluorescence was determined with a plate reader (absorption 485 nm, emission 535 nm).

Statistical Analysis

Data were analyzed using Student's *t*-test and analysis of variance with a Tukey's or Kruskal–Wallis post hoc test as necessary, using GraphPad Prism version 4 software (Graph-Pad Software, San Diego, CA). Data are expressed as means ± SEM.

Results

Eh Induces a Robust Secretory Response in *Muc2*^{-/-} Mice

Although *Eh* colonizes the mucus layer, which acts as an innate host defense mechanism to prevent parasite contact-dependent cytolysis of the underlying mucosa,²³ the specific role of MUC-2 in innate immunity against *Eh* remains unclear. *Muc2*^{-/-} mice offer an excellent opportunity to examine luminal and epithelial barrier functions in the absence of an intact mucus layer. In the present study, *Eh* inoculated into colonic loops induced a differential, time-dependent increase in both gross pathology and secretory responses in *Muc2*^{-/-} mice, compared with WT mice (Figure 1). Gross pathology scores were significantly increased in *Muc2*^{-/-} mice as early as 1 hour after infection, and steadily increased up to 6 hours ($P < 0.001$). At 3 and 6 hours after infection, colons were severely dilated with luminal secretions under intense pressure, and loops occupied >50% of the peritoneal cavity. Mucosal exudates from *Muc2*^{-/-} mice were brownish (bloody) and watery, whereas those from WT mice were clear, thick, and mucoid. Importantly, *Muc2*^{-/-} mice exhibited a progressive increase in pathology over time, such that at 6 hours after infection both the proximal and distal loops were severely dilated in some mice, whereas in WT mice the secretory responses did not worsen significantly over time (Figure 1).

Eh Stimulates Mucin and Nonmucin Glycoprotein Secretion in WT but Not *Muc2*^{-/-} Mice

Because *Eh* stimulates mucin secretion in animal models of amebiasis²⁴ and in human colonic goblet cells *in vitro*,¹⁴ it was of importance to determine the composition of the

secretory responses in *Muc2*^{-/-} mice. To this end, newly synthesized glycoproteins were metabolically labeled with [³H]glucosamine, and the ³H-labeled mucin and nonmucin secretions were quantified in response to *Eh* infection. Surprisingly, *Eh* induced a fivefold increase in total ³H-labeled glycoprotein secretions in WT mice, but in *Muc2*^{-/-} mice the total ³H-labeled glycoprotein pool remained unchanged (Figure 2A).

To determine whether the ³H-labeled glycoproteins were high molecular weight V_0 mucin and/or nonmucin components, proteins were fractionated using Sepharose 4B column chromatography (Figure 2B), which separates the high molecular weight mucins from the nonmucin components.^{10,20} Predictably, the luminal secretions from WT infected mice showed a 1.8-fold and 1.7-fold increase in mucin and nonmucin ³H-labeled glycoproteins, respectively, compared with those from *Muc2*^{-/-} mice. Thus, glycoprotein secretions were dominant for the WT mice, whereas in *Muc2*^{-/-} mice secretions consisted primarily of water, ionic secretions, and/or nonglycosylated proteins (Figure 2B).

Because MUC-2 has been shown to protect against contact-dependent cytolysis *in vitro*,²³ we sought to determine whether mice are more susceptible to *Eh*-induced damage in the absence of MUC-2. Accordingly, colonic tissues were carefully excised, fixed in Carnoy's solution to preserve the mucus layer, and stained to visualize cellular morphology (H&E) and to quantify neutral (PAS) and acidic (AB) mucin. Not surprising, tissues from WT mice showed filled PAS-positive goblet cells throughout the length of the crypts, and secreted mucus was visible overlying the surface IECs (Figure 3). In contrast, control *Muc2*^{-/-} colons showed crypt elongation with branching, and only AB-positive mucin in the deep crypts (data not shown). Moreover, no abnormal inflammatory cellular

Figure 1 *Eh* induces a rapid sustained secretory response with increased gross pathology in colonic loops of *Muc2*^{-/-} mice. WT and *Muc2*^{-/-} mice were anesthetized, and closed colonic loops were infected with 1×10^6 virulent, log-phase trophozoites for 1, 3, or 6 hours after infection. After sacrifice, the colon was excised, and gross pathology scores were determined. **A**: Controls are sham-treatment mice with colon ligation alone, shown for comparison. **B–D**: Excised colons of representative WT and *Muc2*^{-/-} mice, with the gross pathology scoring for each group. Data are expressed as means $\pm$ SEM. $n = 12$ mice per group. ** $P < 0.01$, *** $P < 0.001$. The rulers shown for scale illustrate in millimeters. Ctrl, control; Inf, infected; PI, after infection.

Figure 2 *Eh* stimulates mucin and nonmucin glycoprotein secretion in WT and *Muc2*^{-/-} mice. Mucosal secretions obtained from WT and *Muc2*^{-/-} mice at 3 hours after infection were pooled and precipitated with a 10% trichloroacetic acid and 1% phosphotungstic acid solution. **A:** Significantly more glycoproteins were obtained from mucosal secretions of WT infected mice than from WT control or *Muc2*^{-/-} infected mice. **B:** Sepharose 4B column chromatography profiles obtained from the same ³H-labeled glycoprotein secretions from WT and *Muc2*^{-/-} infected mice. The mucosal secretions of WT infected mice were rich in mucin (*V*₀ fractions) and nonmucin glycoproteins. The column was calibrated with various molecular weight markers as shown. Data in panel A are expressed as means ± SEM of two independent experiments with similar results. *n* = 6 (control) or 7 (infected mice). ***P* < 0.01. B12, vitamin B₁₂; BD, blue dextran; Ova, chicken ovalbumin; TG, thyroglobulin.

infiltrate was observed under basal conditions or after *Eh* infection at 6 hours after infection. Predictably, *Eh* induced a prominent mucus secretagogue effect in WT as early as 1 hour after infection, which had formed a thick, well-organized mucus plug by 6 hours (Figure 3). At 6 hours after infection, the colonic mucosa in *Muc2*^{-/-} mice was severely compressed as a result of increased pressure (due to the intense secretory responses) inside the loop. Amebas did not bind to/or invade the epithelium in either WT or *Muc2*^{-/-} mice at any time point examined. In WT mice, *Eh* trophozoites were found embedded in the thick mucus blanket away from the mucosal surface, whereas in *Muc2*^{-/-} mice trophozoites were found throughout the intestinal lumen, engulfing bacteria and *Trichomonas* spp. organisms.

Effect of *Eh* on Other Goblet Cell Mediators

Previous studies have shown that goblet cells synthesize and secrete MUC-2, mITF, and resistin-like β and that in the absence of MUC-2 these cells lose their characteristic goblet cell phenotype.^{7,25} Because all three goblet cell mediators play critical roles in innate host defense and wound repair, we sought to determine whether there is a compensatory increase in these or other secretory mucins in *Muc2*^{-/-} mice

after *Eh* infection. Surprisingly, there was no increase in *Tff3* or *Retnlb* gene expression in response to *Eh*. Curiously however, *Muc6* mRNA expression was subtly but noticeably increased in WT and *Muc2*^{-/-} mice starting at 3 hours, suggesting that MUC-6 might play a protective role (Supplemental Figure S1).

Eh Induces a Differential Proinflammatory Response in WT and *Muc2*^{-/-} Mice

Goblet and columnar epithelial cells respond to inflammatory stimuli, including *Eh*, by releasing preformed and

Figure 3 *Eh* evokes a robust mucin secretagogue effect in WT mice. Colonic tissue sections from WT and *Muc2*^{-/-} infected mice were fixed in Carnoy's solution and stained with PAS reagent to visualize neutral mucins. In WT sham-treatment mice, PAS-filled goblet cells are visible throughout the length of the crypts and in the intestinal lumen (mucus). In contrast, in *Muc2*^{-/-} mice the tissue is devoid of PAS-positive neutral mucins, and crypts are elongated with branching. Note the PAS-positive (magenta) mucin secretion in WT but not in *Muc2*^{-/-} mice in response to *Eh*. In WT mice at 1 hour after infection, an ameba (arrow) is engulfed by neutral mucins; at 3 hours, mucin released from goblet cells forms a mucus plug (arrow) at the opening of the crypt. At 6 hours, the inner mucus layer is a deep magenta color (arrow) and the outer parts are a lighter magenta. Note the paucity of PAS-positive staining in *Muc2*^{-/-} tissues. Scale bar = 50 μm.

newly synthesized mucins^{1,5,8} and by increased expression and secretion of proinflammatory mediators.²⁶ Thus, it was of interest to determine whether *Eh*-induced inflammatory responses are responsible for the enhanced secretory responses observed in *Muc2*^{-/-} mice. Accordingly, inflammation was assessed by myeloperoxidase activity as a measure of neutrophil influx and activation, qPCR for cytokine gene expression, and ELISA to detect the presence of secreted cytokines in colonic exudates. Surprisingly, even though there was no significant increase in myeloperoxidase activity at 3 and 6 hours after infection (Supplemental Figure S2), increased protein secretion of the IL-8 homologs KC, MIP-2, and MCP-1 was observed in *Muc2*^{-/-} mice (Supplemental Figure S3). In addition, *Eh* infection increased mRNA expression and/or protein release of the proinflammatory cytokines TNF- α , IFN- γ , and IL-13, indicative of a robust but differential inflammatory response in *Muc2*^{-/-} mice, compared with WT mice (Figure 4). In particular, *Eh* induced a robust proinflammatory response characterized by elevated *Il1b* gene expression and protein release in WT mice (Figure 4). In *Muc2*^{-/-} mice, however, this response was characterized by elevated levels of TNF- α , IFN- γ , and IL-13 cytokine release.

Eh Alters TJ Proteins in *Muc2*^{-/-} Mice

Proinflammatory cytokines, including TNF- α , alter TJ permeability in IECs resulting in occludin endocytosis and increased paracellular permeability.²⁷ To investigate whether the increased proinflammatory cytokines observed in *Muc2*^{-/-} mice are associated with altered TJ permeability

and increased secretory responses, TJ mRNA expression was examined by qPCR and protein levels were examined by Western blot and immunofluorescence microscopy. *Eh* significantly increased the expression of the pore-forming claudin-2 gene (*Cldn2*) at 1 and 3 hours after infection in *Muc2*^{-/-} mice, compared with WT mice (Figure 5A), concomitant with no change or variable expression of mRNA of the barrier-tightening claudins 1, 3, 5, and 8 (Supplemental Figure S4). Correspondingly, occludin mRNA expression was significantly decreased in *Muc2*^{-/-} mice at 3 and 6 hours (Figure 5B). The mRNA expression data were consistent with the data on protein levels. WT mice showed low basal expression of claudin-2 and occludin; however, only occludin increased in response to *Eh*, peaking at 6 hours (Figure 5, C and D). In contrast, *Muc2*^{-/-} basal levels of claudin-2 and occludin were consistently higher than WT, and claudin-2 protein increased steadily in response to *Eh* (Figure 5C).

These findings were confirmed by confocal immunofluorescence microscopy in *Eh*-infected *Muc2*^{-/-} colons. Claudin-2 protein expression was markedly increased throughout the cytoplasm at 3 and 6 hours after infection, whereas ZO-1 expression was undetected (Figure 6A). In WT mice, the modest increase in claudin-2 expression was colocalized with ZO-1 at the apical cell surface; at 6 hours after infection, both claudin-2 and ZO-1 were reduced (Figure 6A). In infected WT mice, there was a modest increase in occludin expression colocalized with ZO-1 at 3 and 6 hours after infection. In *Muc2*^{-/-} mice, however, occludin levels were significantly reduced at 3 hours after infection, and ZO-1 appeared disorganized; by 6 hours, the expression of both

Figure 4 *Eh* induces a differential proinflammatory response in WT and *Muc2*^{-/-} mice. Full-thickness colonic loop tissues were processed for proinflammatory gene expression (A) and mucosal exudates were processed for cytokine protein levels (B) at various times after *Eh* infection. Gene expression fold change is relative to sham-treatment controls. Data are expressed as means $\pm$ SEM of two independent experiments with similar results. * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 5 *Eh* alters gene expression of TJ proteins in *Muc2*^{-/-} mice. Intestinal permeability in *Muc2*^{-/-} mice was assessed for claudin-2 and occludin expression. **A:** *Cldn2* gene expression levels were consistently higher in *Muc2*^{-/-} mice at all time points investigated, although this peaked at 3 hours after infection. **B:** Concomitantly, *Eh* induced a time-dependent decrease in occludin levels in *Muc2*^{-/-} mice. **C:** Representative Western blots for WT and *Muc2*^{-/-} mice after *Eh* infection at 1, 3, and 6 hours after infection. **D:** Relative intensity of claudin-2 and occludin protein for the Western blots in **C**. Data are expressed as means $\pm$ SEM. Gene expression fold change is relative to sham-treatment controls. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

of these TJ proteins was reduced (Figure 6B). These findings suggest that WT mice in the presence of an intact mucin barrier have well-organized claudin-2 and occludin proteins, and that occludin is modestly increased after infection. In *Muc2*^{-/-} mice, however, the TJ proteins were basally overexpressed and either increased (claudin-2) or decreased (occludin and ZO-1) after *Eh* infection, which may explain the increased secretory responses observed in these mice.

Eh Infections Cause Paracellular Serum Protein Leakage in *Muc2*^{-/-} Mice

Because *Muc2*^{-/-} are more susceptible to *Eh*-induced pathological responses (as evidenced by gross pathology scores, robust secretory responses, and altered TJ permeability), we sought to determine the composition of the luminal exudates obtained from these mice. In addition to more copious fluid

Figure 6 *Eh* alters TJ proteins in *Muc2*^{-/-} mice. Intestinal permeability in *Muc2*^{-/-} mice was assessed for claudin-2 and occludin using confocal immunofluorescence microscopy. **A:** *Muc2*^{-/-} mice constitutively express elevated claudin-2 protein levels, compared with WT, at the apical surface of the cell, away from ZO-1. In WT mice, claudin-2 was colocalized with ZO-1 (arrows) at 3 hours after infection, but showed a modest reduction in expression at 6 hours (inset). Importantly, the claudin-2 present at the apical surface was colocalized with ZO-1. In *Muc2*^{-/-} mice, at 3 after infection [and at 6 hours (inset)], claudin-2 protein was overexpressed at the apical cell surface, with a concomitant loss of ZO-1 evident at both 3 and 6 hours. **B:** *Muc2*^{-/-} mice constitutively expressed more occludin at the apical cell surface, where it was colocalized with ZO-1 (arrows). A modest increase in occludin was observed in WT mice at 3 hours [and at 6 hours (inset)], colocalized with ZO-1. In *Muc2*^{-/-} mice, in contrast, *Eh* caused a progressive decrease in occludin expression concomitant with a loss of ZO-1 at 3 hours, which was more pronounced at 6 hours (inset). Scale bar = 50 μ m.

secretions, there was a time-dependent increase in serum albumin leakage in *Muc2*^{-/-} mice, compared with their WT counterparts, which was most striking at 6 hours after infection ($P < 0.01$) (Figure 7A). Interestingly, the same was not true for serum IgA or IgG levels, indicating that the increased movement of albumin was the result of paracellular leakage of serum proteins into the intestinal lumen (Figure 7, B and C). To determine whether there was altered intestinal permeability, we quantified the movement of the inert probe FITC-dextran in the serum of mice infected with *Eh* for 3 hours after infection. Surprisingly, although FITC-dextran levels were somewhat higher in *Eh*-infected WT mice, *Muc2*^{-/-} mice showed a decrease in FITC levels (Supplemental Figure S5); however, neither difference reached significance.

Identification of the *Eh* Virulence Factor Involved in Altering Intestinal Permeability

Because *Eh* infection elicited a hypersecretory response in *Muc2*^{-/-} mice, it was of interest to identify the parasite virulence component involved in this response. Based on the results obtained (Figures 4 and 5), we used *Il1b* gene expression to evaluate proinflammatory responses and *Cldn2* and *Ocln* expression as markers for alteration in TJ permeability. We used *Muc2*^{-/-} mice, because the colonic response is robust in these mice. As expected, live *Eh* was most effective at upregulating *Il1b* mRNA expression; however, equivalent amounts of lysed or fixed *Eh* inhibited expression by 89% and 95%, respectively (Supplemental Figure S6A). Similarly, lysed or fixed *Eh* decreased *Cldn2* and *Ocln* expression, but not to the same extent as expression of *Il1b* (Supplemental Figure S6).

Because live *Eh* evoked a proinflammatory response and altered TJ proteins in the absence of direct parasite–cell contact, and because *Eh* cysteine proteinases can stimulate proinflammatory responses in *Muc2*^{-/-} colons,²⁸ we speculated that a cysteine proteinase might be the candidate virulence factor involved in inducing these effects. Consistent with our previous findings, live *Eh* pretreated with the specific cysteine proteinase inhibitor E-64, markedly reduced *Il1b*, *Cldn2*, and *Ocln* mRNA expression (Figure 8, A–C). Moreover, because *Eh* cysteine protease A5 (EhCP-A5) is the major proteinase released by live *Eh*,⁸ it was not surprising that CP-A5-deficient *Eh* amebas (*Eh*^{cpA5-/-}) were refractory for stimulating *Il1b*, *Cldn2*, and *Ocln* mRNA expression in *Muc2*^{-/-} mice (Figure 8). To confirm whether EhCP-A5 alters TJ proteins, we used confocal immunofluorescence microscopy to visualize claudin-2, occludin, and ZO-1. In WT mice infected with *Eh*, claudin-2 expression remained the same, occludin was increased, and ZO-1 was lost; there were no changes in mice infected with *Eh*^{cpA5-/-}. Furthermore, the *Eh*-induced claudin-2 expression at the apical cell surface concomitant with a loss of ZO-1 that was observed in *Muc2*^{-/-} mice was absent in the *Eh*^{cpA5-/-}—infected mice (Figure 9A). Curiously, a loss of occludin and ZO-1 was also observed in the mice infected with *Eh* at

Figure 7 *Eh* induces serum albumin leakage in *Muc2*^{-/-} mice. Serum albumin (A), IgA (B), and IgG (C) levels in colonic loop exudates after *Eh* infection were quantified by ELISA. A marked, time-dependent increase in serum albumin leakage into the intestinal lumen was observed in *Muc2*^{-/-} mice, with no similar alterations in serum IgA or IgG levels. Curiously, the same was not observed in WT mice for any of the proteins examined at the time points investigated. Data are expressed as means $\pm$ SEM of two independent experiments with similar results. $n = 4$ to 6 mice per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3 hours after infection, and (although this was absent in the *Eh*^{cpA5-/-}—infected mice) the degradation of ZO-1 was less pronounced (Figure 9B). Thus, EhCP-A5 appears to be a major virulence factor responsible for eliciting both proinflammatory responses and alterations in TJ proteins.

Discussion

The colonic mucus layer is the first line of innate host defense against intestinal amebiasis, and *Eh* interactions with MUC-2 may hold the key to understanding disease pathogenesis. *Eh* binds epithelial cells as a prerequisite for

Figure 8 Virulent *Eh* but not *Eh*^{CPA5-/-} induces proinflammatory and TJ protein gene expression in *Muc2*^{-/-} mice. WT and *Muc2*^{-/-} mice were infected with *Eh* or with *Eh* pretreated for 12 hours with the cysteine protease inhibitor E-64 (*Eh* + E-64) or with *Eh*^{CPA5-/-} for 3 hours. Gene expression of *Il1b* (A), *Cldn2* (B), and *Ocln* (C) was examined. Gross pathology scores were similar to those presented in Figure 1C. Infection with *Eh* + E-64 or with *Eh*^{CPA5-/-} elicited an overall lower gross pathology score in WT and *Muc2*^{-/-} mice, compared with *Eh*, but the difference was not statistically different (perhaps because of the small sample size). Gene expression fold change is relative to sham-treatment controls. *n* = 4 mice per group. **P* < 0.05.

host-cell cytolysis and disease pathogenesis, and this binding is inhibited when parasites are incubated with purified human colonic mucins.²³ We have previously shown that EhCP-A5 cleaves MUC-2 at the C-terminus to disrupt the mucin polymer and to compromise barrier integrity.²⁹ Curiously, MUC-2 lacks the critical amino acid residues susceptible to EhCP-A5 degradation, which may

explain why rodents are resistant to intestinal amebiasis. In WT mice with an intact mucin barrier, *Eh* colonizes the mucus layer but cannot degrade it. How *Eh* interacts with the colon in the absence of this barrier is unclear. Our present studies show that, with an intact mucus layer, mucin secretion is rapidly induced after *Eh* infections and that this engulfs invading trophozoites, thus preventing the parasites from binding the epithelium. Curiously, *Eh* also elicited a heightened proinflammatory response characterized by elevated levels of IL-1β. In the absence MUC-2, however, *Eh* induced a robust hypersecretory response associated with movement of water into the colon, a differential proinflammatory response characterized by elevated TNF-α and IFN-γ, altered expression of TJ proteins, and increased intestinal permeability to serum proteins. Importantly, in the absence of this protective mucus layer, there were no signs of epithelial damage, suggesting that other innate responses are present in *Muc2*^{-/-} mice that prevent colonization and/or epithelial destruction. Previous studies have shown that aberrant mucin expression is observed in colitis in *Muc2*^{-/-} mice; in that condition, it is thought to serve a protective role.²⁵ Our present studies support these findings; in the distal colon of *Muc2*^{-/-} mice, *Muc6* gene expression was present at low levels and was localized to the base of the crypts in the AB-positive staining region. In response to *Eh*, a time-dependent increase in *Muc6* gene expression was observed in both WT and *Muc2*^{-/-} mice, suggesting that this may represent a previously unnoted innate immune response toward *Eh*.

A curious finding was that *Eh* induced a robust differential proinflammatory response in WT and *Muc2*^{-/-} mice, correlated with changes in IL-1β, TNF-α, IFN-γ, and IL-13 expression. In WT mice, *Eh* induced elevated levels only of the proinflammatory cytokine IL-1β; in *Muc2*^{-/-} mice, in addition to IL-1β, the levels of TNF-α, IFN-γ, and IL-13 proteins also were increased in colonic exudates, despite only modest changes in mRNA expression. It is well known that IECs produce IL-1β *in vivo* after injury and that *Il1b* gene expression plays a critical role in the early events leading to inflammation.³⁰ In our system, however, it is unclear whether the increased IL-1β expression is itself protective or whether it induces protection mediated by other cytokines. Interestingly, in *Muc2*^{-/-} mice, the increased gross pathology and robust secretory responses were concomitant with elevated levels of IL-1β, TNF-α, IFN-γ, and IL-13. Thus, it is likely that this increase represents an aberrant and uncontrolled proinflammatory response. Previous studies have shown that *Muc2*^{-/-} mice constitutively express increased levels of proinflammatory cytokines, chemokines, and immunoglobulins, suggesting that these mice may have ongoing low-grade inflammation. Furthermore, a deficiency in MUC-2 has been shown to result in colonic bacteria in direct contact with epithelial cells, which may lead to elevated levels of proinflammatory cytokines,² and may contribute to the onset and perpetuation of colitis.^{31,25} In addition, *Muc2*^{-/-} mice have increased

Figure 9 *Eh*^{cpA5}^{-/-} does not alter TJ proteins. Mice were infected with virulent *Eh* or *Eh*^{cpA5}^{-/-} in a colonic loop for 3 hours, and localization of the TJ proteins claudin-2, occludin, and ZO-1 was examined. **A:** *Eh* induced no change in claudin-2 in WT mice, although a modest increase was observed in *Muc2*^{-/-} mice (arrows). In both cases, these changes were concomitant with a loss of ZO-1 and this was attenuated in mice infected with *Eh*^{cpA5}^{-/-}. **B:** A different pattern was observed in occludin, which increased in WT but decreased in *Muc2*^{-/-} mice (arrows); however, the concomitant changes in ZO-1 remained consistent with those observed for claudin-2. Scale bar = 50 μ m.

bacterial penetration before dextran sulfate sodium-induced colitis,³² suggesting that epithelial integrity is already compromised in these mice. In light of these reports, it appears that the robust increase in proinflammatory cytokines observed in the present study may be aberrant responses characteristic of a hyperactive immune system, therefore suggesting that not only does MUC-2 act as a physical barrier to prevent colonization, but it also protects against robust acute inflammatory responses to mild injury.

In *Eh* infection, it is unclear what causes the alteration in gut permeability to water, ions, or protein leakage. Elevated levels of proinflammatory cytokines are known to be associated with alterations in TJ permeability, although the precise mechanism underlying this effect is uncertain. Serosal addition of TNF- α to HT-29/B6 colonic cells resulted in an 81% decrease in transepithelial electrical resistance, reduced complexity and depth of TJ strands, and increased sodium and mannitol flux.³³ Similarly, IFN- γ

reduced transepithelial electrical resistance in monolayer T84 cells after 48 hours,³⁴ with redistribution of occludin, claudins 1 and 4, and JAM-A away from the TJs via protein endocytosis.³⁵ Furthermore, a synergistic effect was observed when cells were treated with a combination of IFN- γ and TNF- α .³⁵ Although the mechanisms are not clear, it appears that TNF- α and IFN- γ promote barrier dysfunction by altering transepithelial electrical resistance and TJ permeability through decreased expression of the barrier-tightening claudins 1, 5, and 7, as well as of occludin.³⁶ Thus, it is tempting to speculate that the proinflammatory responses observed in *Muc2*^{-/-} mice may be responsible for the alterations in gut permeability. Indeed, previous studies have shown that IECs secrete TNF- α , which may explain the elevated levels of this cytokine in the intestinal lumen, and TNF- α is a chemotactic and chemokinetic stimulus for *Eh*.³⁷ Furthermore, TNF- α is associated with gut inflammation³⁸ and with the diarrheal response typically observed in *Eh* infections in children.³⁹ Thus, it is likely that TNF- α is an important player in driving the secretory responses observed in these studies.

An emerging concept in understanding of the movement of water, ions, and high molecular weight molecules between serosal and luminal cell surfaces is that of the pore and leak pathways.⁴⁰ The pore pathway, a high-capacity paracellular route of transport, is size- and charge-selective, allowing movement of molecules of ≤ 0.4 nm. This pathway is thought to be under the control of claudins, and IL-13 has been shown to activate it.⁴⁰ The leak pathway, a low-capacity channel, allows the flux of larger ions and molecules, regardless of their charge, and ZO-1 and occludin have been implicated in the control of this pathway.⁴⁰ Curiously, the TNF- α -induced changes in barrier integrity have been associated with alterations in the leak pathway.⁴⁰ Furthermore, pathogens can increase intestinal permeability by altering TJ localization and function and activation of these pathways, and we have previously shown that the intestinal barrier is compromised in *Muc2*^{-/-} mice infected with *Citrobacter rodentium*.⁴¹ Here, we show for the first time that *Eh* alters TJ permeability *in vivo*, by increasing claudin-2 expression and localization, which may be the result of increased IL-13 expression, while at the same time altering occludin and ZO-1 expression, which may be mediated by changes in TNF- α . Claudin-2 is a small protein (20 to 27 kDa) localized to the crypts but it is rarely expressed in the normal epithelium. It is increased in inflamed tissue such as celiac disease⁴² and ulcerative colitis,⁴³ and is associated with increased ion and water transport across the membrane and decreased transepithelial electrical resistance.⁴⁴ Occludin is a 65-kDa protein, and siRNA silencing increased the flux of large probes (including mannitol and dextran) and the expression of claudin-2 in enterocytes.⁴⁵ Occludin expression is down-regulated by IL-1 by an NF- κ B-dependent mechanism,⁴⁶ and TNF- α has been shown to induce occludin internalization. The function and stability of claudin and occludin are post-transcriptionally modified,

and phosphorylation of these proteins leads to increased protein internalization, indicative of either increased protein turnover or disruption of the interaction between scaffolding proteins,⁴⁷ including ZO-1. Both claudin and occludin associate with the plasma membrane protein ZO-1 (a 220-kDa protein linked to the actin cytoskeleton). Interestingly, studies *in vitro* have demonstrated that *Eh* trophozoites can increase paracellular permeability through the degradation of ZO-1.⁴⁸

Here, we have shown that *Eh* infection differentially regulates the expression of TJ proteins depending on the presence or absence of an intact mucin barrier. In our interpretation of the data, in WT mice the elevated levels of claudin-2 open the pore pathway, facilitating the movement of water and ions into the intestinal lumen hydrating the mucus layer. Once hydrated, this layer effectively traps *Eh* trophozoites, thereby preventing direct contact with the underlying IECs. Furthermore, *Eh* degrades the barrier forming protein ZO-1, which would normally lead to increased intestinal permeability through the leak pathway. In the presence of an intact mucin barrier, however, *Eh* increases occludin expression; the occludin is localized to the TJ, where it reinforces the barrier and prevents increased epithelial permeability and penetration by other potential pathogens. In *Muc2*^{-/-} mice, however, *Eh* induces a markedly different response. Our data suggest that the high basal and increased claudin-2 expression at the apical surface of enterocytes could lead to increased flux of water and ions in a similar manner to that induced in WT mice. However, in the absence of an intact mucin barrier, the markedly reduced expression of both occludin and ZO-1 after *Eh* infection in *Muc2*^{-/-} mice leads to a loss of barrier integrity, facilitating the movement of serum proteins into the intestinal lumen. Our data further indicate that changes in intestinal permeability in the absence of direct contact are mediated primarily by the virulence factor EhCP-A5, which is secreted by live parasites.

In summary, using *Eh* as a model of a noninvasive pathogen in WT and *Muc2*^{-/-} mice, we have unraveled novel information on how colonic MUC-2 plays a critical role in both luminal and epithelial barrier functions. In WT mice with an intact mucin barrier, *Eh* stimulated mucin secretion and proinflammatory cytokines aimed at sequestering the parasites away from the epithelium. In *Muc2*^{-/-} mice, however, *Eh* induced an aberrant proinflammatory response concomitant with altered expression of claudin-2, occludin, and ZO-1 leading to activation of the pore and leak pathways, resulting in a robust secretory response (presumably aimed at flushing out the parasites). Although the net effect may function to prevent parasite colonization and/or invasion in *Muc2*^{-/-} mice, the mechanisms by which this was achieved may have contributed to the initial pathological responses. Thus, with the present study we have defined for the first time a mechanistic role for MUC-2 in conferring epithelial barrier function in innate host defenses against *Eh*.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2012.11.035>.

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