Colonic MUC2 mucin regulates the expression and antimicrobial activity of β-defensin 2

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In this study we identified mechanisms at the colonic mucosa by which MUC2 mucin regulated the production of β-defensin in a proinflammatory milieu but functionally protected susceptible bacteria from its antimicrobial effects. The regulator role of MUC2 on production of β-defensin 2 in combination with the proinflammatory cytokine interleukin-1β (IL-1β) was confirmed using purified human colonic MUC2 mucin and colonic goblet cells short hairpin RNA (shRNA) silenced for MUC2. In vivo, Muc2−/− mice showed impaired β-defensin mRNA expression and peptide localization in the colon as compared with Muc2+/− and Muc2+/+ littermates. Importantly, purified MUC2 mucin abrogated the antimicrobial activity of β-defensin 2 against nonpathogenic and enteropathogenic Escherichia coli. Sodium metaperiodate oxidation of MUC2 removed the capacity of MUC2 to stimulate β-defensin production and MUC2's inhibition of defensin antimicrobial activity. This study highlights that a defective MUC2 mucin barrier, typical in inflammatory bowel diseases, may lead to deficient stimulation of β-defensin 2 and an unbalanced microbiota that favor the growth of β-defensin-resistant microbes such as Clostridium difficile.

INTRODUCTION

The colonic mucosa is covered by gel-forming glycosylated MUC2 mucin secreted by goblet cells that is organized as an inner sterile layer firmly attached to the epithelium and an outer layer with an expanded volume and colonized by bacteria.1 Embedded in colonic mucin are host defensin peptides, a group of small molecules (12–50 amino acids) divided into subgroups on the basis of their amino-acid composition and structure. Cationic defensin peptides are classified as α or β based on their size and intramolecular disulfide bond patterns between six cysteines.2 Defensins are of interest because they are effective in killing a broad spectrum of microbes, including Escherichia coli,3 and have immune modulatory functions including chemotaxis of dendritic and T cells.4 In the colon, human β-defensin 2 (hBD-2) secreted by epithelial cells are the main representative of the host defensin peptides and are modulated by inflammatory and bacterial stimuli.5,6

The microbiota establishes a symbiotic and healthy relationship in the colonic lumen,7 although it is constantly exposed to mucin and antimicrobial peptides. Modulation between colonic MUC2 mucin and β-defensin and their influence on the microbiota is unknown, although their dysfunction contributes to the pathogenesis of inflammatory bowel diseases. In ulcerative colitis, expression of β-defensin mRNA is increased in epithelial cells,8,9 and the adherent mucus gel is thin and even denuded in patches10,11 with lower goblet cell numbers.12 In Crohn’s disease, the colonic mucin layers appear normal but glycosylation patterns and functional properties are altered10,11 and β-defensin expression is diminished.8,9 Moreover, microbiota dysbiosis with decreased complexity of the microbial ecosystem is characteristic of both Crohn’s disease and ulcerative colitis.13 These studies suggest that there is a fine balance in the colon between MUC2 mucin and antimicrobial peptides that function together to prevent colonization by pathogens and at the same time protect the indigenous microbiota. To gain insights into the innate host defense mechanisms at the surface of the colonic mucosa, we studied the interaction between MUC2 mucin and β-defensin 2 in association with commensal microbiota or disease. Here we show that MUC2 mucin plays a dual functional role in the colon to stimulate β-defensin production in the presence of the proinflammatory cytokine interleukin-1β (IL-1β) and adenosine triphosphate (ATP) and at the same time protects...
otherwise β-defensin susceptible enteropathogenic E. coli (EPEC) or nonpathogenic E. coli.

RESULTS

MUC2 mucin in combination with proinflammatory IL-1β stimulates hBD-2 in colonic epithelial cells

The modulatory effect of MUC2 mucin on β-defensin production was determined in cultured colonic cells under conditions that represent proinflammatory or physiological states. Inflammatory conditions were represented by IL-1β, a proinflammatory cytokine released by macrophages and dendritic cells associated with colitis, and extracellular ATP, a danger molecule either from stressed, injured, and necrotic intestinal cells or from bacteria. Physiological conditions were represented by sodium butyrate, a normal colonic probiotic product and primary fuel of colonocytes associated with prevention of colorectal diseases. These stimulatory conditions were assessed in human colonic epithelial adenocarcinoma HT-29 cells that express hBD-2 in response to inflammatory and bacterial stimuli and constitutively express low levels of MUC2 mRNA and secrete small amounts of mucin.

To determine whether MUC2 mucin can regulate hBD-2 expression and peptide production, cells were stimulated with purified secreted MUC2 mucin isolated from human colon adenocarcinoma LS 174T goblet cells (Supplementary Figure S1A–C online). The purified LS 174T MUC2 mucin showed no contaminating MUC5AC or MUC5B or other proteins (Supplementary Figure S1D,E). Purified MUC2 mucin alone (up to 40 μg ml⁻¹) caused a modest increase in hBD-2 mRNA (Figure 1a) and a greater effect on hBD-2 peptide release in HT-29 cells (Figure 2a) but none of these differences were significant. However, from preliminary studies, we have established that stimulating HT-29 cells with graded dosages of IL-1β (0–40 ng ml⁻¹), butyrate (0–40 mM), and ATP (0–10 mM) alone for up to 16 h show that 20 ng ml⁻¹ of IL-1β and 20 mM of butyrate induced modest hBD-2 mRNA expression (~4- and 2.5-fold change, respectively), whereas ATP did not (data not shown). Based on these observations we then investigated whether simultaneous stimulation with these mediators plus exogenously added MUC2 mucin could influence hBD-2. Our results showed that simultaneous addition of MUC2 mucin (≥ 10 μg ml⁻¹) and IL-1β (20 ng ml⁻¹) for 16 h induced significantly elevated hBD-2 mRNA expression (eighthfold change; Figure 1b) and peptide release (Figure 2b). Similarly, ATP (7.5 mM) simultaneously cocultured with MUC2 mucin (with 0.7–3 μg ml⁻¹ of MUC2) for 16 h increased hBD-2 mRNA and peptide release (with ≥ 25 μg ml⁻¹ of MUC2; Figures 1c and 2c). In contrast, cells incubated with the highest tested dose of butyrate (20 mM) plus exogenously added MUC2 had no effect on hBD-2 (Figures 1d and 2d). The stimulatory effect on hBD-2 expression by IL-1β (20 ng ml⁻¹) or ATP (7.5 mM) in combination with MUC2 (40 and 3 μg ml⁻¹ for IL-1β and ATP, respectively) was completely inhibited by bisindolylmaleimide I, a highly selective, cell-permeable, and reversible protein kinase C (PKC) inhibitor (Figure 1e,f).

By confocal immunofluorescence, low intracellular localization of hBD-2 was homogenously seen in the cytoplasm of control HT-29 cells. However, cells treated with MUC2 mucin (20 μg ml⁻¹) and either IL-1β (20 ng ml⁻¹) or ATP (7.5 μM), but not butyrate (20 mM), showed relatively more intense hBD-2 immunoreactivity as compared with untreated cells, IgG controls, or cells treated individually with MUC2, IL-1β, or ATP (Figure 3).

To confirm specificity for MUC2 in regulating hBD-2, we used a high mucin-producing clone of HT-29 (clone HT-29 C16) designated MUC2 H and a derivate clone silenced for MUC2 by short hairpin RNA (shRNA) designated MUC2 L. MUC2 L showed 90% reduction in MUC2 mRNA expression as compared with MUC2 H cells with no significant concomitant compensatory effects on MUC5AC and MUC5B mRNA expression (Supplementary Figure S2A). As predicted, MUC2 H expressed higher basal hBD-2 mRNA levels (Figure 4a,c,d), and stimulation with 20 or 30 ng ml⁻¹ of IL-1β for 16 h induced significantly elevated hBD-2 mRNA expression (eighthfold change) as compared with MUC2 L cells (Figure 4a). These data were corroborated by immunofluorescence studies that demonstrated higher basal levels of intracytoplasmic accumulation of hBD-2 in MUC2 H cells that increased after stimulation with IL-1β (20 μg ml⁻¹) for 16 h (Figure 4b). The hBD-2 mRNA levels or peptide cytoplasmic accumulation was absent or low in resting or IL-1β-stimulated MUC2 L cells (Figure 4a,b). Stimulation with 20 mM of sodium butyrate (0–40 mM) for 16 h induced modest hBD-2 mRNA expression in MUC2 H cells (threefold change; Figure 4c), whereas stimulation with ATP (0–10 μM) did not affect hBD-2 mRNA levels in neither MUC2 H nor MUC2 L cells (Figure 4d).

MUC2 mucin allows pathogenic and nonpathogenic E. coli to resist the antimicrobial effects of β-defensin 2

Human hBD-2 has broad antimicrobial effects against Gram-positive and Gram-negative bacteria, and thus it was of interest to determine whether MUC2 mucin could regulate the activity of hBD-2 against commensal and/or pathogenic bacteria. As shown, nonpathogenic E. coli (HB101; Figure 5a) and EPEC (Figure 5b) were killed with as little as 1 μM of hBD-2, whereas the probiotic anaerobe Bacteroides vulgatus (Figure 5c) and pathogenic Clostridium difficile (Figure 5d) were resistant even at concentrations up to 4 μM. Interestingly, increasing the concentration of MUC2 mucin allowed E. coli to survive under otherwise lethal dosages of hBD-2 (Figure 5a,b). To exclude the possibility of steric interference and specificity for MUC2 mucin, we used pig gastric mucin at equimolar concentrations as MUC2 and showed partial protection at inhibiting E. coli against hBD-2 (Figure 5a,b). In particular, higher concentrations of MUC2 mucin (100 μg) significantly protected E. coli against hBD-2, whereas pig gastric mucin did not (Figure 5a,b). MUC2 mucin had no effect against B. vulgatus (Figure 5c) and C. difficile (Figure 5d). As the antimicrobial effect of cationic peptides such as hBD-2 on E. coli is mostly based on the capacity to cause membrane pore
formation and ultrastructural alterations, we investigated whether MUC2 mucin could prevent defensin-induced damage. To do this, EPEC was treated with hBD-2 and/or MUC2 mucin, and scanning electron microscopy showed that untreated bacteria (Figure 6a) or bacteria treated with MUC2 mucin alone (Figure 6b) or simultaneously with MUC2 mucin and hBD-2 (Figure 6d) showed normal rod-shaped elongated bacteria with smooth intact surfaces. In stark contrast, E. coli exposed to hBD-2 alone showed shortened and severe membrane alterations (Figure 6d), including multiple protrusions, dimples, and blisters on the surface (Figure 6e) that eventually ended in shrunken and dead bacteria in a debris field (Figure 6f). Microscopic quantification of 100 bacteria exposed to hBD-2 alone showed significant \( P<0.05 \) signs of membrane damage as compared with bacteria treated with hBD-2 + MUC2 mucin or MUC2 mucin alone or those that were left untreated.

The role of colonic mucin favoring bacterial growth was confirmed \textit{ex vivo} by coculturing EPEC with crude colonic mucosal contents of mice. In this assay, colonic luminal secretions from Muc2\(^{+/+}\) and Muc2\(^{+/-}\) but not from Muc2\(^{-/-}\) mice significantly increased the number of surviving E. coli after incubation for 2 h (Figure 7a). To investigate the length of time that MUC2 needed to be in contact with hBD-2
Figure 2  Interleukin-1β (IL-1β) and adenosine triphosphate (ATP) but not butyrate stimulate β-defensin secretion in the presence of MUC2 mucin. HT-29 cells were incubated with (a) purified secreted colonic MUC2 mucin only or in combination with either (b) IL-1β (20 ng ml⁻¹), (c) ATP (7.5 μM), or (d) butyrate (20 mM) for 16 h in serum-free medium as detailed in Figure 1. Secretion of human β-defensin 2 (hBD-2) peptide was quantified by enzyme-linked immunosorbent assay (ELISA) from the supernatants of colonic cells. Samples were duplicated and experiments were replicated at least two times. One-way analysis of variance (ANOVA) and post Tukey’s multiple comparison tests were run to determine significant differences among groups. P-values for all significant comparisons with control (*P<0.05) are represented.

Figure 3  Confocal intracellular localization of β-defensin induced by interleukin-1β (IL-1β), adenosine triphosphate (ATP), and MUC2 mucin in colonic cells. HT-29 cells were incubated with IL-1β (20 ng ml⁻¹), ATP (7.5 μM), or butyrate (20 mM) only (upper panel) or in combination with MUC2 mucin (20 μg ml⁻¹, lower panel) for 16 h in serum-free medium. β-Defensin was stained with an antibody against anti-hBD-2 (yellow) and the nucleus with 4',6-diamidino-2-phenylindole (DAPI; blue). Image is from one of four independent experiments. Scale bar = 10 μm.
to abrogate its antimicrobial effects, EPEC was exposed to hBD-2 and MUC2 mucin simultaneously for up to 60 min (Figure 7b). As shown, although the number of EPEC steadily increased in the presence of MUC2 either with or without hBD-2, hBD-2 alone gradually killed E. coli in the first 60 min in the absence of MUC2 (Figure 7b).

**MUC2 oligosaccharides are the determinant of bacterial antimicrobial protection and β-defensin induction**

Mucin O-linked glycans make up ~80% of the MUC2 mucin mass that serves as binding sites for bacteria. Thus, to determine the importance of MUC2 oligosaccharide moieties in protecting bacteria against β-defensin, mucin carbohydrates were disrupted by gentle oxidization with sodium metaperiodate that cleaves adjacent carbon atoms that contain hydroxyl groups (cis-glycols). As predicted, alterations of MUC2 glycans and perhaps some amino acids by oxidation significantly abrogated the protective effects of MUC2, allowing hBD-2 to effectively kill EPEC (Figure 8a) as compared with untreated MUC2. The role of mannose and sialic acid mucin residues in protecting bacteria from hBD-2 was also investigated because mannose-type N-linked oligosaccharides of rat and rabbit intestinal mucin serve as binding sites for E. coli O157:H7 expressing type 1 mannose-sensitive fimbriae and intestinal mucin oligomannosyl receptors for type 1 pili are covered by noncovalently bound lipid that may involve terminal sialic acid residues. Surprisingly, mannose and sialic acid residues on MUC2 did not significantly protect bacteria against hBD-2, as enzymatic removal of these oligosaccharides did not reduce the number of surviving EPEC in the presence of 1 μM of hBD-2 (Figure 8b). Interestingly, MUC2 mucin oligosaccharides were also found to be critically important in regulating β-defensin as the addition of oxidized MUC2 mucin induced significantly lower hBD-2 mRNA expression (Figure 8c) and peptide release (Figure 8d) in HT-29 cells simultaneously stimulated with IL-1β as compared with native untreated MUC2. To determine the physical interactions between mucin and β-defensin, we coated enzyme-linked immunosorbent assay (ELISA) plates with purified MUC2 mucin and quantified the binding of recombinant hBD-2. Regardless of the concentration of MUC2 used, there was no significant increase in the binding of hBD-2 toward immobilized MUC2 (Supplementary Figure S2B).

**Muc2-deficient mice have reduced β-defensin expression**

To determine the importance of the colonic mucin barrier in the production of defensins, we investigated the expression and production of β-defensins in the mucosa of the proximal colon.

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**Figure 4** Interleukin-1β (IL-1β) and butyrate stimulate human β-defensin 2 (hBD-2) in high MUC2-producing colonic cells. HT-29 H and L cells were incubated with (a, b) IL-1β, (c) butyrate, or (d) adenosine triphosphate (ATP) for 16 h in serum-free medium. (a, c, d) Expression of hBD-2 mRNA was quantified by real-time qRT-PCR and represented as fold change relative to the housekeeping gene. Samples were triplicated and experiments were replicated at least two times. One-way analysis of variance (ANOVA) and post Tukey’s multiple comparison tests were run to determine significant differences among groups. P-values for all significant comparisons with control (*P < 0.05) are represented. (b) HT-29 H and L cells were incubated with IL-1β (20 ng ml⁻¹) for 16 h and β-defensin was stained with antibody anti-hBD-2 (yellow) and nucleus with 4’,6-diamidino-2-phenylindole (DAPI; blue). Image is from one of four independent experiments. Scale bar = 10 μm.
of $\text{Muc}2^{+/+}$ and $\text{Muc}2^{+-}$ mice with a well-defined and intact mucus layer and in $\text{Muc}2^{-/-}$ littermates in the absence of mucus (Figure 9a). β-defensin peptide was distributed uniformly along the lumen and apical surface of the colonic cells and not restricted to goblet cells in the mucosa of $\text{Muc}2^{+/+}$ and $\text{Muc}2^{+-}$ mice (Figure 9b and Supplementary Figure S2C), whereas it was
Samples were triplicated and experiments were conducted using bacterial adenosine triphosphate (ATP) and represented by the logarithm of the number of bacteria against the antimicrobial effects of MUC2 mucin (20 μg/mL) or control MT-LB buffer for 2 h. P-values for all significant comparisons (*P < 0.05) are represented.

(b) In vitro coculture of EPEC with human β-defensin 2 (hBD-2; 2 μM) and Muc2 mucin (20 μg/mL) for 1 h revealed that mucin rapidly protects bacteria against the antimicrobial effects of β-defensin. The numbers of surviving colony-forming units (CFUs) were quantified by measuring bacterial adenosine triphosphate (ATP) and represented by the logarithm to base 10 (log10). Samples were triplicated and experiments were replicated at least three times. One-way analysis of variance (ANOVA) and post Tukey’s multiple comparison tests were run to determine significant differences among groups. P-values for all significant comparisons (*P < 0.05) are represented.

Figure 7 The survival of E. coli against hBD-2 in the presence of colonic contents or purified MUC2 mucin. (a) Colonic contents from mice with an intact mucin barrier enhance the growth of *Escherichia coli*. Enteropathogenic *Escherichia coli* (EPEC) were incubated with luminal mucosal contents derived from the colons of Muc2+/+, Muc2+/−, and Muc2−/− littermates (total 20 μg protein) or control MT-LB buffer for 2 h. P-values for all significant comparisons (*P < 0.05) are represented. (b) In vitro coculture of EPEC with human β-defensin 2 (hBD-2; 2 μM) and Muc2 mucin (20 μg/mL) for 1 h revealed that mucin rapidly protects bacteria against the antimicrobial effects of β-defensin. The numbers of surviving colony-forming units (CFUs) were quantified by measuring bacterial adenosine triphosphate (ATP) and represented by the logarithm to base 10 (log10). Samples were triplicated and experiments were replicated at least three times. One-way analysis of variance (ANOVA) and post Tukey’s multiple comparison tests were run to determine significant differences among groups. P-values for all significant comparisons (*P < 0.05) are represented.

Restrained to a minimum presence in the colonic lumen of Muc2−/− littermates (Figure 9b). Similarly, basal mRNA expression of β-defensin 4 (orthologous of hBD-2)28,29 and 14 (orthologous of hBD-3)30 was significantly reduced in the colonic mucosa of Muc2−/− mice as compared with Muc2+/− and Muc2+/+ littermates (Figure 9c,d).

DISCUSSION

Our results unraveled an important regulatory role for MUC2 mucin in hBD-2 mRNA and peptide production in a pro-inflammatory milieu. The hBD-2 mRNA expression, peptide release, and intracellular localization in colonic HT-29 cells that constitutively express low mucin20,21 was enhanced following stimulation with the proinflammatory cytokine IL-1β and/or ATP in association with exogenously added colonic MUC2 mucin. The MUC2 mucin used in this study purified from colonic LS 174T cells31 contained very high levels of immunoreactive MUC2. Although the presence of small amounts of contaminating proteins in this preparation cannot be excluded, our studies showed no major contaminating proteins visualized by silver staining and no immunoreactive MUC5AC or MUC5B. The participatory role of MUC2 in inducing β-defensin was confirmed using high MUC2-producing HT-29 cells where IL-1β increased hBD-2 mRNA expression and intracellular peptide accumulation. Previous studies have shown that hBD-2 expression was upregulated in the colonic epithelium in response to IL-1β,32,33 but as shown in our study, β-defensin induction by IL-1β may require a healthy MUC2 mucin barrier. Although the synergistic mechanism between MUC2 and defensins is still unknown, we found that MUC2 oligosaccharides facilitated the induction of β-defensin as alteration of MUC2 by oxidation impaired hBD-2 stimulation by IL-1β. As sodium metaperiodate can also oxidize certain amino acids, we cannot exclude those as having an effect in our study. This study also shows that ATP in combination with MUC2 mucin could induce β-defensins in colonic cells. This is important as extracellular ATP, either from stressed, injured, or necrotic intestinal cells19 or from bacteria,17 constitutes a danger signal that, based on our finding, may regulate hBD-2 in the colon in coordination with MUC2 mucin. Similar amounts of intestinal extracellular ATP were noted in the intestine of mice with epithelial cells deficient in ectonucleoside triphosphate diphosphohydrolases.16 Thus, severe inflammation or necrosis or defect in ATP hydrolysis in the colon may induce enough extracellular ATP that induces hBD-2 in coordination with MUC2. Intestinal commensal bacteria can also produce luminal ATP17 but are insufficient at low concentrations to stimulate hBD-2 based on our findings. We also found a nonsynchronized dose response between hBD-2 mRNA and peptide secretion following stimulation with MUC2 and IL-1β/ATP. This implies differential mechanisms of expression and synthesis in response to the respective agonists and possible roles of ATP as a goblet cell secretagogue that increases the amount of mucin and the synergic effect. Furthermore, high doses of ATP may have a negative impact in MUC2 H cells to induce MUC2 production and it was dependent on the addition of exogenous mucin. Surprisingly, sodium butyrate, a physiological metabolite produced by colonic probiotic bacteria, showed only a limited effect on hBD-2 in MUC2 H cells, perhaps as a result of the ability of butyrate to induce cell differentiation and the production of even more MUC2, but it was refractory to further stimulation with exogenously added MUC2. We speculate that hBD-2 was not induced by butyrate as a mechanism to prevent exaggerated or harmful reactions to the normal microbiota. In agreement, sodium butyrate was shown to exhibit other anti-inflammatory functions, by inhibiting the expression of proinflammatory cytokines.33
nuclear factor (NF)-κB have been implicated in the expression of hBD-2 in human lung epithelial cells (A549) stimulated with IL-1β. The involvement of diacyl glycerol and calcium-responsive PKC and NF-κB in the regulation of hBD-2 seems to be common to various stimuli as it was also reported in mononuclear phagocytes in response to lipopolysaccharide and A549 cells infected with Mycobacterium bovis bacillus Calmette–Guerin. At present, we do not know the exact mechanism of how exogenous MUC2 mucin regulates hBD-2 mRNA expression. Although PKC/NF-κB mechanisms are expected for IL-1β, ATP in synergy with mucin seems also to be at least, in part, mediated through activation of similar signaling proteins.

Our in vivo mouse model underscores the importance of MUC2 for proper β-defensin stimulation as Muc2-deficient animals expressed less β-defensin mRNA and peptide expression than heterozygous and wild-type (WT) littermates with an intact mucin barrier. The reduced baseline production of β-defensin in Muc2-deficient mice correlates with an impaired response to proinflammatory mediators (e.g., IL-1β) as showed in vitro in human colonic MUC2 L cells (Figure 4). Altered colonic β-defensin expression with decreased antimicrobial capacity was also described in Nlrp3-deficient mice in a dextran sulfate sodium colitis model. Other host antimicrobial peptides may respond distinctively in the colon of Muc2−/− mice as the expression of cathelicidins did not differ among Muc2−/− and WT mice, whereas the expression of anti-microbial C-type lectin Reg3b, Reg3c, and Ang4 peptides was higher in the distal colon of Muc2−/− compared with WT but similar in the proximal colon. The Muc2-deficient murine model is relevant to study the biology of mucin in epithelial barrier function and the influence of MUC2 in the expression of host defense peptides. This intestinal mucin model suggests that the patched and incomplete adherent mucus gel seen in ulcerative colitis accompanied by high proinflammatory cytokines (e.g., IL-1β) can increase the expression of hBD-2 in epithelial cells.

At the functional level, we found that MUC2 mucin protected enteric bacteria from the antimicrobial effect of β-defensin. EPEC and nonpathogenic E. coli are highly

Figure 8 Mucin oligosaccharide moieties are critical in regulating β-defensin induction and in protecting bacteria. Enteropathogenic Escherichia coli (EPEC) were incubated with (a) human β-defensin 2 (hBD-2; 1 μM) or with MUC2 mucin (20 μg ml −1) oxidized by sodium metaperiodate or (b) enzymatically cleaved of sialic acid and mannose. The numbers of surviving colony-forming units (CFUs) were quantified by measuring bacterial adenosine triphosphate (ATP) and represented by the logarithm to base 10 (log 10). P-values for all significant comparison with no mucin control (*P<0.05) are represented. Expression of hBD-2 mRNA quantified by real-time qRT-PCR and represented as fold change relative to the housekeeping gene, and (d) hBD-2 peptide release measured by enzyme-linked immunosorbent assay (ELISA) was determined in HT-29 cells stimulated with interleukin-1β (IL-1β; 20 ng ml −1) and native or oxidized MUC2 mucin (20 μg ml −1). Samples were triplicated and experiments were replicated at least two times. One-way analysis of variance (ANOVA) and post Tukey’s multiple comparison tests were run to determine significant differences among groups. P-values for all significant comparisons with control cells without IL-1β (*P<0.05) are represented.
susceptible to hBD-2, yet they survived in the presence of MUC2 mucin. Importantly, we showed that bacterial membrane alterations and subsequent death caused by the electrostatic interactions between cationic β-defensin and negatively charged surface of EPEC were inhibited in the presence of MUC2 mucin. Bacteria and mucin association was likely determined by bacterial lectins with high affinity for MUC2 N- and O-linked oligosaccharides receptors as the protective effects of mucin was abrogated following sodium metaperiodate oxidation. This agrees with studies in ulcerative colitis patients with an impaired mucus layer that showed severely reduced population of Enterobacteriaceae mostly represented by commensal E. coli.40 In addition, MUC2 mucin allowed the survival of hBD-2-resistant bacteria, including commensal B. vulgatus and C. difficile, the causative agent of antibiotic-associated diarrhea and pseudomembranous colitis.40 Thus, defects in the colonic mucin layer may favor the growth of microbes that resist β-defensin (e.g., C. difficile) over bacteria vulnerable to β-defensin that survived because of the protective effects of mucin. In agreement, C. difficile is more prevalent in patients with ulcerative colitis that have a thinner or depleted mucin layer with concomitant worst prognosis and mortality.41,42 The beneficial effect of colonic mucin on bacterial growth was confirmed ex vivo in which EPEC increased in numbers in the presence of the colonic contents of Muc2+/+ and Muc2−/− but remained stable when cocultured with the colonic contents of Muc2−/− mice. Although the crude mucosal preparations from Muc2+/+ and Muc2−/− littomates may contain other inflammatory factors (e.g., reactive oxygen species) that could influence bacterial growth, our findings agree with studies where cationic peptide extracted from colonic biopsies from patients with ulcerative colitis with an impaired mucin layer were more effective at killing B. vulgatus and E. coli compared with colonic contents from patients with Crohn’s disease with a preserved mucin layer.43

Mechanistically, we showed that MUC2 mucin quickly protected E. coli against a lethal dose of hBD-2 (30–60 min bacteria). This rapid protection was needed as hBD-2 by itself or in association with lysozymes can disrupt bacterial membranes as early as at 30 min.44 Other complex carbohydrates found in the gastrointestinal lumen may also confer partial protective effects on bacteria as E. coli partially survived hBD-2 in the presence of equimolar concentrations of mucin preparation from pig stomach. The crude pig gastric mucin likely contains MUC5AC, MUC6, as well as other glycoproteins (not specified by the manufacturer). Hypothetically, the antimicrobial activity of defensin peptides could be blocked
by electrostatic interactions with mucin in the outer mucin layer that harbors commensal bacteria. In support of this, hBD-1 and hBD-3 were shown to be bound to rectal mucus extracts and had partially reduced antimicrobial efficacy.46 Thus, an impaired mucin layer may lead to more free active antimicrobial peptides, although the lack of MUC2 will progressively affect proper hBD-2 stimulation. However, colonic MUC2 mucin, unlike rectal mucus, did not bind hBD-2, at least under in vitro condition. We hypothesize that the “protective effect” of MUC2 is because bacteria use it as source of energy that can overcome the killing effect of hBD-2. This is based on increased bacterial growth in the presence of MUC2 only or with MUC2/hBD-2 in combination (Figure 5a,b). Moreover, that bacteria is protected by MUC2 is shown by our studies in which periodate treatment abrogated the mucin protective effect and bacteria became more susceptible to be killed by hBD-2 (Figure 5a). Furthermore, the in vivo role of β-defensins in the colonic lumen may serve to prevent harmful inflammatory responses and not merely antimicrobial as exogenous cathelicidin (other major host defense peptide) reduced tissue levels of IL-1β and tumor necrosis factor-α in the colon of dextran sulfate sodium-challenged cathelicidin knockout (Crapm⁻/−) mice.46 Taken together, our studies have unraveled a complex interplay at the mucosal surface of the colon where MUC2 mucin and antimicrobial β-defensin peptides regulate the microbiota. Colonic MUC2 provides a matrix to sustain β-defensin induction in the presence of IL-1β to functionally protect susceptible bacteria from the anti-microbial effects of β-defensin. Defective expression of MUC2 may modify the composition of the commensal microbiota by eliminating a major source of energy and exposing the normal microbiota to the lethal effects of β-defensin. Changes in microbiota composition plus the lack of MUC2 mucin that regulates induction of β-defensin could lead to opportunistic microbial invasion and/or impaired innate responses.

METHODS

Ethics statement. All studies were approved under the University of Calgary Animal Care Committee that adheres to the principles and policies on “Guide to the Care and Use of Experimental Animals” by the Canadian Council on Animal Care.

Purification of human colonic mucin. Secreted mucin was purified from human LS 174T goblet cells as previously described.31 LS 174T cells constitutively express and secrete high levels of MUC2 mRNA (four times more than HT-29)23 and low expression of MUC1 with no MUC2 mucin20,21 were maintained in Dulbecco’s modified Eagle’s medium (12430, Gibco, LifeTechnology, Burlington, ON, Canada) with 10% fetal bovine serum (Benchmark Gemini Bio-Products, Sacramento, CA), sodium pyruvate (1 mM) (11360, Gibco) and penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). To neutralize the enzymatic activity, samples were boiled for 15 min and washed several times with PBS (pH 7.3). Finally, samples were concentrated in Amicon Ultra 3K centrifuge collector tubes (Ultracel Millipore, EMD Millipore, Etobicoke, ON, Canada).

Human colonic epithelial cells. Human colonic adenocarcinoma HT-29 cells (a gift from Dr CI Laboisse Bioetadys, Faculté de Médecine, Université de Nantes, France) that constitutively express low mucin20,21 were maintained in Dulbecco’s modified Eagle’s medium (12430, Gibco, LifeTechnology, Burlington, ON, Canada) with 10% fetal bovine serum (Benchmark Gemini Bio-Products, Sacramento, CA), sodium pyruvate (1 mM) (11360, Gibco) and penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). To neutralize the enzymatic activity, samples were boiled for 15 min and washed several times with PBS (pH 7.3). Finally, samples were concentrated in Amicon Ultra 3K centrifuge collector tubes (Ultracel Millipore, EMD Millipore, Etobicoke, ON, Canada).
MUC5B (sense 5′-TTCTCAGGTTCCAGGTCATC-3′); hBD-2 (DEFB4A NM_004942.2, PPH11010A, RT2 qPCR Primer Assay, Qiagen) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′-TGCA CCACCAACTGCTTACG-3′; antisense, 5′-GGCATGAGCTTGTTGCT ATGAG-3′). For mouse: murine β-defensin 4 (Defb4 NM_019728.4, PPM2917A, RT2 qPCR Primer Assay, Qiagen), murine β-defensin 14 (Defb14 NM_183026.2, PPM41918A, RT2 qPCR Primer Assay, Qiagen), and murine actin (sense, 5′-CTCAAAATGCTGATTCT-3′; antisense, 5′-TGGGGTGTGGAAGCTTC-3′). The reaction mixtures were incubated for 95 °C for 5 min, followed by denaturation for 5 s at 95 °C and combined annealing/extension for 10 s at 60 °C for a total of 40 cycles. Values of target mRNA were corrected relative to the housekeeping gene coding for human GAPDH or murine actin. Data were analyzed using the 2−ΔΔCT methods and expressed as fold changes (mean ± s.e.).

Confocal immunofluorescence microscopy of human colonic cells and murine colon. Murine colonic tissues fixed in Carnoy’s were sectioned (7 μm) and deparaffinized by xylene substitute (Neo-Clear, Millipore), followed by decreasing concentrations of ethanol and running tap water. Slides were boiled in 10 mM sodium citrate (Sigma) plus 0.05% Tween-20 (pH 6) for 20 min in microwave oven for antigen retrieval and then cooled at room temperature (RT) and rinsed in PBS. Free aldehydes were blocked by 0.1 M glycine in PBS for 10 min at RT. Human HT-29 cells grown in 12-well plate were rinsed with PBS and fixed in 4% paraformaldehyde with 150 mM NaCl, 4 mM NaH₂PO₄, and 5 mM KCl (pH 7.4) for 10 min at RT. Murine and human sections were rinsed in cold PBS plus Tween 0.05% (pH 7.2) (PBS-Tw), permeabilized with PBS-Tw plus 0.25% Triton X-100 for 10 min at RT, and rinsed in cold PBS-Tw. Preparations from murine colon and human HT-29 cells were blocked with PBS-Tw, 1% bovine serum albumin, 10% goat serum, 0.3 M glycine, and 0.05% saponin for 2 h at RT and rinsed with PBS-Tw. Murine tissue was blotted with affinity-purified rabbit IgG anti-hBD-2 (Abcam, Cambridge, MA) or with affinity-purified rabbit IgG anti-LS 174T MUC2 antibodies.31 Human HT-29 cells were blotted with affinity-purified rabbit IgG anti-hBD-2 (Abcam) and affinity-purified goat IgG anti-human MUC2 (Santa Cruz) antibodies. Sections were rinsed with cold PBS-Tw. As secondary antibodies, human cells were blotted with DyLight 647-affinity pure donkey anti-rabbit IgG F(ab′)2 fragment-specific antibodies and DyLight 594-affinity pure donkey anti-goat IgG (Fab′)2 fragment-specific antibodies, and murine tissues with DyLight 647-affinity pure donkey anti-rabbit IgG F(ab′)2 fragment-specific antibodies. Secondary antibodies were diluted 1:1,000 in PBS-Tw plus 1% bovine serum albumin and incubated for 1 h at RT. Preparations were rinsed in cold PBS-Tw and nuclei were counterstained with 4,6-diamidino-2-phenylindole. Sections were rinsed with cold PBS-Tw and mounted with FluorSave reagent (Calbiochem, EMB Millipore, Etobicoke, ON, Canada). Slides were examined using a Fluoview FV1000 confocal immunofluorescence microscope (Olympus, Toronto, ON, Canada).

Determination of secreted human β-defensin in human epithelial colonic cells. Secreted hBD-2 peptides were quantified in the supernatant of cultured cells by ELISA as per the manufacturer’s specifications (Peprotech, Cedarlane, Burlington, ON, Canada). Briefly, 96-well ELISA plates were coated with 100 μl of capture antibody diluted at 0.5 μg ml⁻¹ overnight at RT and blocked with 1% bovine serum albumin in PBS for 2 h at RT. Plates were incubated in quadruplicate with 100 μl of supernatant of cultured cells for 2 h at RT and then with 100 μl of detection antibody diluted at 0.5 μg ml⁻¹ for 2 h at RT. After incubation with 100 μl of avidin peroxidase diluted 1:2,000 for 30 min at RT, the color was developed by incubating plates with 100 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma) for 15 min at RT and substrate stopped by the addition of 50 μl of 2 M H₂SO₄. Absorbance was read at 450/550 nm on an ELISA microplate reader (Bio-Rad Laboratories, Hercules, CA). Several washes with PBS-Tw were done after each step.
An ELISA kit was modified to detect binding of hBD-2 peptides to mucin. ELISA plates were coated with different concentrations of purified MUC2 mucin diluted in 100 μl of coating buffer (50 mM NaHCO₃/Na₂CO₃, pH 9.6) in 96 wells overnight at 4 °C, washed, then incubated with 200 pg of rhBD-2 for 2 h. The supernatant (i.e., containing unbound hBD-2 peptides) was collected and immediately processed for detection of hBD-2 peptides as described above. That MUC2 effectively attached to the wells was verified in previous studies in which only 10% of the added MUC2 was recovered after incubation overnight at 4 °C. For all ELISAs, a standard curve comprised recombinant hBD-2 (Peprotech) and doubling dilutions from 1 to 200 pg ml⁻¹ was included to calculate the hBD-2 peptide concentration in the samples.

**Bacterial cultures.** Nonpathogenic K-12 derivate HB101 E. coli and enteropathogenic O127:H6 (E2348/69 EPEC) were cultured in LB agar. *B. vulgaris* (ATCC 8482) and *Clostridium difficile* (R20291) were cultured in anaerobiosis to fastidious anaerobe agar (Lab909, LabM, Lancashire, UK).

**Antimicrobial activity of β-defensins.** Fresh *E. coli* colonies were grown in tryptic soy broth (2209 Fluka, Sigma) in aerobic conditions for 2 h at 37 °C with vigorous shaking (300 r.p.m.). Colonies of *B. vulgaris* and *C. difficile* were grown in brain heart infusion (BD Canada, Mississauga, ON, Canada, Difco) in anaerobiosis for 48 h at 37 °C. For the antimicrobial test, *E. coli* was diluted by a 600 spectrophotometry to a final concentration of 1 x 10⁶ colony-forming units per ml in a MT-LB buffer containing 50 mM NaHCO₃ (Sigma), 10 mM disodium hydrogen phosphate, 5 mM sodium dihydrogen phosphate, plus 1% LB broth. *B. vulgaris* and *C. difficile* were diluted to 60 x 10⁶ colony-forming units in the same buffer medium but with 18% brain heart infusion broth. The bacterial suspensions were incubated with recombinant hBD-2 (Peprotech) alone or coincubated with MUC2 mucin or type III mucin from porcine stomach (Sigma M1778) for 2 h at 37 °C with constant agitation. Surviving colony-forming units were quantified by incubating the bacterial suspension (8 μl) with CellTiter-Glo Luminescent Cell Viability assay substrate (Promega, Madison, WI) (35 μl) in white solid 96-well plates (Greiner Bio-One, Monroe, NC) and luminescence measured in a luminometer (Victor2, Wallac 1420 3.0, PerkinElmer, Guelph, ON, Canada). The number of bacteria was calculated by a standard curve with known number of bacteria and checked by counting colonies in dilutions plated directly onto LB agar or fastidious anaerobe agar after incubation for 1 to 2 days at 37 °C.

**Murine mucin secretion in vivo studies.** To quantify an in vivo role for colonic mucin in protecting bacteria against β-defensin, fresh colonic contents were obtained from Muc2+/−, Muc2−/−, and Muc2−/− littermates. Mice were fasted overnight and killed and the colon was excised and opened longitudinally. Colonic mucosal secretion was obtained by gently scraping and flushing with PBS. Samples were vortexed and centrifuged (1,000 g, 10 min, 4 °C) to remove cellular debris and immediately frozen at −70 °C. Surviving EPEC after incubation with murine colonic contents (total 20 μg protein) was calculated by CellTiter-Glo Luminescent Cell Viability assay (Promega) as described above.

**Scanning electron microscopy.** EPEC (10⁶ per ml) incubated with MT-LB buffer only or in combination with hBD-2 (2 μM) and/or MUC2 mucin (20 μg ml⁻¹) for 2 h were rinsed and spin at 4,000 g with PBS. Bacteria were fixed with 2.5% in 0.2 M cacodylate buffer for 45 min at 4 °C and washed three times with 0.2 M cacodylate buffer. Bacteria were dehydrated through ethanol gradient (30, 50, 70, 80, 90, 95, 100%, and 100%) and the critical-point dry achieved by increasing gradient with hexamethyldisilazane (HMDS) (100% ethanol; 2:1 ethanol: HMDS; 1:2 ethanol: HMDS and 100% HMDS). Bacteria were sputter coated with gold palladium and imaged using a FEI XL30 scanning electron microscope. Profiles showing membrane damage and shortening was estimated by examining 100 bacteria/condition in microscopic fields randomly chosen through observation and statistically analyzed by nonparametric χ² tests.

**Statistical analysis.** Differences between groups were analyzed by unpaired t-test or analysis of variance and Tukey’s post-tests (GraphPad Prism 5.0 Mac, GraphPad Software Inc, La Jolla, CA). Graphs represent two to three independent experiments, and error bars represent means ± s.e. Differences in values were considered significant when P was <0.05.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at http://www.nature.com/mi

**ACKNOWLEDGMENTS**

We thank Michael Schoel from the Microscopy and Imaging facility at University of Calgary for his assistance in the SEM studies. This work was supported by a grant from the Canadian Institute of Health Research (CIHR) to K.C. E.R.C. was supported by an Alberta Innovates-Health Solutions Fellowship Award. The Live Cell Imaging Facility is funded by an equipment and infrastructure grant from the Canadian Foundation Innovation (CFI) and the Alberta Science and Research Authority.

**AUTHOR CONTRIBUTIONS**

E.R.C. and K.C. conceived, designed, and performed the experiments and analyzed the data. V.K.-S. performed the animal experiments. F.M. performed mucin isolation and purification. E.R.C. and K.C. wrote the manuscript.

**DISCLOSURE**

The authors declared no conflict of interest.

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