JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Hen Egg Lysozyme Attenuates Inflammation and Modulates Local Gene Expression in a Porcine Model of Dextran Sodium Sulfate (DSS)-Induced Colitis

MAGGIE LEE, JENNIFER KOVACS-NOLAN, CHENGBO YANG, TANIA ARCHBOLD, MING Z. FAN, AND YOSHINORI MINE*

Department of Food Science and Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Inflammatory bowel disease (IBD) is a chronic and recurring inflammation of the gastrointestinal tract, associated with a dysregulation of the mucosal immune system. There is an increasing prevalence of IBD; however, current pharmaceutical treatments are only moderately effective and have been associated with potential long-term toxicity. Lysozyme, a well-known antimicrobial protein found in large quantities in hen egg white, is a promising alternative for the treatment of IBD. A porcine model of dextran sodium sulfate (DSS)-induced colitis was used to examine the effect of hen egg lysozyme (HEL) supplementation on intestinal inflammation. Treatment with DSS resulted in weight loss, severe mucosal and submucosal inflammation, colonic crypt distortion, muscle wall thickening, down-regulation of mucin gene expression, and increased gastric permeability, but these symptoms were attenuated following supplementation with HEL and restored to basal levels observed in untreated control animals. Treatment with HEL also significantly reduced the local expression of pro-inflammatory cytokines TNF-α, IL-6, IFN-γ, IL-8, and IL-17 while increasing the expression of the anti-inflammatory mediators IL-4 and TGF-β, indicating that HEL may function as a potent anti-inflammatory and immunomodulator. Furthermore, the concomitant increases in TGF-β and Foxp3 levels suggest that HEL may aid in restoring gut homeostasis by activating regulatory T cells, which are important in the regulation of the mucosal immune system. These results suggest that HEL is a promising novel therapeutic for the treatment of IBD.

KEYWORDS: Inflammatory bowel disease (IBD); dextran sodium sulfate (DSS); colitis; lysozyme; anti-inflammatory

INTRODUCTION

Inflammatory bowel disease (IBD), a chronic and relapsing inflammation of the gastrointestinal tract, affects millions of people worldwide (*1*). The two forms of IBD include Crohn's disease (CD) and ulcerative colitis (UC). Although the causes of IBD are still unknown, genetic and environmental factors, infectious agents, impairment of local tolerance, and mucosal imbalance leading to an ongoing activation of the mucosal immune system have been suggested to play a role (*1, 2*). The resulting imbalance of the mucosal immune system causes an overproduction of inflammatory cytokines, reactive oxygen metabolites, growth factors, adhesion molecules, and trafficking of effector leukocytes into the intestine, thus leading to uncontrolled intestinal inflammation and tissue damage (*3, 4*).

At present, the pharmacologic agents available for the treatment of IBD include corticosteroids and immunosuppressive agents; however, they have limited therapeutic efficacy and have been associated with severe side effects and long-term toxicity (*5*). In fact, population-based studies have shown that a significant portion of corticosteroid-treated patients develop steroid dependency or even steroid-refractory illness, while the need for surgical intervention has remained unchanged (*5*). Therefore, there is a need for novel safe and effective therapeutic agents for the treatment of IBD. Dietary nutrients may be involved in the modulation of the immune response, and it has been suggested that dietary management may be an attractive alternative to drug therapy of IBD (*4*).

Hen egg lysozyme (HEL) is well-known for its antimicrobial properties (*6*) and acts as a muramidase, hydrolyzing the $\beta 1-4$ linkage between *N*-acetylmuramate and *N*-acetylglycosamine in the peptidoglycan layer of Gram-positive bacteria (*7*). It is a unique protein, with a small compact globular structure and a net positive charge at physiological pH. It has 19 positively charged amino acid residues and an isoelectric point (p*I*) of 10.7. Lysozyme and lysozyme-derived peptides have demonstrated

* Corresponding author [telephone (519) 824-4120, ext. 52901; fax (519) 824-6631; e-mail ymine@uoguelph.ca.

10.1021/jf803133b CCC: $40.75
Published on Web 02/20/2009

DNA-binding activity, and it has been suggested that the interaction of lysozyme with DNA molecules may interfere with DNA replication, modulate gene expression, and block bacterial and viral infections, contributing to its innate immune modulating activities (*8*). HEL belongs to the family of host defense proteins known as defensins, antimicrobial proteins that play an important role in host innate immunity. Recent evidence has indicated that patients with IBD have a reduced capacity to express mucosal defensins (*9, 10*). Furthermore, HEL has been shown to protect against oxidative stress-induced tissue damage, by suppressing the generation of reactive oxygen species and decreasing the expression of oxidative stress genes (*11*), and to stimulate gastric mucus production and prevent mucosal tissue damage in atherosclerotic rats (*12*), suggesting that HEL may be an attractive candidate as a novel therapeutic for the treatment of IBD.

Dextran sodium sulfate (DSS) is commonly used in rodent models to chemically induce intestinal inflammation and is characterized by weight loss, bloody diarrhea, epithelial cell damage, mucosal ulcers, and neutrophil infiltration, as well as an increased production of inflammatory cytokines including interleukin (IL)-12, interferon (IFN)-γ, and IL-1β (*13, 14*). Although it does not induce the characteristic T helper (Th) 2-biased immune response typically found in ulcerative colitis (*15*), it has been widely used to evaluate therapeutic agents and study disease pathogenesis. Recently, a porcine model of DSS-induced colitis was described (*16, 17*). Pigs share a similar gastrointestinal morphology and physiology with humans (*18*) and, therefore, may be a more suitable model for the evaluation of IBD therapeutics. Furthermore, using an outbred animal model, such as pigs, should allow for a better indication of potential efficacy in humans.

In the present study we examined the ability of HEL to reduce DSS-induced colitis symptoms and pathology and evaluated the effect of HEL supplementation on local gene expression using a porcine model of experimental colitis.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise specified.

Animals and Experimental Design. Four-day-old Yorkshire piglets were obtained from the University of Guelph Arkell Swine Research Station (Guelph, ON, Canada). During the study piglets were housed individually in steel cages equipped with heating lamps and fed three times a day with a commercial milk replacement formula (Soweena Litter Life; Merrich's Inc., Middleton, WI). Animals were surgically fitted with an intragastric catheter (Micro-Renathane, o.d. = 0.8 mm, Braintree Scientific, Inc., Braintree, MA). Following a three-day recovery period, animals were randomly assigned into one of three groups (negative control (Neg), $n = 6$; positive control (Pos), $n = 8$; HEL treatment (HEL), $n = 7$) and infused with dextran sodium sulfate (DSS) (MP Biomedicals, Solon, OH) or saline for 5 days, followed by infusion with HEL or saline. All procedures were carried out in accordance with the Canadian Council of Animal Care's Guide to the Care and Use of Experimental Animals and were approved by the University of Guelph Animal Care Committee.

Induction of Colitis and HEL Administration. To induce experimental colitis, animals were infused with DSS dissolved in saline at 1.25 g of DSS/kg of body weight (BW) via an intragastric catheter for a period of 5 days. The DSS dose was determined from previous studies using DSS in piglets (*19*). Negative control animals received only sterile saline during this period. Following 5 days of DSS infusion, animals in the HEL group were infused with hen egg lysozyme (Inovatech Egg Products, Abbotsford, BC, Canada) at a dose of 150 mg/kg of BW dissolved in saline, for 5 days. The dose of HEL was determined to be the minimal dose required to achieve therapeutic effects as estimated from previous in vitro studies (*11*).

Animals in the Neg and Pos groups received sterile saline. All of the infusions were heated to 37 °C to minimize discomfort. Animals were euthanized, and colon tissues were collected and rinsed with protease inhibitor phenylmethanesulfonyl fluoride (PMSF) in saline. Colon tissues were stored in 10% formalin for histological analysis or flash frozen in liquid nitrogen for enzyme-linked immunosorbent assay (ELISA) and real-time RT-PCR analyses.

Physical Assessment of Inflammation. Animals were monitored daily, and body weight (BW), food intake, and stool consistencies were recorded.

In Vivo Gut Permeability Analysis. Gut permeability was measured as described previously by Thymann et al. (*20*), with modifications. Briefly, on the last day of treatment pigs were infused with 0.6 g/kg of BW D-mannitol. Blood was collected at 0, 35, and 70 min postinfusion via the suborbital sinus into heparinized tubes, centrifuged at 800*g* for 5 min to obtain plasma, and stored at −20 °C until further analysis.

The measurement of plasma D-mannitol concentrations was adapted from previous studies (*21, 22*). Briefly, plasma was boiled for 5 min and centrifuged at 15000 rpm for 60 min, and the supernatant was combined with β-nicotinamide adenine dinucleotide sodium salt (β-NAD) and D-mannitol dehydrogenase (Megazyme International, Wicklow, Ireland), at final concentrations of 20 μmol/mL and 0.1 unit/mL, respectively. Samples were incubated for 150 min at 40 °C, and NADH production was measured spectrophotometrically at 340 nm. D-Mannitol concentrations were determined from a standard curve.

Histological Analysis. Immediately following sacrifice, colon tissues were placed into 10% formalin for 24 h and transferred to 70% ethanol. Approximately five to six tissue cross sections of 2−3 mm thickness were cut and placed into histology cassettes in 70% ethanol. Tissues were fixed onto slides and stained with hemotoxylin and eosin (H&E). Slides were examined using a Leica DMR microscope (Leica Microsystems GmbH, Wetzlar, Germany), and muscle thickness was analyzed using Openlab 4.0.4 software (Improvision, Coventry, U.K.).

Measurement of TNF-α and IL-6 Concentrations in Colonic Tissue. Colon tissues were manually ground in liquid nitrogen using a mortar and pestle. Pulverized tissue (2 g) was homogenized in 3 mL of HBSS containing 2 μg/mL *N*-tosyl-L-phenylalanine chloromethyl ketone, 2 μg/mL *N*-α-*p*-tosyl-L-lysine ketone, 2 μg/mL leupeptin, 2 μg/mL hemisulfate, 2 μg/mL aprotinin, 2 μg/mL pepstatin A, and 100 mM PMSF using a PowerGen 700D homogenizer (Thermo Fisher Scientific, Waltham, MA). The homogenized tissues were centrifuged at 12000*g* for 15 min, and the supernatant was collected and analyzed by ELISA. IL-6 and tumor necrosis factor (TNF)-α concentrations were measured using porcine IL-6 and TNF-α Quantikine ELISA Kits according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN).

RNA Isolation and Analysis of Gene Expression by Real-Time RT-PCR. Real-time RT-PCR analysis was used to measure the gene expression of various biomarkers in the colon. Total RNA was extracted from pulverized colon tissue using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Inc., Hercules, CA), and cDNA synthesis was carried out using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions. Real-time PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using the following conditions: denaturation for 15 s at 95 °C, annealing for 15 s at 56 °C, and extension for 30 s at 72 °C. Porcine primers were designed using Primer3 v.0.4.0 (*23*) and synthesized by the University of Guelph Laboratory Services Molecular Biology Section (Guelph, ON) (**Table 1**). The relative mRNA expression of the genes was calculated using the $2^{-\Delta Ct}$ formula (*24*), using porcine β-actin as the housekeeping gene.

Statistical Analysis. All values are expressed as mean ± SE. Statistical analysis was carried out using ANOVA followed by Tukey's multiple-comparison test. P values of <0.05 were considered to be significant unless otherwise stated.

RESULTS

HEL Supplementation Attenuates DSS-Induced Weight Loss and Colitis Symptoms. To determine the effect of HEL

Table 1. Porcine Primers Used for Real Time RT-PCR

gene	forward primer (5′−3′)	reverse primer (5′−3′)	product (bp)	accession no.
β-actin	GGATGCAGAAGGAGATCACG	ATCTGCTGGAAGGTGGACAG	130	U07786
IL-8	TGGCAGTTTTCCTGCTTTCT	CAGTGGGGTCCACTCTCAAT	154	M86923
IFN-γ	CCATTCAAAGGAGCATGGAT	GAGTTCACTGATGGCTTTGC	146	AY188090
IL-1β	CAAAGGCCGCCAAGATATAA	GAAATTCAGGCAGCAACAT	147	NM_214055
IL-17	TCATGATCCCACAAAGTCCA	AGTCCATGGTGAGGTGAAGC	146	NM_001005729
IL-4	TCTCACCTCCCAACTGATCC	AGCTCCATGACGAGTTCTT	147	L12991
IL-10	TGATGGGGAGGATATCAAGG	TGGAGCTTGCTAAAGGCACT	150	NM_214041
TGF-β	CGAGCCCTGGATACCAACTA	AGGCTCCAGATGTAGGGACA	164	Y00111
IL-12p40	TTTCAGACCCGACGAACTCT	CATTGGGGTACCAGTCCAAC	160	NM_214013
Foxp3	CTGACAAGGGTTCCTGCTGT	GAAATCTGGGAACGTGCTGT	149	NM_001128438
MUC1	ACCAAGTCCCCTAACCCATC	TTGGAATTTTCCAGGCAGTC	101	XM_001926883
MUC2	ACCCGCACTATGTCACCTTC	GGGATCGCAGTGGTAGTTGT	131	NM_002457

supplementation on colitis symptoms, nutrient absorption, and growth performance, animals were monitored daily for changes in body weight, food intake, and stool consistency. Following DSS infusion, severe and bloody diarrhea was observed in all animals; however, following HEL treatment animals were free of diarrhea and had improved stool consistency when compared to animals in the Pos group. The ratio of BW to food intake of each animal was analyzed to assess nutrient absorption and appetite. HEL treatment significantly improved the BW gain to feed ratios ($P < 0.01$) when compared to untreated Pos animals (**Figure 1**). There were no significant differences in the BW gain to feed ratios of HEL and Neg animals, indicating that HEL supplementation attenuated the DSS-induced weight loss and restored nutrient absorption and metabolism to basal levels observed in the negative control animals.

HEL Reduces DSS-Induced Gastrointestinal Permeability and Restores Gut Barrier Function. The presence of plasma D-mannitol was assessed as an indicator of gut permeability (**Figure 2A**). In all groups, the relationship between plasma D-mannitol concentrations over time was found to be linear. This linear relationship was used to determine the rate of increase of plasma D-mannitol levels, defined as the slope of the line for each group. The rate of D-mannitol uptake in the Pos group (2.23 ± 0.57 μmol/mL·min, $r^2 = 0.94$) was significantly greater than that of animals in the Neg control group (1.22 ± 0.03 μmol/mL·min, $r^2 = 0.99$) ($P = 0.01$) and the HEL-treated group (1.67 ± 0.11 μmol/mL·min, $r^2 = 0.99$) ($P = 0.008$), suggesting that the colonic permeability induced by DSS treatment was reduced with HEL supplementation, and gut barrier integrity was restored to levels similar to those of the negative control animals. To further assess epithelial integrity, relative expression of the mucin genes MUC1 and MUC2 was examined. The expression of MUC1 was decreased

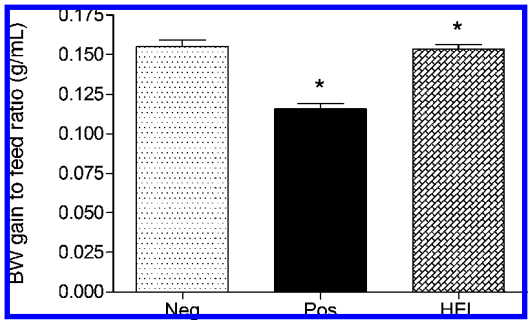

Figure 1. Body weight gain to feed ratios of Neg, Pos, and HEL-treated piglets. Animals were intragastrically infused with saline or DSS for 5 days, followed by 5 days of saline or HEL. Body weights and food intake were measured daily, and gain to feed ratios (g/mL) were determined. Values shown are means ± SE. *, $P < 0.05$ relative to Pos group.

in Pos animals when compared to untreated (Neg) animals, but was significantly increased in animals supplemented with HEL ($P < 0.01$). Little difference was observed in MUC2 gene expression (**Figure 2B**).

HEL Supplementation Ameliorates DSS-Induced Colitis Pathology and Muscle Thickness. Representative histological images from each group are shown in **Figure 3A**. Histological grading of H&E-stained sections of the colon from Pos animals showed severe mucosal and submucosal inflammation, as well as extensive crypt distortion, which was not observed in colon sections from animals in the Neg or HEL groups, suggesting extensive colon ulceration and inflammatory cell infiltration characteristic of IBD. To assess the extent of inflammation and resulting muscle thickening, colon smooth muscle thickness was also measured. Animals in the Pos group showed significantly increased muscle thickness ($P < 0.01$) when compared to untreated control animals, which was significantly reduced following treatment with HEL ($P < 0.01$) (**Figure 3B**).

HEL Supplementation Suppresses TNF-α and IL-6 Secretion in the Colon. TNF-α and IL-6 are important mediators of inflammation. Concentrations of both of these pro-inflammatory cytokines were significantly elevated in Pos control animals ($P < 0.05$) relative to the Neg control animals (**Figure 4**). Following administration of HEL, TNF-α and IL-6 levels were significantly decreased ($P < 0.05$) to basal levels similar to those observed in the untreated (Neg) animals, thereby reducing inflammatory tissue damage.

HEL Modulates Local Gene Expression and Restores Mucosal Immune Homeostasis. To further study the mechanisms by which HEL attenuated DSS-induced inflammation, gene expression of several important mediators involved in inflammation or immune regulation was measured. When compared to animals in the Neg group, DSS-induced colitis was characterized by a marked increase in the expression of pro-inflammatory cytokines IFN-γ ($P < 0.05$), IL-8 ($P < 0.05$), IL-1β ($P < 0.05$), IL-12p40 ($P < 0.05$), and IL-17 (**Figure 5A**), as measured in the Pos control animals. HEL supplementation resulted in significant decreases ($P < 0.05$) in IFN-γ, IL-8, and IL-17 gene expression, whereas IL-1β and IL-12p40 mRNA levels did not change following HEL treatment. DSS treatment suppressed expression of the anti-inflammatory cytokine IL-4 ($P < 0.05$), when compared to the Neg group, which was restored upon supplementation with HEL (**Figure 5B**). In contrast, expression of the anti-inflammatory cytokine IL-10 was increased in positive control (Pos) animals compared to both the Neg and HEL-treated groups ($P < 0.05$). Transforming growth factor (TGF)-β and forkhead box p3 (Foxp3) gene expression were both significantly increased in animals supplemented with HEL ($P < 0.01$ and $P < 0.05$, respectively).

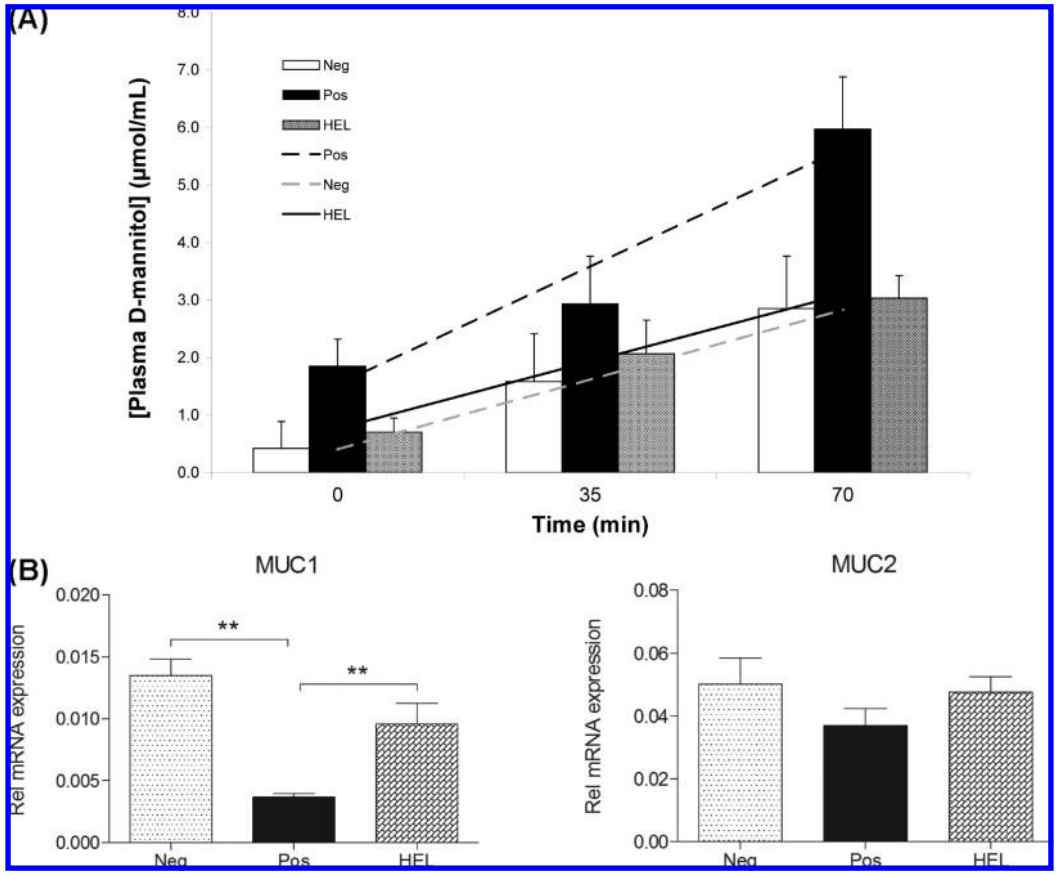

Figure 2. Effect of HEL on in vivo gastrointestinal permeability and mucin gene expression. (**A**) Prior to sacrifice, animals were intragastrically infused with D-mannitol, and plasma D-mannitol concentrations were measured at 0, 35, and 70 min postinfusion. (**B**) Relative gene expression of the mucin genes MUC1 and MUC2 in the colon tissues was determined by real-time RT-PCR. Values shown are means ± SE. **, $P < 0.01$ relative to Pos group.

DISCUSSION

The incidence of IBD is on the rise; however, current IBD therapeutic agents such as corticosteroids and aminosalicylates show limited efficacy and potential long-term toxicity. Therefore, dietary components, such as HEL, are attractive alternatives for IBD treatment. There is also increasing evidence that antimicrobial proteins, such as HEL, may play an important role in controlling infectious and inflammatory diseases, and defensin dysregulation has recently been linked with IBD pathophysiology (*25*). We have demonstrated here that HEL supplementation successfully attenuated DSS-induced intestinal inflammation and modulated local gene expression to restore immune homeostasis.

In humans, colonic inflammation can cause weight loss due to increased metabolic rate, decreased dietary intake, and malabsorption (*26*). In the present study, pigs in the Pos group showed significantly lower weight gain to feed ratios when compared to the negative control animals. In contrast, the animals that were treated with HEL maintained ratios that were similar to those of the Neg group. The increased gut permeability may have also contributed to the reduced weight gain observed in the Pos animals. It has been suggested that therapeutic restoration of epithelial barrier function could improve pathophysiology and clinical outcomes in IBD; however, there are few data regarding the effects of IBD therapeutics on intestinal barrier function (*27*). HEL supplementation was able to significantly reduce the gut permeability caused by DSS and improve intestinal epithelial barrier function. This may be due in part to the capacity of HEL to modulate cytokines involved in inflammation. Inflammatory cytokines such as TNF-α, IFN-γ, and IL-1β increase intestinal permeability; thus, once inflammation is established, altered epithelial permeability continues and contributes to the severity of pathology and inability to resolve inflammation and repair wounds (*28*, *29*).

One of the hallmarks of DSS-induced colitis is profound colonic inflammation characterized by crypt destruction, mucosal ulceration, erosions, and infiltration of lymphocytes into the mucosal tissue (*30*). Whereas Pos animals showed mucosal and submucosal inflammation and thickening as well as marked crypt hyperplasia, diminished inflammation characterized by a reduction in cell infiltration and recovery of intact epithelium was evident in HEL-supplemented animals, indicating that HEL may have been able to reverse DSS-induced ulceration and crypt destruction. Smooth muscle thickness is commonly used to evaluate inflammation along the gastrointestinal tract. Previous research has shown that inflammation causes proliferation of the intestinal smooth muscle cells, leading to increases in muscle mass and muscle cell numbers (*31*), and an accumulation of collagen leading to a thickening of the intestinal wall (*32*). This is consistent with the results observed here, where HEL supplementation reduced the significant muscle thickening seen in the Pos group, and provides further evidence that HEL was able to attenuate the DSS-induced damage to the colon.

In the colon, the mucus layer acts as a physical barrier to protect and maintain epithelium integrity (*33*). It has been found that mice deficient in mucin production spontaneously developed a colitis-like phenotype (*34*). Furthermore, decreased expression of mucin genes (MUC1, MUC2, MUC3, and MUC4) has been observed during DSS-induced colitis in mice (*33*). In the present study, relative mucin gene expression was indeed found to be

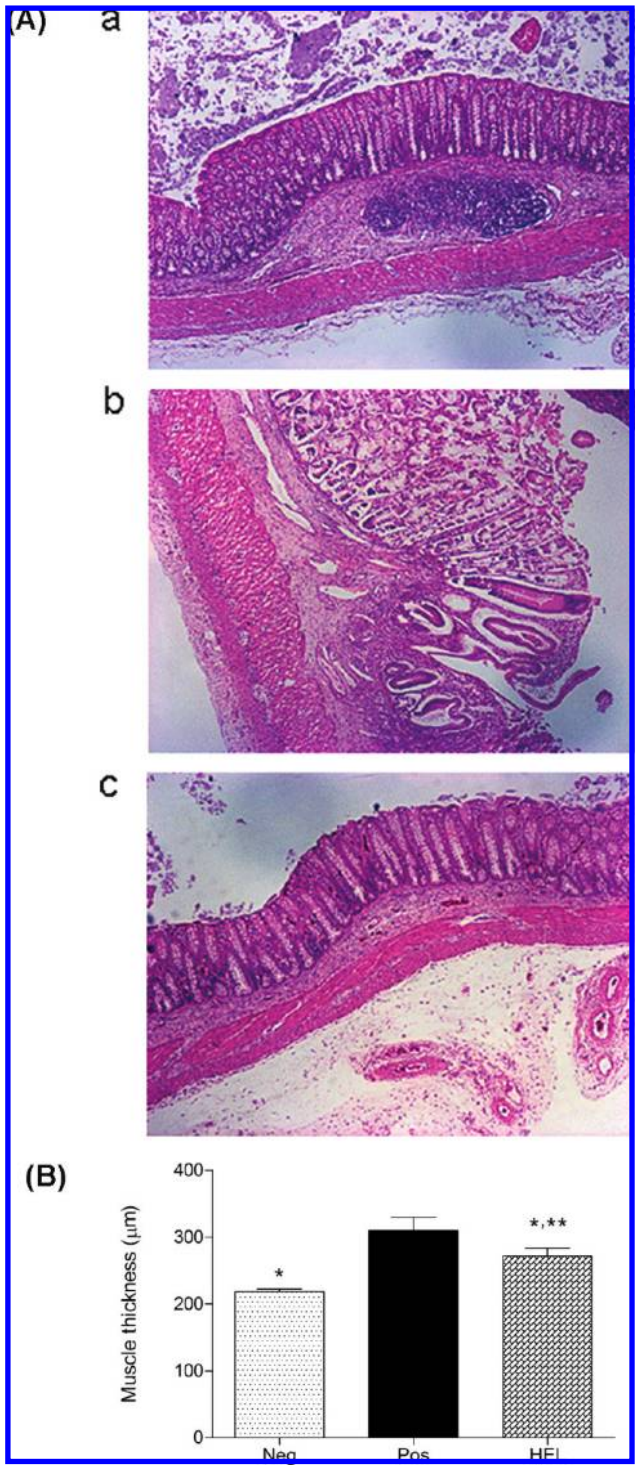

Figure 3. Effect of HEL on DSS-induced colitis histology and muscle thickness. (**A**) Representative H&E-stained colon sections from (**a**) Neg control, (**b**) Pos control, and (**c**) HEL-supplemented animals. (**B**) Muscle thickness of colon sections from Neg, Pos, and HEL animals was measured as an indicator of the extent of inflammation. Values shown are means ± SE. **, $P < 0.01$ relative to Pos group; **, $P < 0.01$ relative to Neg group.

reduced as a result of DSS treatment, but was restored upon administration of HEL. This effect was most pronounced with the membrane-associated mucin MUC1, as opposed to MUC2, which is secreted, and was previously shown to be down-regulated in mouse models of colitis (*27*), indicating that the membrane-associated MUC1 may also be important in IBD pathology. It has been noted that even though therapeutics that

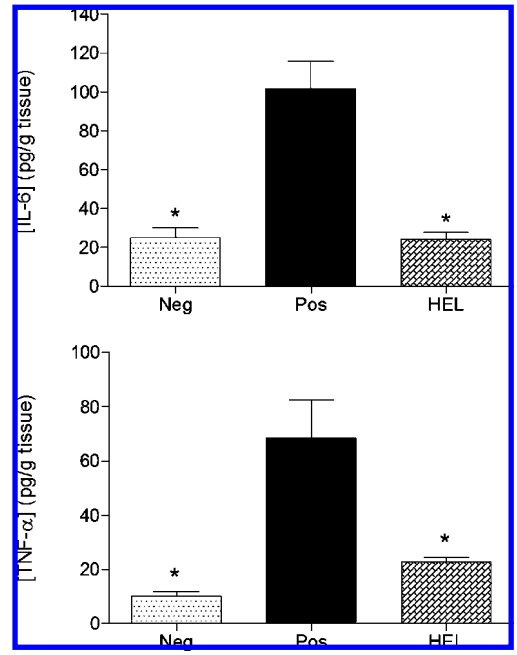

Figure 4. Concentrations of TNF-α and IL-6 in the colon. Colon tissues from Neg, Pos, and HEL-treated animals were homogenized in HBSS containing protease inhibitors, and TNF-α and IL-6 concentrations in the supernatants were determined by ELISA. Results are expressed as picograms per gram of colon tissue. Values shown are means ± SE. **, $P < 0.05$ relative to Pos group.

directly stimulate production of constituents of the epithelial barrier may not resolve advanced lesions, they may have promise in maintaining patients in remission (*27*), suggesting a further advantage of HEL as a therapeutic in IBD.

Although the etiology of IBD remains unknown, it is known that cytokines play an important role in disease progression. Increased levels of pro-inflammatory cytokines TNF-α, IL-6, IL-8, and IFN-γ have been detected in the colon of patients with IBD and mice with DSS-induced colitis (*35*, *36*). IL-6 and TNF-α, in particular, are key pro-inflammatory cytokines contributing to the pathogenesis of IBD (*37*, *38*), and their clinical relevance has been highlighted by the success of experimental therapies targeting these cytokines and their respective pathways (*39*). A significant reduction was observed here in the concentrations of IL-6 and TNF-α in the colon of HEL-treated animals, providing strong evidence that HEL may exert its anti-inflammatory effects by directly modulating the colonic expression of genes involved in inflammatory processes, thereby contributing to the improved gut histology and permeability. Likewise, HEL treatment significantly reduced local gene expression of IFN-γ, as well as IL-8, a chemokine that contributes to IBD-mediated pathology via the recruitment of neutrophils to the intestinal mucosa (*40*). The persistent up-regulation of IL-1β and IL-12p40 in response to HEL administration suggests that HEL may also play a role in modulating innate immune responses, and it has been suggested that defects or alterations in innate immune responses may play a crucial role in the pathogenesis of IBD (*41*), further highlighting the potential immunomodulatory activity of HEL. The anti-inflammatory cytokine IL-4 is typically found at low levels in the colonic mucosa of IBD patients (*42*), and similarly, IL-4 gene expression has been found to be reduced in rodent models of IBD (*43*). Indeed, we found that IL-4 levels were significantly lower in Pos control animals when compared to the Neg control group, but were increased by HEL supplementation. Surprisingly, expression levels of the anti-inflammatory cytokine IL-

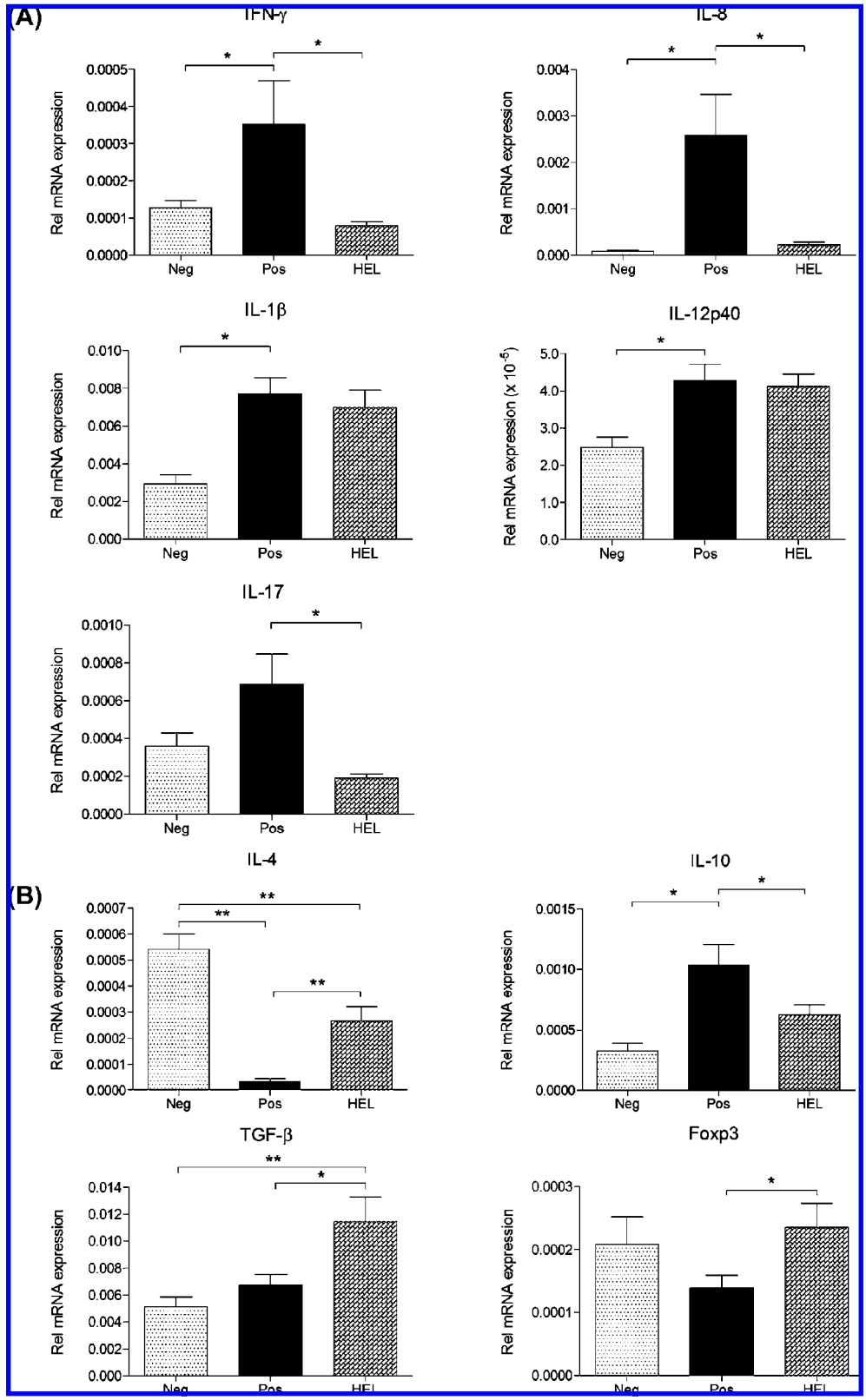

Figure 5. Effect of HEL supplementation on (**A**) pro-inflammatory cytokine and (**B**) anti-inflammatory cytokine and regulatory mRNA levels in the colon. mRNA was extracted from colon tissues of Neg, Pos, and HEL-treated animals, and real-time RT-PCR was carried out as described under Materials and Methods. Values shown are means ± SE. **, $P < 0.05$; **, $P < 0.01$.

10 were elevated in animals in the Pos group and decreased with HEL supplementation. Elevated levels of IL-10 have been detected in IBD patients (*44*), as well as in mice (*45*), as a response to chronic inflammation. The results observed here suggest that IL-10 may have remained up-regulated in the Pos group in an attempt to overcome DSS-induced inflammation, whereas the HEL-treated animals underwent a more rapid return to normal gut homeostasis and a subsequent decrease in IL-10.

One of the major concepts previously used to explain the different pathogenic mechanisms in CD and UC was the Th1/Th2 paradigm. It has become increasing clear, however, that the IL-17 pathway is critical in IBD (*46*). Th17 cells produce

IL-17, IL-6, and TNF-α, which in turn act on fibroblasts, macrophages, and endothelial and epithelial cells to elicit inflammatory mediator and chemokine release. This environment recruits neutrophils and creates a general state of tissue inflammation (*47*). The reduction in these cytokines in response to HEL supplementation suggests that HEL may be capable of down-regulating the Th17 pathway. Although TGF-β has also been implicated in the differentiation of Th17 cells (*48*, *49*), recent evidence suggests that this may occur in a concentration-dependent manner. Zhou et al. (*50*) demonstrated that at low levels, TGF-β synergizes with IL-6 to produce Th17 cells. At high concentrations, however, TGF-β induces up-regulation of the transcription factor Foxp3, inhibiting Th17 cell differentiation and favoring the development of Foxp3$^+$ regulatory T (T_{reg}) cells, which are important for maintaining immune homeostasis (*51*). Accordingly, we observed an increase in both TGF-β and Foxp3 gene expression and a concomitant decrease in IL-17 gene expression in animals that were supplemented with HEL. These results are in line with previous observations that targeting rapidly expanding populations of effector T cells, as evidenced here by the decrease in effector cytokines, and sparing T_{reg} cell function may allow T_{reg} cells to re-establish mucosal homeostasis and may be a novel strategy for inducing remission in IBD patients (*52*).

There are numerous reports of bioactive food components; however, their effectiveness after oral administration may be limited by their sensitivity to enzymatic digestion in the gastrointestinal tract. Whereas the enzymatic (muramidase) activity of HEL is reduced by proteolytic digestion, a number of reports have demonstrated antimicrobial activity that is independent of HEL enzymatic activity and could in fact be enhanced by digestion with pepsin and trypsin (*53*). Moreover, we have demonstrated that digestion of HEL with pepsin and trypsin increased its anti-inflammatory activity when compared to native HEL, reducing LPS-induced IL-8 secretion in vitro (unpublished results). Further work will be required to elucidate the anti-inflammatory and immune-modulating mechanisms of these HEL peptides.

Our data clearly demonstrate that HEL has a therapeutic effect in a porcine model of DSS-induced colitis. HEL was able to attenuate DSS-induced inflammation and restore epithelial barrier function. Furthermore, HEL supplementation reduced pro-inflammatory cytokine expression and increased the expression of anti-inflammatory and regulatory genes, indicating that HEL may be a promising new alternative for IBD treatment.

ACKNOWLEDGMENT

We thank Dr. A. M. Hayes, Department of Pathobiology, University of Guelph, for histopathological analyses and Anil Puttaswamy for assistance with real-time RT-PCR.

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Received for review October 7, 2008. Revised manuscript received December 17, 2008. Accepted January 16, 2009. This work was supported by the Advanced Foods and Materials Network (AFMNet), Canada.

JF803133B