Interleukin-10 is Differentially Expressed in the Small Intestine and the Colon Experiencing Chronic Inflammation and Ulcerative Colitis Induced by Dextran Sodium Sulfate in Young Pigs

D. LACKEYRAM[1,2], D. YOUNG[3], C.J. KIM[3], C. YANG[1,4], T.L. ARCHBOLD[1], Y. MINE[3], M.Z. FAN[1]

[1]Department of Animal Biosciences, [2]Open Learning and Educational Support, University of Guelph, Guelph, Ontario, Canada N1G 2W1, [3]Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1, [4]Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

Short title: Expression of IL-10 in Young Pigs with Ulcerative Colitis

Key words

Chronic bowel inflammation • Cytokine • Gut permeability • IL-10 • Colonic ulcerative colitis

Corresponding author

M.Z. Fan, Department of Animal Biosciences, Building #70, University of Guelph, Guelph, Ontario, Canada N1G 2W1. E-mail: mfan@uoguelph.ca

Summary

Intestinal inflammation induced with dextran sodium sulfate (DSS) is used to study acute or chronic ulcerative colitis in animal models. Decreased gut tissue anti-inflammatory cytokine IL-10 concentration and mRNA abundance are associated with the development of chronic bowel inflammation. Twelve piglets of 3 days old were fitted with an intragastric catheter and randomly allocated into control and DSS groups by administrating either sterile saline or 1.25 g of DSS/kg body weight (BW) in saline per day, respectively, for 10 days. Growth rate and food conversion efficiency were reduced ($p<0.05$) in the DSS piglets compared with the control group. Quantitative histopathological grading of inflammation in the jejunum and colon collectively showed that the DSS treatment resulted in 12 fold greater ($p<0.05$) inflammation severity scoring in the colon than in the jejunum, indicative of chronic ulcerative colitis in the colon. Upper gut permeability endpoint was 27.4 fold higher ($p<0.05$) in the DSS group compared with the control group. The DSS group had higher concentrations and mRNA abundances ($p<0.05$) of TNF-α and IL-6 in the jejunal and colonic tissues compared with the control group. Colonic concentration and mRNA abundance of IL-10 were reduced ($p<0.05$), however, jejunal IL-10 mRNA abundance was increased ($p<0.05$) in the DSS group compared with the control group. In conclusion, administration of DSS at 1.25 g/kg BW for 10 days respectively induced acute inflammation in the jejunum and chronic inflammation and ulcerative colitis in the colon with substantially decreased colonic concentration and mRNA abundance of IL-10 in the young pigs, mimicking the IL-10 expression pattern in humans associated with chronic bowel inflammation.

Introduction

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract that causes abdominal pain and discomfort together with altered bowel habits such as diarrhea (Murch *et al*. 1991, Cohen *et al*. 2004, Longstreth *et al*. 2006). Two major forms of IBD that have been well characterized are ulcerative colitis and Crohn's disease. While ulcerative colitis is always restricted to the colon and involves the rectum, Crohn's disease can occur at any location in the intestinal tract with the highest incidences reported in ileum, cecum and colon (Leenen and Dieleman 2007).

Acute ulcerative colitis could be established within 4-5 days of consumption of an effective dose of DSS in rodents and pigs (Tsune *et al*. 2003, Kim *et al*. 2009, Sánchez-Fidalgo *et al*. 2010, Young *et al*. 2010). Chronic ulcerative colitis was induced after administration of DSS for 3-5 weeks in rodents (Okayasu *et al*. 1990, Sánchez-Fidalgo *et al*. 2010). No studies have been conducted to establish the DSS induction of chronic bowel inflammation associated with chronic ulcerative colitis through profiling IL-10 gene expression. It has been shown that the gut microflora in monogastric animals can be re-established within 10 days (Sonnenburg *et al*. 2005). A life span for intestinal epithelial cells, including intraepithelial lymphocytes, of young pigs was estimated to be within 5-10 days from our previous studies (Fan *et al*. 2001). Thus, we hypothesized that administration of an effective dose of DSS for 10 days would be a minimal time period to induce chronic bowel inflammation responses and chronic ulcerative colitis in the colon of young pigs. Therefore, the primary objectives of this study were to investigate if chronic inflammation responses associated with chronic ulcerative colitis in the colon of young pigs could be induced through intra-gastric (ig) infusion of an effective dose of DSS for 10 days

through comparatively profiling IL-10 and representative pro-inflammatory cytokine gene expression in both the colon and the small intestine.

Materials and Methods

Animals, diets, surgery and experimental design

A total of 12 purebred Yorkshire gilts at the age of day 3 were obtained from the University of Guelph Arkell Swine Research Station and were transferred into the Animal Wing in the Department of Animal Biosciences at the university. After two days of adaptation to a commercial milk replacer (Soweena® Litter Life – Merrick's Inc., WI, UAS) (Fan *et al*. 2001), piglets were surgically fitted with an ig catheter (Micro-Renathane®, O.D. 0.8 mm, Braintree Scientific, Inc., MA, USA), and allowed a 3-day post surgical recovery for regaining their normal level of food intake. Each ig catheter was anchored to a trimmed inert silicone anchor (about 8 x 12 mm; Access Technologies, Skokie, IL) that was further sutured onto the gastric wall with the catheter end of about 30 mm inserted inside the gastric environment. Each piglet was then dressed in a custom-made infusion jacket with a dorsal pocket for temporary storage of the exterior segment of the catheter. The piglets were then randomly assigned into a control group (n = 6) and a DSS-treatment (n = 6) group. One piglet was lost due to surgical complication and/or adaptation to DSS infusion, resulting in five observations for the DSS group. The piglets were fed close to their *ad libitum* intake level three times daily at 0900, 1200 and 1600 h. Daily food intake and initial and final body weights (BW) were measured for all the piglets. All animal handling procedures were approved by the Animal Care Committee at the University of Guelph. The animal trial in this study was conducted in accordance with the

guidelines established by Canadian Council of Animal Care (Candian Council on Animal Care 1993).

Intra-gastric injection protocols

Piglets in both groups were infused via the ig catheter with an equal volume (10 ml/piglet.day) of either sterile saline (control group) or 1.25 g of DSS/kg BW.day with DSS (MW, 36,000-50,000; MP Biomedicals, Solon, OH, USA) solubilized in sterile saline (DSS group). The DSS dose used in this study was based from studies reported in piglets (Mackenzie *et al*. 2003, Bassaganya-Riera and Hontecillas 2006, Kim *et al*. 2009, Young *et al*. 2010). Both saline and DSS solutions were pre-warmed at 37 °C in a water bath. The ig injections were consistently administered twice daily following the morning and the afternoon meals for a period of 10 days.

In vivo stomach-small intestine-specific permeability test, sample collection and processing

In vivo gut permeability was measured by using a non-metabolizable monomer permeability marker D-mannitol according to an established protocol (Thymann *et al*. 2006). A linear increase in D-mannitol concentration in plasma within approximately 90 min after a bolus injection of D-mannitol was observed in the previous study with piglets (Thymann *et al*. 2006). Previous studies demonstrated that a total digesta retention time in the stomach and the small intestine of suckling piglets is between 6 and 9 hours (Kidder and Manners 1978, Snoeck *et al*. 2004). Thus, 70-min time course was chosen to probe the stomach-small intestine-specific *in vivo* permeability to D-mannitol in this study. At the end of the DSS-treatment period, both groups of piglets were injected with a bolus dose (0.6 g D-mannitol/kg BW solubilized in saline)

of D-mannitol (Sigma/Aldrich, St. Louis, MO, USA) in a total injection volume at about 13 ml/kg BW via an ig catheter. Blood samples were collected by puncture of the orbital sinus into pre-chilled centrifuge tubes containing heparin (Sigma/Aldrich) immediately prior to the injection of D-mannitol and this sample collection was designated to be the zero time point. Blood was also collected at 35 and 70 min post-injection, respectively. In order to minimize background interference, aliquot plasma samples were further cleared out, prior to D-mannitol analysis, for polymer organic compounds, including proteins, RNA and DNA. This was conducted by boiling glass sample test tubes in a water bath for 3-5 min followed by centrifugation at 21,000 x *g* for 60 min on a micromax microcentrifuge (International Equipment Company, Needham Heights, MA, USA). Plasma D-mannitol analysis was adapted from previous methods (Lunn *et al*. 1989, Graefe *et al*. 2003). D-Mannitol (0-40 nmol/incubation) in standards and plasma samples was analyzed by spectrophotometry at 340 nm by monitoring NADH yield after incubations with D-mannitol dehydrogenase (Megazyme International Ireland LTD., Co. Wicklow, Ireland) at pH 8.6 and 40 ºC.

Subsequent to the bolus injection of D-mannitol and blood sampling for the *in vivo* upper gut permeability test, piglets were sedated by inhalation of anesthetic isoflurane for tissue sampling and were euthanized by using sodium pentobarbital (Graefe *et al*. 2003). Intestinal tissues were immediately removed and rinsed thoroughly in an ice-cold saline (154 mM NaCl at pH 7.4) containing a protease inhibitor phenylmethysulfonyl fluoride (PMSF) (0.1 mM; Sigma/Aldrich). Representative tissues were taken for gut mucosal morphological and histopathological measurements. Gut tissues were also immediately sampled and flash frozen in liquid nitrogen. The flash frozen tissue samples were further pulverized to be homogenous under

136 liquid nitrogen using a pair of mortar and pestle and were sub-sampled and stored at –80 °C for
137 further analyses (Lackeyram *et al*. 2010).
138
139 *Histology and histopathology measurements*
140 Cross sections of both proximal jejunal and colonic tissues were fixed in 10 % formalin for
141 12 hours, paraffin-embedded, and then stained with haematoxylin and eosin (Fan *et al*. 2001).
142 The villous height, crypt depth and smooth muscle thickness were measured using the Openlab™
143 software (Improvision, Coventry, UK) (Lackeyram *et al*. 2010). Three jejunal and colonic tissue
144 sections from each piglet were histopathologically blind-graded by two trained individuals. The
145 quantitative histopathological grading used to assess the degree of inflammation was adopted
146 from previous studies (Dieleman *et al*. 1998; Kim *et al*. 2009, 2010, Young *et al*. 2010). The
147 scoring of the endpoints is described briefly as follows: occurrence of inflammation (0 = none, 1
148 = slight, 2 = moderate and 3 = transmural); extent of inflammation (0 = none, 1 = mucosa, 2 =
149 mucosa and submucosa and 3 = transmural); degree of tissue regeneration (0 = complete
150 regeneration or appearance of normal tissue, 1 = almost complete regeneration, 2 = basal 2/3
151 damaged, 3 = only surface epithelium intact and 4 = entire crypt and epithelium lost), and crypt
152 damage (0 = no damage, 1 = basal 1/3 damage, 2 = basal 2/3 damaged, 3 = only surface
153 epithelium intact and 4 = entire crypt and epithelium lost) (Dieleman *et al*. 1998). The
154 percentage contribution of each endpoint was scored (1 = up to 25 %, 2 = 26-50 %, 3 = 51-75 %
155 and 4 = 76-100 %) (Dieleman *et al*. 1998). Each of the 4 graded endpoints was then multiplied
156 by the percentage contribution score (1-4) to yield scoring ranges (0-12) for inflammation and
157 the extent of inflammation, and scoring ranges (0-16) for regeneration and crypt damage
158 (Dieleman *et al*. 1998).

159

160 *ELISA for cytokines*

161 Porcine specific kits for the analyses of TNF-α, IL-6 and IL-10 cytokine contents in the
162 intestinal tissues and plasma samples were purchased from Quantikine® (R&D Systems Inc.,
163 Minneapolis, MN, USA). Crude tissue homogenate was prepared by homogenizing 0.5 g of
164 jejunal and colonic tissues with a Powergen 125 hand-held tissue homogenizer (Fisher,
165 Pittsburgh, PA, USA) in 1 ml of Hank's buffer of balanced salt mixture without phenol red,
166 calcium and magnesium (HyClone, Logan, UT, USA) but with 1 µl of protease inhibitor
167 cocktail. The cocktail included the following protease inhibitors (mM): 0.02 N-tosyl-L-
168 phenylalanine chloromethyl-ketone; 0.02 N-α-p-tosyl-L-lysine ketone; 0.02 leupeptin
169 hemisulfate; 0.02 apoprotinin; 0.02 pepstatin A and 0.1 PMSF (Sigma/Aldrich). The
170 homogenate was immediately centrifuged at 12,000 x *g* at 4 ºC and 100 µL of the supernatant
171 (about 0.800 mg protein/sample) was used to initiate the ELISA incubation according to the
172 instructions on the specific kit and absorbance was read by using a BioRad Model 550
173 Microplate reader (BioRad, Hercules, CA, USA). Protein contents in the supernatant and plasma
174 samples were measured by using a Bio-Rad commercial kit (Lackeyram *et al*. 2010).

175

176 *RNA preparation and real time RT-PCR for cytokine mRNA*

177 Total cellular RNA was extracted from pulverized porcine proximal jejunal and colonic
178 tissue samples using TRIzol reagent (Invitrogen Corporation – Life Technologies, Carlsbad, CA,
179 USA). The RNA quality was checked through our established procedures including by 1 %
180 agarose gel electrophoresis stained with 10 µg/ml ethidium bromide and had an OD260:OD280
181 ratio between 1.8 and 2.0 (Lackeyram *et al*. 2010). About 1 µg RNA per sample was treated

182 with DNase (Invitrogen) according to the manufacturer's instructions and quantitative real time
183 RT-PCR (0.1 μg cDNA/sample) was performed in a Smart Cycler (Cepheid, Sunnyvale, CA,
184 USA) using Quantitect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA, USA) according
185 to instructions to the kit. Primers for the porcine TNF-α, IL-6, IL-10 and β-actin (housekeeping
186 gene control) were designed with the Primer 3 software (http://frodo.wi.mit.edu) and shown in
187 Table 1. Equal amounts of DNase I-treated RNA were added to a total volume of 25 μl
188 containing 12.5 μl SYBR Green mixture, 0.25 μl RT mixture and 1 μM of each of the forward
189 and the reverse primers. We used the following protocol: (i) denaturation program (15 min at 95
190 °C); (ii) amplification and quantification program, repeated 45 cycles (15 s at 95 °C, 15 s at 58
191 °C, 15 s at 72 °C); and (iii) melting curve program (60-99 °C with a heating rate of 0.1 °C/s and
192 fluorescence measurement). Negative controls were performed in which water was substituted
193 for RNA. Melting curve analysis was conducted to confirm the specificity of each product, and
194 the size of product was verified on ethidium bromide-stained 2 % agarose gels in Tris-acetate–
195 EDTA buffer (Rideout *et al*. 2007).
196
197 *Calculations and statistical analyses*
198 The ratio of the expression of the target cytokine genes relative to the housekeeping gene
199 β-actin was calculated (Kelta *et al*. 2004; Lackeyram *et al*. 2010) as:
200 $$R = 2^{-Ct\ (target\ -\ housekeeping)},$$
201 where R is the relative expression ratio value of the target cytokine genes; and Ct is the cycle
202 number at the threshold at which the target cytokine genes and the housekeeping gene β-actin are
203 amplified beyond the of 30 fluorescence units. We normally set 45 cycles as the maximal cycle
204 number, unless for a target gene with a very low expression level, when we observed a trend to

reach the threshold during 45 cycles we would extend the cycle number to 50 or more. Optimal real-time PCR efficiencies were acquired by amplification of dilution series of RNA according to the equation $10^{(-1/slope)}$ and were consistent between each of the target cytokine genes and the housekeeping gene β-actin (Lackeyram *et al*. 2010).

Differences in our reported endpoints between the control and the DSS groups were compared by the analysis of variance (ANOVA). The ANOVA was conducted by using the PROC MIXED model of SAS (SAS Institute, Cary, NC, USA). Homogeneity of variances was examined and confirmed by the Levene's test for the endpoints, including, histology measurements, cytokine concentrations and cytokine mRNA abundances, by using SAS. Related linear regression analyses were conducted by using the Fig.P program (Fig.P, 1993, Biosoft, Cambridge, UK). Comparison of the simple linear regression slopes between the control and DSS groups associated with the *in vivo* upper gut permeability measurements was conducted by using the pooled *t*-test (Byrkit *et al*. 1987). Where appropriate, data are presented as means ± SE. p values < 0.05 were considered significant.

Results

Growth performance

The DSS and the control groups of piglets had a similar ($p>0.05$) initial BW at the beginning of the study (Table 2). Both the DSS and the control groups of piglets also consumed a similar ($p>0.05$) amount of the milk replacer over the course of the experimental period. Despite this, the DSS group had lower ($p<0.05$) average daily BW gain and gain to food ratio

227 than the control group (Table 2). Furthermore, piglets receiving the DSS treatment were all
228 associated with severe diarrhea.
229
230 *Changes in morphological and histopathological endpoints*
231 Histological analyses of the proximal jejunal cross-sections showed decreases ($p<0.05$) in
232 the villous height and increases ($p<0.05$) in the crypt depths and the smooth muscle thickness in
233 the DSS group compared with the control group (Table 3, Fig. 1A and 1B). The calculated
234 villous height to crypt depth ratio was lower ($p<0.05$) in the DSS group in comparison with the
235 control group. In colon, a decrease ($p<0.05$) in the entire mucosal thickness and an increase
236 ($p<0.05$) in the smooth muscle thickness were observed in the DSS group compared with the
237 control group (Table 3; Fig. 1C and 1D). Quantitative histopathological grading of inflammation
238 in the jejunum and the colon has been summarized in Table 4 and these data collectively indicate
239 that DSS treatment resulted in 12 fold greater ($p<0.05$) inflammation severity scoring in the
240 colon than in the jejunum.
241
242 *In vivo upper gut permeability*
243 There were linear relationships ($p<0.05$) between plasma D-mannitol concentrations and
244 post-injection time within 70 min of a bolus ig injection of 0.6 g D-mannitol/kg BW in both the
245 DSS and the control groups (Fig. 2). The slopes of these linear relationships represent the
246 increase in plasma D-mannitol concentration per min in the piglets in both the DSS and the
247 control groups, and are used as an indicator of *in vivo* upper gut permeability. The estimated
248 indicator value (parameter estimates ± SE) for the *in vivo* upper gut permeability was 27.4 fold

249 higher ($p<0.05$) in the DSS-treated piglets (DSS, 0.1389 ± 0.0130 vs. control, 0.0049 ± 0.0014
250 μmol D-mannitol per mL plasma/min) compared with the control piglets.
251
252 *Cytokine levels and tissue cytokine mRNA abundances*
253 When expressed as pg/g fresh tissue, IL-10 concentration was reduced ($p<0.05$) by 2.4 fold
254 in the colon (DSS, 50.2 ± 4.4 vs. control, 169.6 ± 6.4; $p=0.0008$) and by 15% in the jejunum
255 (DSS, 190.4 ± 4.8 vs. control, 224.8 ± 6.8; $p=0.0470$) in the DSS group, respectively, compared
256 with the control group. When expressed as pg/mg extractable tissue protein for removal of the
257 potentially differential water content effect, IL-10 concentration was also considerably decreased
258 ($p<0.05$) in the colon of the DSS group; however, IL-10 concentration was not significantly
259 affected in the jejunum of the DSS group compared with the control group (Fig. 3).
260 Furthermore, when expressed as pg/mL, circulating plasma IL-10 concentration was reduced
261 ($p<0.05$) by 1.5 fold in the DSS group (DSS, 110.4 ± 12.6 vs. control, 273.8 ± 21.7; $p=0.0040$)
262 compared with the control group. When expressed as pg/mg extractable protein, circulating
263 plasma IL-10 concentration was still decreased ($p<0.05$) in the DSS group compared with the
264 control group (Fig. 3). Real time RT-PCR analyses for the cytokine gene expressions revealed
265 that colonic IL-10 mRNA abundance was decreased to be undetectable, and there was not a trend
266 to reach the threshold even after 45 cycles in the DSS group, while colonic IL-10 mRNA was
267 abundantly expressed in the control group (Table 5). On the contrary, jejunal IL-10 mRNA
268 abundance was 14 fold higher ($p<0.05$) in the DSS group than in the control group (Table 5).
269 When expressed as pg/mg extractable tissue protein for removal of the potentially
270 differential water content effect, the DSS group had higher concentrations ($p<0.05$) of both TNF-
271 α and IL-6 cytokines in the jejunal and colonic tissues compared with the control group (Fig. 3).

Furthermore, TNF-α and IL-6 cytokine concentrations (pg/mg extractable tissue protein) were 2.9 and 5.4 fold higher ($p<0.05$) in the colon than in the jejunum within the DSS group. However, no differences ($p>0.05$) in TNF-α and IL-6 cytokine concentrations were observed between the colon and the proximal jejunum within the control group, when these were expressed as pg/mg extractable tissue protein. In addition, there were higher ($p<0.05$) circulating plasma TNF-α and IL-6 cytokine concentrations (pg/mg plasma protein) in the DSS group than in the control group (Fig. 4). Real time RT-PCR analyses for the cytokine gene expressions revealed higher ($p<0.05$) mRNA abundances of TNF-α and IL-6 in both the jejunal and the colonic tissues in the DSS group compared with the control group (Table 5).

Discussion

Although the exact etiology of IBD is unknown, the pathology has been associated with relapsing intestinal inflammation (Sartor 1997, Fiocchi 1998) linking to increased epithelial permeability (Podolsky 2002), psychological stress (Mawdsley and Rampton 2007), environmental factors such as smoking (Somerville *et al*. 1984), and diets and nutrition (Ainley *et al*. 1991). Thus, biological mechanisms as well as preventive and therapeutic strategies need to be further investigated. Intestinal inflammation is the hallmark of IBD and a number of experimental models have been developed to investigate roles of innate immunity, adaptive immune system and regulatory immune mechanisms playing in the pathogenesis of IBD through cytokines (Papadakis and Targan 2000, Strober *et al*. 2002, Elson *et al*. 2005). Earlier studies showed that administration of DSS induced colonic mucosal immune responses due to development of pathogenic microflora and phagocytosis of DSS by macrophages in the mucosa

295 (Okayasu *et al*. 1990). Both acute and chronic ulcerative colitis, as induced by DSS, were
296 investigated in several animal species, including mice (Okayasu *et al*. 1990, Dieleman *et al*.
297 1998), rats (Tsune *et al*. 2003), guinea pigs (Iwanaga *et al*. 1994) and more recently with piglets
298 (Mackenzie *et al*. 2003, Bassaganya-Riera and Hontecillas 2006, Kim *et al*. 2009, Young *et al*.
299 2010). A convenient route of DSS delivery for inducing intestinal inflammation in smaller
300 laboratory animals such as mice, rats and guinea pigs is through drinking water. However, pigs
301 are sensitive to the flavor of their diets and water, so ig infusion of DSS via a catheter is effective
302 when using the pig model (Mackenzie *et al*. 2003, Bassaganya-Riera and Hontecillas 2006, Kim
303 *et al*. 2009, Young *et al*. 2010). Thus, DSS has been widely used to induce acute and chronic
304 bowel inflammation associated ulcerative colitis in animal models.

305 Acute bowel inflammation associated with acute ulcerative colitis is mediated by innate
306 immunity through rapid responses of cytokine and IgA secretions from immune cells localized in
307 the colon, including intraepithelial lymphocytes, dendritic cells, and lymphocytes and
308 macrophages in Peyer's patches, lamina propria and mesentery lymphoid nodes (Strober *et al*.
309 2002, Elson *et al*. 2005). Increased colonic apical expression of toll-like receptor-4 (TLR-4), as
310 mediated by lipopolysaccharide endotoxin, is shown to induce innate immunity and DSS-
311 induced acute ulcerative colitis (Fukata *et al*. 2005, Fukata and Abreu 2008). Because of
312 absence of adaptive immunity responses, a DSS-induced acute ulcerative colitis model would not
313 suitable for studying cellular and molecular events and immunological mechanisms involved in
314 developing chronic bowel inflammation (Grisham 2008). Therefore, a DSS-induced acute
315 ulcerative colitis model is only particularly useful for testing preventive strategies and for
316 understanding cellular events and molecular mechanisms associated with epithelial adaptation
317 during the onset of bowel inflammation.

318 Chronic bowel inflammation associated with chronic bowel diseases such as chronic
319 ulcerative colitis in the colon is mediated by adaptive immunity and regulatory immune
320 mechanisms through recruiting effector T and B immune cells from the primary immune organs
321 (Strober *et al*. 2002, Elson *et al*. 2005). Elevated expression of pro-inflammatory cytokines,
322 such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), occurs under both acute
323 and chronic bowel inflammation (Papadakis and Targan 2000). Thus, profiling pro-
324 inflammatory gene expression alone is not suitable to serve as a benchmark for evaluating
325 chronic bowel inflammation. The anti-inflammatory role of IL-10 was classically demonstrated
326 in the IL-10-deficient mouse study (Kühn *et al*. 1993). Colonic expression of anti-inflammatory
327 cytokine IL-10 is uniquely different between the onset of acute bowel inflammation and the
328 progression of chronic bowel inflammation. Colonic IL-10 gene expression, as marked by IL-10
329 mRNA abundances, was enhanced under acute bowel inflammation via innate immunity (Egger
330 *et al*. 2000, Braat *et al*. 2003, Kim *et al*. 2009, Young *et al*. 2010). However, the IL-10 level was
331 shown to be very low in chronic inflamed mucosal tissues contributed primarily by recruited
332 effector T cells and macrophages via adaptive immunity and regulatory immune mechanisms
333 (Autschbach *et al*. 1998, Gasche *et al*. 2000). Therefore, decreases in IL-10 concentration and
334 IL-10 mRNA abundance are recognized biomarkers for the status of chronic bowel
335 inflammation.

336 The primary objective of this study was to investigate if chronic colonic inflammation
337 responses associated with chronic ulcerative colitis in the colon of young pigs could be induced
338 through administration of an effective dose of DSS for a time period of 10 days through
339 comparatively profiling IL-10 gene expression in both the colon and the small intestine. We are
340 interested in the development of a DSS-induced chronic ulcerative colitis model in young pigs

341 through profiling cytokine gene expression for the following reasons. Chronic inflammatory
342 bowel diseases do occur in infants and children (Kappelman and Grand 2008, Kelsen and
343 Baldassano 2008). A young pig based chronic ulcerative colitis model will allow studies of IBD
344 with implications to both children and adults. Although active immunity in piglets is not fully
345 developed to its peak maturity until 7 weeks of age (Fan 2003), it is rapidly developing in young
346 pigs. Thus, DSS challenge can theoretically provoke both innate and adaptive immunity and
347 chronic bowel inflammation in young pigs. Furthermore, pigs are now widely recognized as a
348 relevant animal model for studying human gastrointestinal physiology and nutrition and a young
349 pig based chronic ulcerative colitis model will allow the use of a much smaller quantity of
350 bioactive compounds at their early stage of development for therapeutic oriented research.
351 The 2.4 fold dramatic reduction of the IL-10 concentration (Fig. 3) and declining to the
352 undetectable level (Table 5) of the relative IL-10 mRNA abundance in the colon of the DSS
353 group from this study supported our original research hypothesis that chronic colonic
354 inflammation occurred in the young pigs following the 10-day period of the DSS administration.
355 These results are in line with reported human clinic studies in showing that the colonic IL-10
356 level was declined to a very low level in chronic inflamed mucosa contributed primarily by
357 recruited effector T cells and macrophages via adaptive immunity (Autschbach *et al*. 1998,
358 Gasche *et al*. 2000). Significant decreases in circulating plasma levels of IL-10 were also
359 observed in the DSS group in this study (Fig. 4). However, blood circulation of systemic levels
360 of IL-10 was not always necessarily shown to be linked to status of chronic bowel inflammation
361 (Braat *et al*. 2003). Gut mucosal IL-10 is known to be contributed by many cell types, including
362 T cells, mast cells, epithelial cells, macrophages and dendritic cells (Unutmaz and Pulendran
363 2009). It has been further revealed that lamina propria-recruited macrophages are the major

364 cellular contributor to the mucosal IL-10, and interstitial IL-10, acted, in a paracrine manner, in
365 promoting regulatory T cells to express transcriptional factor Foxp3 for suppressing autoimmune
366 responses (Murai *et al*. 2009, Unutmaz and Pulendran 2009). Considering that colonic
367 monocytes such as macrophages only represented a very small fraction of cell populations in the
368 colonic mucosa, expression of total IL-10 mRNA abundance relative to the housekeeping gene
369 β-actin in the homogenized colonic mucosa obtained for the DSS group might have been
370 dramatically reduced to be undetectable in this study (Table 5). On the other hand, the jejunal
371 IL-10 mRNA relative abundance was drastically enhanced, in contrast to, the colon within the
372 DSS group in this study (Table 5). These observations have two implications. Firstly, the DSS
373 administration did not cause chronic inflammation to the jejunal mucosa in the young pigs in this
374 study. Secondly, adaptive immunity and regulatory immune mechanisms responded
375 differentially to the jejunal and colonic mucosal tissues under the DSS-induced chronic
376 ulcerative colitis in the colon of young pigs in this study. Under this context, it should be
377 pointed out that only acute inflammation and acute ulcerative colitis in the colon were induced
378 upon administration of the same dose of DSS (1.25 g/kg BW) for 5 days, as indicated by the
379 enhanced colonic IL-10 mRNA abundances in our previous studies with young pigs (Kim *et al*.
380 2009, Young *et al*. 2012). Therefore, it can be concluded that administration of DSS (1.25 g/kg
381 BW) for 10 days was effective in inducing a marked reduction in the colonic concentration and
382 mRNA abundance of IL-10 and the development of chronic inflammation associated with
383 chronic ulcerative colitis in the colon of young pigs.

384 It has been well established that expressions of the pro-inflammatory cytokines TNF-α and
385 IL-6 are recognized major biomarkers of intestinal inflammation (Braegger *et al*. 1992, Rugtveit
386 *et al*. 1997, Mudter and Neurath 2007). Significant increases in both TNF-α and IL-6 cytokine

387 concentrations and relative mRNA abundances in the jejunal and colonic tissues were observed
388 in the DSS group when compared with the control group (Table 5; Fig. 3). Furthermore, TNF-α
389 and IL-6 concentrations in circulating blood were higher in the DSS group than in the control
390 group (Fig. 4). These significant intestinal local and systemic responses in the TNF-α and IL-6
391 concentrations could have further attributed to either a decreased protection of the small intestine
392 to enteral antigens and/or the angiogenic responses involved in tissue repair (Chidlow *et al*.
393 2007), further suggesting the contribution of adaptive immunity responses in this study.
394 Furthermore, within the DSS treatment group, the TNF-α and IL-6 cytokine levels were much
395 higher in the colonic than in the jejunal tissue (Fig. 3), suggesting that the DSS administration
396 caused much more severe inflammatory responses in the colon. These results were consistent
397 with the histopathological grading of the proximal jejunum and colon, suggesting that the DSS
398 treatment resulted in considerable transmural inflammatory damage in the colon (Table 4). Our
399 morphological data in Table 3 showed that the DSS administration and the associated
400 inflammation caused considerable villous atrophy, crypt hyperplasia and stimulated smooth
401 muscle growth, which is not uncommon to the injured gut. Both TNF-α and IL-6 have been
402 shown to reduce mucosal hypertrophic growth and cell maturation by inhibiting the mammalian
403 target of rapamycin-signaling pathway mediated cellular protein synthesis (Yang *et al*. 2008). In
404 addition, both TNF-α and IL-6, are known to affect crypt cell proliferation and apoptosis, thus
405 contributing to hyperplasic crypt cellular growth (Seidelin 2004). This is compounded by the
406 ability of IL-6 to recruit angiogenic adhesion molecules that contribute to classic IBD mucosal
407 lesions when unregulated during inflammation (Romano *et al*. 1997, Ito *et al*. 2002). Hence, our
408 data further suggested that administration of DSS at 1.25 g/kg BW for 10 days resulted in
409 significant bowel inflammation with a particular severity in the colon, demonstrating chronic

410 ulcerative colitis in the colon of young pigs in this study by taking together of the IL-10
411 expression data.

412 It has been well demonstrated that abnormal upper gut permeability, especially in the
413 stomach and the small intestinal region, predisposes the gut mucosal local immune system and
414 the body primary immune organs to interact with antigens originated and presented from the gut
415 lumen, leading to contribution of adaptive immune responses to the pathogenesis of chronic
416 bowel inflammation and IBD (Meddings 2008, Arrieta *et al*. 2009, Su *et al*. 2009). The dramatic
417 27.4 fold increase (Fig. 2) in the rate of plasma D-mannitol concentration rising in the DSS
418 group highlights the compromised permeability of the stomach and the small intestine due to
419 DSS administration in the young pigs in this study. It should be pointed out that D-mannitol
420 used in this study is a transcellular permeability marker. Nevertheless, our *in vivo* stomach-small
421 intestine-specific permeability data supported the notion of abrogation of the upper bowel barrier
422 function is a pre-requisite for the occurrence of chronic bowel inflammation and the
423 development of IBD (Meddings 2008, Arrieta *et al*. 2009, Su *et al*. 2009). Therefore,
424 compromised upper gut permeability due to the DSS administration contributed to the
425 development of the acute inflammation in the jejunum and the chronic inflammation in
426 association with chronic ulcerative colitis in the colon of the young pigs in this study.

427 Interestingly, feed intake was not significantly reduced in the DSS-infused young pigs,
428 even though severe intestinal inflammation was present. This result is, in contrast to, the
429 previous observations of a reduced food intake associated with IBD due to cytokine-induced
430 anorexia and food avoidance (Hoshino *et al.* 1991, Rigaud *et al.* 1994). Differences in
431 experimental conditions such as age or species of test animals or human subjects and the
432 duration of inflammation might have affected pro-inflammatory cytokine levels in the blood

433 circulation and food intake responses between the studies. The significantly lower BW gain and
434 feed conversion efficiency in the DSS-treated piglets in comparison with the control group
435 observed in this study are typical symptoms of chronic ulcerative colitis (Leenen and Dieleman
436 2007), and may be explained by several reasons. Firstly, active colitis enhances the whole body
437 energy metabolic rate (Klein *et al*. 1988, Azcue *et al.* 1997). Secondly, the small intestinal
438 villous atrophy associated with IBD demonstrated by DSS challenge in this study might have
439 resulted in a degree of compromised final phase nutrient digestion in the small intestine
440 (Lackeyram *et al*. 2012), which could exacerbate IBD symptoms in the large intestine such as
441 food intolerance (Atkinson *et al.* 2004, Rigaud *et al*. 1994), bacterial overgrowth and bloating
442 (Pimentel *et al*. 2000). Thirdly, absorbed amino acids are first-pass utilized by the gut and other
443 visceral organs such as liver in the young pig (Stoll *et al.* 1998). Under intestinal inflammation,
444 a much larger proportion of the absorbed amino acids are shifted for their local utilization and
445 metabolism in the gut and the other visceral organs, resulting in a much reduced availability of
446 the absorbed amino acids for the peripheral muscle protein synthesis and deposition (Fan *et al*.
447 2006). Finally, it has been well documented that bowel inflammation such as IBD alters whole
448 body protein metabolism by reducing protein synthesis and increasing protein degradation in the
449 skeletal muscle, and enhancing amino acid supply to the increased demands of visceral organ
450 protein synthesis (Heys *et al*. 1992, Farges *et al*. 2002, Mercier *et al.* 2002). Therefore, growth
451 rate and efficiency of dietary nutrient utilization were decreased during the chronic bowel
452 inflammation in association with chronic ulcerative colitis in the colon induced by DSS in the
453 young pigs in this study.

454 In summary, administration of DSS at 1.25 g/kg BW for 10 days was effective in inducing
455 a marked reduction in the concentration and mRNA abundance of IL-10 and elevated

456 concentrations and mRNA abundances of TNF-α and IL-6 in the colon of the young pigs. Taken
457 together, our results suggest that this study regimen represented a time period (i.e., 10 days) in
458 the DSS administration at 1.25 g/kg BW for the development of chronic inflammation in
459 association with chronic ulcerative colitis in the colon of young pigs. Furthermore, we
460 demonstrated that the upper gut permeability was compromised in the young pigs under the
461 chronic inflammation with chronic ulcerative colitis in the colon as induced by DSS. Therefore,
462 this DSS-induction based chronic ulcerative colitis young pig model is useful for studying
463 mechanisms and therapeutic strategies associated with the regulation of IL-10 gene expression in
464 the colon as well as cellular and molecular events responsible for the compromised upper gut
465 permeability under chronic bowel inflammation linked to ulcerative colitis in humans.
466

467 ## Conflict of Interest

468 There is no conflict of interest.
469

470 ## Acknowledgements

471 Supported by research grants from the Natural Sciences and Engineering Research Council
472 (NSERC) of Canada Discovery Program and the Ontario Ministry of Agriculture, Food and
473 Rural Affairs (OMAFRA) – the University of Guelph Partnership Research Program (to M.Z.
474 Fan).
475

476 ## References

477

478 AINLEY C, CASON J, SLAVIN BM, WOLSTENCROFT RA, THOMPSON RP: The influence
479 of zinc status and malnutrition on immunological function in Crohn's disease.
480 *Gastroenterology* **100:** 1616-25, 1991.

481 ARRIETA MC, MADSEN K, DOYLE J, MEDDINGS J: Reducing small intestinal permeability
482 attenuates colitis in the IL10 gene-deficient mouse. *Gut* **58:** 41-8, 2009.

483 ATKINSON W, SHELDON TA, SHAATH N, WHORWELL PJ: Food elimination based on
484 IgG antibodies in irritable bowel syndrome: a randomised controlled trial. *Gut* **53:** 1459-
485 64, 2004.

486 AUTSCHBACH F, BRAUNSTEIN J, HELMKE B, ZUNA I, SCHÜRMANN G, NIEMIR ZI,
487 WALLICH R, OTTO HF, MEUER SC: In situ expression of interleukin-10 in
488 noninflamed human gut and in inflammatory bowel disease. *Am J Pathol* **153:** 121-30,
489 1998.

490 AZCUE M, RASHID M, GRIFFITHS A, PENCHARZ PB: Energy expenditure and body
491 composition in children with Crohn's disease: effect of enteral nutrition and treatment
492 with prednisolone. *Gut* **41:** 203-8, 1997.

493 BASSAGANYA-RIERA J, HONTECILLAS R: CLA and n-3 PUFA differentially modulate
494 clinical activity and colonic PPAR-responsive gene expression in a pig model of
495 experimental IBD. *Clin nutr (Edinburgh, Scotland)* **25:** 454, 2006.

496 BRAAT H, PEPPELENBOSCH MP, HOMMES DW: Interleukin-10-based therapy for
497 inflammatory bowel disease. *Expert Opin Biol Ther* **3:** 725-31, 2003.

498 BRAEGGER CP, NICHOLLS S, MURCH SH, MACDONALD TT, STEPHENS S: Tumour
499 necrosis factor alpha in stool as a marker of intestinal inflammation. *The Lancet* **339:** 89-
500 91, 1992.
501 BYRKIT DR, SCHAEFER RL, SKILLINGS JH: *Statistics today: a comprehensive*
502 *introduction*Benjamin/Cummings Pub Co, Menlo Park, Calif., 1987.
503 CANDIAN COUNCIL ON ANIMAL CARE: *Guide to the care and use of experimental*
504 animals. MCWILLIAM A, CROSS B, OLFERT E (eds), Ottawa, ON, 1993.
505 CHENG H, LEBLOND CP: Origin, differentiation and renewal of the four main epithelial cell
506 types in the mouse small intestine. I. Columnar cell. *Am J Anat* **141:** 461-79, 1974.
507 CHIDLOW JH, SHUKLA D, GRISHAM MB, KEVIL CG: Pathogenic angiogenesis in IBD and
508 experimental colitis: new ideas and therapeutic avenues. *Am J Physiol Gastrointest Liver*
509 *Physiol* **293:** G5-G18, 2007.
510 COHEN SL, MOORE AM, WARD WE: Interleukin-10 knockout mouse: a model for studying
511 bone metabolism during intestinal inflammation. *Inflamm Bowel Dis* **10:** 557-63, 2004.
512 DIELEMAN LA, PALMEN MJ, AKOL H, BLOEMENA E, PEÑA AS, MEUWISSEN SG,
513 VAN REES EP: Chronic experimental colitis induced by dextran sulphate sodium (DSS)
514 is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol* **114:** 385-91, 1998.
515 EGGER B, BAJAJ-ELLIOTT M, MACDONALD TT, INGLIN R, EYSSELEIN VE,
516 BÜCHLER MW: Characterisation of acute murine dextran sodium sulphate colitis:
517 cytokine profile and dose dependency. *Digestion* **62:** 240-8, 2000.
518 ELSON CO, CONG Y, MCCRACKEN VJ, DIMMITT RA, LORENZ RG, WEAVER CT:
519 Experimental models of inflammatory bowel disease reveal innate, adaptive, and

regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* **206:** 260-76, 2005.

FAN MZ: Growth and Ontogeny of the Gastrointestinal Tract. *The neonatal pig: gastrointestinal physiology and nutrition*Nottingham University Press, Thrumpton, Nottingham, 2003, pp 31-60.

FAN MZ, CHIBA LI, MATZAT PD, YANG X, YIN YL, MINE Y, STEIN HH: Measuring synthesis rates of nitrogen-containing polymers by using stable isotope tracers. *J Anim Sci* **84 Suppl:** E79-93, 2006.

FAN MZ, STOLL B, JIANG R, BURRIN DG: Enterocyte digestive enzyme activity along the crypt-villus and longitudinal axes in the neonatal pig small intestine. *J Anim Sci* **79:** 371-381, 2001.

FARGES MC, BALCERZAK D, FISHER BD, ATTAIX D, BÉCHET D, FERRARA M, BARACOS VE: Increased muscle proteolysis after local trauma mainly reflects macrophage-associated lysosomal proteolysis. *Am J Physiol Endocrinol Metab* **282:** E326-35, 2002.

FIOCCHI C: Inflammatory bowel disease: etiology and pathogenesis *Gastroenterology* **115:** 182-205, 1998.

FUKATA M, ABREU MT: What are toll-like receptors and what role may they have in IBD? *Inflamm Bowel Dis* **14:** S90-S92, 2008.

FUKATA M, MICHELSEN KS, ERI R, THOMAS LS, HU B, LUKASEK K, NAST CC, LECHAGO J, XU R, NAIKI Y: Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis *Am J Physiol* **288:** G1055-G1065, 2005.

543 GASCHE C, BAKOS S, DEJACO C, TILLINGER W, ZAKERI S, REINISCH W: IL-10
544 secretion and sensitivity in normal human intestine and inflammatory bowel disease. *J*
545 *Clin Immunol* **20:** 362-70, 2000.
546 GRAEFE H, GÜTSCHOW B, GEHRING H, DIBBELT L: Sensitive and specific photometric
547 determination of mannitol in human serum. *Clin Chem Lab Med* **41:** 1049-55, 2003.
548 GRISHAM MB: Do different animal models of IBD serve different purposes? *Inflamm Bowel*
549 *Dis* **14 Suppl 2:** S132-3, 2008.
550 HEYS SD, PARK KG, MCNURLAN MA, KEENAN RA, MILLER JD, EREMIN O,
551 GARLICK PJ: Protein synthesis rates in colon and liver: stimulation by gastrointestinal
552 pathologies. *Gut* **33:** 976-81, 1992.
553 HOSHINO E, PICHARD C, GREENWOOD CE, KUO GC, CAMERON RG, KURIAN R,
554 KEARNS JP, ALLARD JP, JEEJEEBHOY KN: Body composition and metabolic rate in
555 rat during a continuous infusion of cachectin. *Am J Physiol* **260:** E27-36, 1991.
556 ITO H, HIROTANI T, YAMAMOTO M, OGAWA H, KISHIMOTO T: Anti-IL-6 receptor
557 monoclonal antibody inhibits leukocyte recruitment and promotes T-cell apoptosis in a
558 murine model of Crohn's disease. *J Gastroenterol* **37:** 56, 2002.
559 IWANAGA T, HOSHI O, HAN H, FUJITA T: Morphological analysis of acute ulcerative colitis
560 experimentally induced by dextran sulfate sodium in the guinea pig: some possible
561 mechanisms of cecal ulceration *J Gastroenterol* **29:** 430-438, 1994.
562 KAPPELMAN MD, GRAND RJ: Does inflammatory bowel disease develop in infants? *Inflamm*
563 *Bowel Dis* **14 Suppl 2:** S6-8, 2008.
564 KELSEN J, BALDASSANO RN: Inflammatory bowel disease: the difference between children
565 and adults. *Inflamm Bowel Dis* **14 Suppl 2:** S9-11, 2008.

566 KIDDER DE, MANNERS MJ: Passage of Food. *Digestion in the* pig. Scientechnica Bristol, UK,
567 1978,
568 KIM CJ, KOVACS-NOLAN J, YANG C, ARCHBOLD T, FAN MZ, MINE Y: L-cysteine
569 supplementation attenuates local inflammation and restores gut homeostasis in a porcine
570 model of colitis. *Biochim Biophys Acta* **1790:** 1161-9, 2009.
571 KIM CJ, KOVACS-NOLAN JA, YANG C, ARCHBOLD T, FAN MZ, MINE Y: L-Tryptophan
572 exhibits therapeutic function in a porcine model of dextran sodium sulfate (DSS)-induced
573 colitis. *J Nutr Biochem* **21:** 468-475, 2010.
574 KLETA R, ROMEO E, RISTIC Z, OHURA T, STUART C, ARCOS-BURGOS M, DAVE MH,
575 WAGNER CA, CAMARGO SR, INOUE S, MATSUURA N, HELIP-WOOLEY A,
576 BOCKENHAUER D, WARTH R, BERNARDINI I, VISSER G, EGGERMANN T, LEE
577 P, CHAIROUNGDUA A, JUTABHA P, BABU E, NILWARANGKOON S, ANZAI N,
578 KANAI Y, VERREY F, GAHL WA, KOIZUMI A: Mutations in SLC6A19, encoding
579 B^0AT1, cause Hartnup disorder. *Nat Genet* **36**: 999-1002, 2004.
580 KLEIN S, MEYERS S, O'SULLIVAN P, BARTON D, LELEIKO N, JANOWITZ HD: The
581 metabolic impact of active ulcerative colitis. Energy expenditure and nitrogen balance. *J*
582 *Clin Gastroenterol* **10:** 34-40, 1988.
583 KÜHN R, LÖHLER J, RENNICK D, RAJEWSKY K, MÜLLER W: Interleukin-10-deficient
584 mice develop chronic enterocolitis. *Cell* **75:** 263-74, 1993.
585 LACKEYRAM D, MINE Y, ARCHBOLD A, FAN MZ: The small intestinal apical hydrolase
586 activities are decreased in the piglet with bowel inflammation induced by dextran sodium
587 sulfate. *J Anim Sci* **90**:287-289, 2012.

588 LACKEYRAM D, YANG C, ARCHBOLD T, SWANSON KC, FAN MZ: Early weaning
589 reduces small intestinal alkaline phosphatase expression in pigs. *J Nutr* **140:** 461-8,
590 March, 2010.
591 LEENEN CH, DIELEMAN LA: Inulin and oligofructose in chronic inflammatory bowel
592 disease. *J Nutr* **137:** 2572S-2575S, 2007.
593 LONGSTRETH GF, THOMPSON WG, CHEY WD, HOUGHTON LA, MEARIN F, SPILLER
594 RC: Functional bowel disorders *Gastroenterology* **130:** 1480-1491, 2006.
595 LUNN PG, NORTHROP CA, NORTHROP AJ: Automated enzymatic assays for the
596 determination of intestinal permeability probes in urine. 2. Mannitol. *Clin Chim Acta*
597 **183:** 163-70, 1989.
598 MACKENZIE ML, WARREN MR, WYKES LJ: Colitis increases albumin synthesis at the
599 expense of muscle protein synthesis in macronutrient-restricted piglets. *J Nutr* **133:** 1875-
600 81, 2003.
601 MAWDSLEY JE, RAMPTON DS: The role of psychological stress in inflammatory bowel
602 disease. *Neuroimmunomodulation* **13:** 327-336, 2007.
603 MEDDINGS J: What role does intestinal permeability have in IBD pathogenesis? *Inflamm*
604 *Bowel Dis* **14 Suppl 2:** S138-9, 2008.
605 MERCIER S, BREUILLÉ D, MOSONI L, OBLED C, PATUREAU MIRAND P: Chronic
606 inflammation alters protein metabolism in several organs of adult rats. *J Nutr* **132:** 1921-
607 8, 2002.
608 MUDTER J, NEURATH MF: Il-6 signaling in inflammatory bowel disease: pathophysiological
609 role and clinical relevance. *Inflamm Bowel Dis* **13:** 1016-23, 2007.

610 MURAI M, TUROVSKAYA O, KIM G, MADAN R, KARP CL, CHEROUTRE H,
611 KRONENBERG M: Interleukin 10 acts on regulatory T cells to maintain expression of
612 the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol*
613 **10:** 1178-84, 2009.
614 MURCH SH, LAMKIN VA, SAVAGE MO, WALKER-SMITH JA, MACDONALD TT: Serum
615 concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel
616 disease. *Gut* **32:** 913-7, 1991.
617 OKAYASU I, HATAKEYAMA S, YAMADA M, OHKUSA T, INAGAKI Y, NAKAYA R: A
618 novel method in the induction of reliable experimental acute and chronic ulcerative colitis
619 in mice. *Gastroenterology* **98:** 694-702, 1990.
620 PAPADAKIS KA, TARGAN SR: Role of cytokines in the pathogenesis of inflammatory bowel
621 disease. *Ann Rev Med* **51:** 289-298, 2000.
622 PIMENTEL M, CHOW EJ, LIN HC: Eradication of small intestinal bacterial overgrowth
623 reduces symptoms of irritable bowel syndrome. *Am J Gastroenterol* **95:** 3503-6, 2000.
624 PODOLSKY DK: Inflammatory bowel disease. *N Engl J Med* **347:** 417-29, August 8, 2002.
625 RIDEOUT TC, YUAN Z, BAKOVIC M, LIU Q, LI RK, MINE Y, FAN MZ: Guar gum
626 consumption increases hepatic nuclear SREBP2 and LDL receptor expression in pigs fed
627 an atherogenic diet. *J Nutr* **137:** 568-72, 2007.
628 RIGAUD D, ANGEL LA, CERF M, CARDUNER MJ, MELCHIOR JC, SAUTIER C, RENÉ E,
629 APFELBAUM M, MIGNON M: Mechanisms of decreased food intake during weight
630 loss in adult Crohn's disease patients without obvious malabsorption. *Am J Clin Nutr* **60:**
631 775-81, 1994.

632 ROMANO M, SIRONI M, TONIATTI C, POLENTARUTTI N, FRUSCELLA P, GHEZZI P,
633 FAGGIONI R, LUINI W, VAN HINSBERGH V, SOZZANI S, BUSSOLINO F, POLI
634 V, CILIBERTO G, MANTOVANI A: Role of IL-6 and its soluble receptor in induction
635 of chemokines and leukocyte recruitment. *Immunity* **6:** 315-25, 1997.
636 RUGTVEIT J, NILSEN EM, BAKKA A, CARLSEN H, BRANDTZAEG P, SCOTT H:
637 Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages
638 from inflammatory bowel disease. *Gastroenterology* **112:** 1493-505, 1997.
639 SARTOR RB: Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases
640 *Am J Gastroenterol* **92:** 5S-11S, 1997.
641 SÁNCHEZ-FIDALGO S, CÁRDENO A, VILLEGAS I, TALERO E, DE LA LASTRA CA:
642 Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice.
643 *Eur J Pharmacol* **633:** 78-84, 2010.
644 SEIDELIN JB: Colonic epithelial cell turnover: possible implications for ulcerative colitis and
645 cancer initiation. *Scand J Gastroenterol* **39:** 20111, 2004.
646 SNOECK V, HUYGHEBAERT N, COX E, VERMEIRE A, SAUNDERS J, REMON JP,
647 VERSCHOOTEN F, GODDEERIS BM: Gastrointestinal transit time of
648 nondisintegrating radio-opaque pellets in suckling and recently weaned piglets. *J Control*
649 *Release* **94:** 143-53, 2004.
650 SOMERVILLE KW, LOGAN RF, EDMOND M, LANGMAN MJ: Smoking and Crohn's
651 disease. *Br Med J (Clin Res ed.)* **289:** 954, 1984.
652 SONNENBURG JL, XU J, LEIP DD, CHEN C-H, WESTOVER BP, WEATHERFORD J,
653 BUHLER JD, GORDON JI: Glycan foraging in vivo by an intestine-adapted bacterial
654 symbiont *Science* **307:** 1955-1959, 2005.

655 STOLL B, HENRY J, REEDS PJ, YU H, JAHOOR F, BURRIN DG: Catabolism dominates the
656 first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed
657 piglets. *J Nutr* **128:** 606-14, 1998.
658 STROBER W, FUSS IJ, BLUMBERG RS: The immunology of mucosal models of
659 inflammation. *Annu Rev Immunol* **20:** 495-549, 2002.
660 SU L, SHEN L, CLAYBURGH DR, NALLE SC, SULLIVAN EA, MEDDINGS JB,
661 ABRAHAM C, TURNER JR: Targeted epithelial tight junction dysfunction causes
662 immune activation and contributes to development of experimental colitis.
663 *Gastroenterology* **136:** 551-63, 2009.
664 THYMANN T, BURRIN DG, TAPPENDEN KA, BJORNVAD CR, JENSEN SK, SANGILD
665 PT: Formula-feeding reduces lactose digestive capacity in neonatal pigs. *Br J Nutr* **95:**
666 1075-81, 2006.
667 TSUNE I, IKEJIMA K, HIROSE M, YOSHIKAWA M, ENOMOTO N, TAKEI Y, SATO N:
668 Dietary glycine prevents chemical-induced experimental colitis in the rat.
669 *Gastroenterology* **125:** 775-85, 2003.
670 UNUTMAZ D, PULENDRAN B: The gut feeling of Treg cells: IL-10 is the silver lining during
671 colitis. *Nat Immunol* **10:** 1141-1143, 2009.
672 VICARIO M, AMAT C, RIVERO M, MORETÓ M, PELEGRÍ C: Dietary glutamine affects
673 mucosal functions in rats with mild DSS-induced colitis. *J Nutr* **137:** 1931-7, 2007.
674 YANG X, YANG C, FARBERMAN A, RIDEOUT TC, DE LANGE CF, FRANCE J, FAN MZ:
675 The mammalian target of rapamycin-signaling pathway in regulating metabolism and
676 growth. *J Anim Sci* **86:** E36-50, 2008.

677 YOUNG D, IBUKI M, NAKAMORI T, FAN MZ**,** MINE Y: Soy-derived di- and tripeptides
678 alleviate colon and ileum inflammation in pigs with dextran sodium sulfate-induced
679 colitis. *J Nutr* **142**:363-368, 2010.
680

681 **Tables:**

682

683 **Table 1.** Primer sequences used for the real time RT-PCR analyses.[1]

Gene	Sequence (5'-3')	Primer size	Tm value	Location	Product size	Accession number
TNF-α	FP: atggatgggtggatgagaaa	20bp	60.13	Exon 8	151bp	X54001
	RP: tggaaactgttggggagaag	20bp	60.08	Exon 10		
IL-6	FP: aaggtgatgccacctcagac	20bp	60.12	Exon 2	151bp	M86722
	RP: tctgccagtacctccttgct	20bp	60.01	Exon 3		
IL10	FP:tgatggggaggatatcaagg	20bp	59.70	Exon 4	150bp	NM_214041
	RP: tggagcttgctaaaggcact	20bp	60.15	Exon 5		
β-actin	FP:ggatgcagaaggagatcacg	20bp	60.77	Exon 4	130bp	U07786
	RP: atctgctggaaggtggacag	20bp	60.26	Exon 5		

684 [1]Primers for the porcine TNF-α, IL-6, IL-10, and β-actin (the housekeeping gene) were designed

685 with Primer 3 (http://frodo.wi.mit.edu).

686

687 **Table 2.** Comparison of growth performance of control and DSS-treated young pigs fed a milk
688 replacer.[1]

Items	**Control**	**DSS**	***p values***
Initial BW, kg	3.20 ± 0.18	3.39 ± 0.04	0.355
Final BW, kg	4.90 ± 0.06[a]	4.55 ± 0.08[b]	0.025
Average daily gain, g/d	170.40 ± 9.17[a]	116.36 ± 16.18[b]	0.014
Average formula intake, ml/d	1165.32 ± 22.20	1216.90 ± 29.70	0.27
Gain to feed ratio, g/ml	0.15 ± 0.01[a]	0.09 ± 0.01[b]	0.012

689 [1]Values are means ± SEM, n = 6, control group; n = 5, DSS group. [a,b]Values in the same row not
690 sharing a superscript letter are different, $p<0.05$.
691
692
693

694 **Table 3.** Comparison of jejunal and colonic morphological endpoints in control and DSS-treated
695 young pigs fed a milk replacer.[1]

Items	**Control**	**DSS**	***p values***
Proximal jejunum			
Villous height, µm	448.56 ± 7.45[a]	257.04 ± 10.53[b]	0.001
Crypt depth, µm	125.09 ± 2.40[a]	168.57 ± 5.78[b]	0.002
Villous to crypt ratio	3.87 ± 0.09[a]	1.39 ± 0.07[b]	0.002
Muscle thickness, µm	228.82 ± 2.42[a]	453.58 ± 15.50[b]	0.001
Colon			
Mucosal thickness, µm	641.06 ± 20.45[a]	346.23 ± 11.35[b]	0.001
Muscle thickness, µm	283.30 ± 22.11[a]	398.70 ± 26.92[b]	0.008

696 [1]Values are means ± SEM, n = 6, control group; n = 5, DSS group. [a,b]Values in the same row not
697 sharing a superscript letter are different, $p<0.05$.
698
699

700 **Table 4.** Comparison of histopathological grading of colonic and jejunal inflammation
701 occurrence in control and DSS-treated young pigs fed a milk replacer.[1]

Feature graded[2]	Control	DSS	*p* values
Proximal Jejunum			
Inflammation	0.06 ± 0.01^{a}	1.53 ± 0.002^{b}	0.001
Extent	0.06 ± 0.01^{a}	1.07 ± 0.02^{b}	0.001
Regeneration	0.00^{a}	1.02 ± 0.001^{b}	0.001
Crypt damage	0.00^{a}	0.40 ± 0.04^{b}	0.001
Total Score	0.11 ± 0.01^{a}	4.0 ± 0.05^{b}	0.001
Large Intestine			
Inflammation	0.09 ± 0.02^{a}	10.67 ± 0.17^{b}	0.001
Extent	0.09 ± 0.02^{a}	10.67 ± 0.17^{b}	0.001
Regeneration	0.00^{a}	14.13 ± 0.23^{b}	0.001
Crypt damage	0.00^{a}	15.16 ± 0.24^{b}	0.001
Total Score	0.19 ± 0.04^{a}	50.64 ± 0.79^{b}	0.001

702 [1]Values are means ± SEM, n = 6, control group; n = 5, DSS group. [2]Quantitative histological
703 grading of intestinal colitis occurrence was adopted from a previous study (Dieleman et al.,
704 1998). [a,b]Values in the same row not sharing a superscript letter are different, $p<0.05$.
705
706
707

708 **Table 5.** Comparison of relative mRNA expressions[1] of TNF-α, IL-6 and IL-10 in the proximal
709 jejunum and colon measured by real time RT-PCR in control and DSS-treated young pigs fed a
710 milk replacer.

Items	**Control**	**DSS**	***p* values**
Proximal jejunum			
TNF-α	0.004 ± 0.0002[a]	5.367 ± 0.289[b]	0.001
IL-6	0.006 ± 0.0005[a]	0.5753 ± 0.018[b]	0.001
IL-10	2.195 ± 0.244 [a]	33.350 ± 7.080 [b]	0.001
Colon			
TNF-α	0.011 ± 0.002[a]	1.210 ± 0.510[b]	0.001
IL-6	0.049 ± 0.008[a]	15.958 ± 0.824[b]	0.001
IL-10	2.500 ± 0.050	UD[2]	-

711 [1]Values are means ± SEM, n = 6, control group; n = 5, DSS group. β-actin was used as the
712 housekeeping gene for data normalization. [2]UD: undetectable by real time RT-PCR analysis.
713 [a,b]Values in the same row not sharing a superscript letter are different, $p<0.05$.

714
715
716
717
718
719

720 **Figure Legends:**

721

722 **Fig. 1.** Pictograph – 10 X magnification of haematoxylin and eosin stained cross sections of
723 intestinal tissues in control and DSS-treated young pigs fed a milk replacer. (**A**) Proximal
724 jejunum of control piglets. (**B**) Proximal jejunum of DSS-treated piglets. (**C**) Colon of control
725 piglets. (**D**) Colon of DSS-treated piglets.

726

727 **Fig. 2.** Linear relationships between plasma D-mannitol concentration (umol/ml) and the time
728 (min) of post-ig infusion of D-mannitol in the control compared with DSS-treated young pigs fed
729 a milk replacer. Values represent mean ± SE, n = 6, control; n = 5, DSS group, for each of the
730 marked time points. For the control group, y = 0.0049 (± 0.0014)*x, r^2 = 0.28, n = 36; for the
731 DSS group, y = 0.1389 (± 0.0130)*x, r^2 = 0.8022, n = 30; $p<0.05$ for all the parameter estimates.
732 Differences in the slopes of the linear relationships were observed between the control and the
733 DSS groups, p=0.001.

734

735 **Fig. 3.** Concentrations (pg/mg protein) of TNF-α (upper panel), IL-6 (middle panel) and IL-10
736 (lower panel) analyzed by ELISA in the jejunum and colon of control and DSS-treated young
737 pigs fed a liquid formula. Values are means ± SEM, n = 6, control; n = 5, DSS group. *Indicate
738 differences from control, $p<0.05$.

739

740 **Fig. 4.** Concentrations (pg/mg protein) of TNF-α (upper panel), IL-6 (middle panel) and IL-10
741 (lower panel) peptides (pg/mg protein) analyzed by ELISA in the plasma of control and DSS-

742 treated young pigs fed a liquid formula. Values are means ± SEM, n = 6, control; n = 5, DSS
743 group. *Indicate differences from control, $p<0.05$.
744

FIGURE 1: Lackeyram et al.

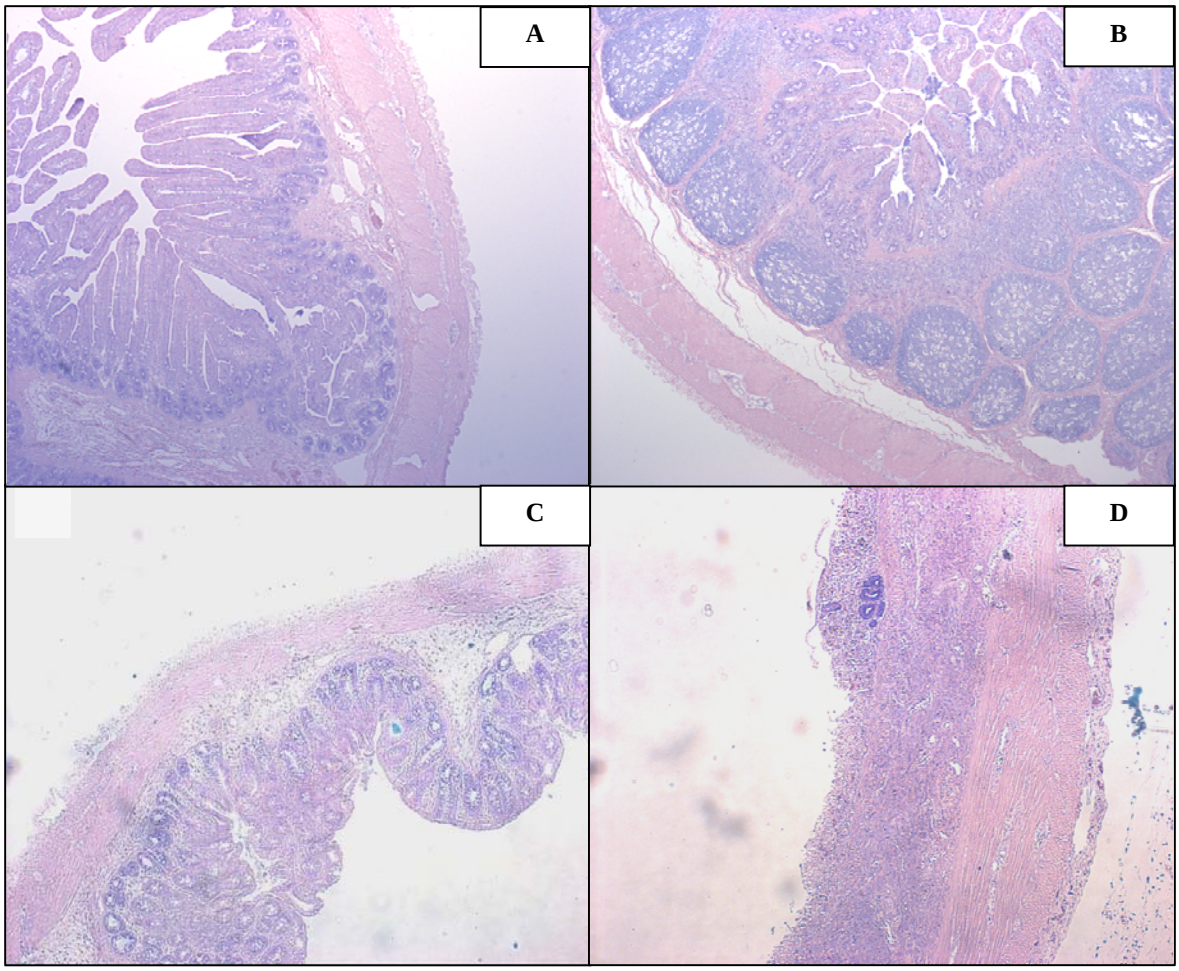

FIGURE 2: Lackeyram et al.

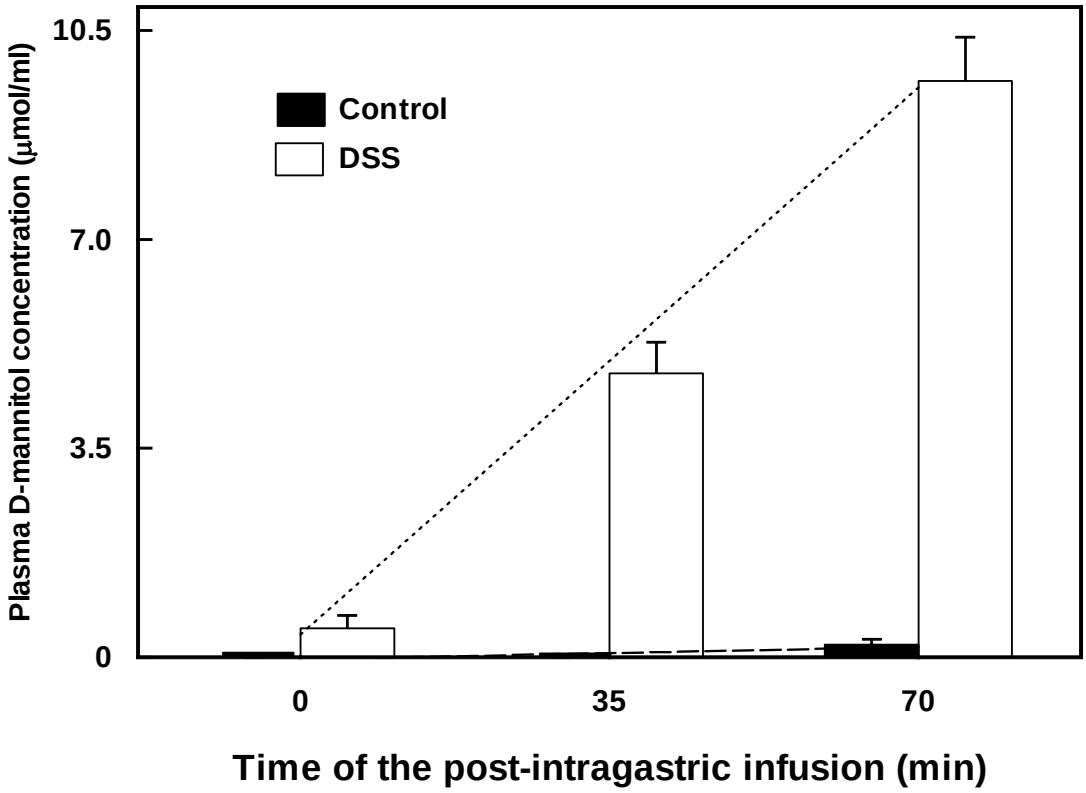

FIGURE 3: Lackeyram et al.

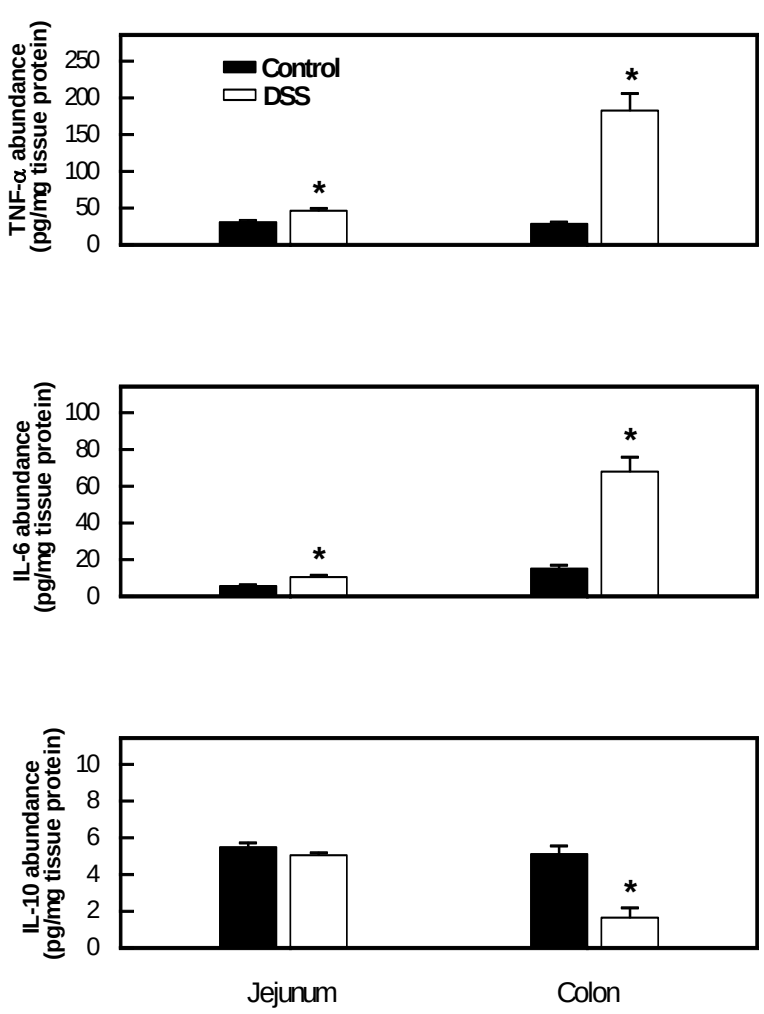

FIGURE 4: Lackeyram et al.