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3 **Ursodeoxycholic acid and lithocholic acid exert anti-inflammatory actions in the colon**

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20 **Running Title:** UDCA and LCA prevent colonic inflammation

21

22 ABSTRACT

23 Inflammatory bowel diseases (IBD) are a group of common and debilitating chronic intestinal
24 disorders for which currently-available therapies are often unsatisfactory. The naturally-
25 occurring secondary bile acid, ursodeoxycholic acid (UDCA), has well-established anti-
26 inflammatory and cytoprotective actions and may therefore be effective in treating IBD.
27 Here, we aimed to investigate regulation of colonic inflammatory responses by UDCA and to
28 determine the potential impact of bacterial metabolism on its therapeutic actions. The anti-
29 inflammatory efficacy of UDCA, a non-metabolisable analogue, 6-methyl-UDCA (6-
30 MUDCA), and its primary colonic metabolite, lithocholic acid (LCA), were assessed in the
31 murine DSS model of mucosal injury. The effects of bile acids on cytokine release (TNF- α ,
32 IL-6, IL-1 β , IFN- γ) from cultured colonic epithelial cells and mouse colonic tissue *in vivo*
33 were investigated. Luminal bile acids were measured by GC-MS. UDCA attenuated release
34 of proinflammatory cytokines from colonic epithelial cells *in vitro* and was protective against
35 the development of colonic inflammation *in vivo*. In contrast, although 6-MUDCA mimicked
36 the effects of UDCA on epithelial cytokine release *in vitro*, it was ineffective in preventing
37 inflammation in the DSS model. In UDCA-treated mice, LCA became the most common
38 colonic bile acid. Finally, LCA treatment more potently inhibited epithelial cytokine release
39 and protected against DSS-induced mucosal inflammation than did UDCA. These studies
40 identify a new role for the primary metabolite of UDCA, LCA, in preventing colonic
41 inflammation and suggest that microbial metabolism of UDCA is necessary for the full
42 expression of its protective actions.

43

44 NEW AND NOTEWORTHY

45 Based on its cytoprotective and anti-inflammatory actions, the secondary bile acid,
46 ursodeoxycholic acid (UDCA), has well-established uses in both traditional and Western
47 medicine. Here, we identify a new role for the primary metabolite of UDCA, lithocholic acid,
48 as a potent inhibitor of intestinal inflammatory responses and we present data to suggest that
49 microbial metabolism of UDCA is necessary for the full expression of its protective effects
50 against colonic inflammation.

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52 **Keywords:** Bile acid; Epithelium; Inflammatory Bowel Disease; Cytokine, Barrier Function

53 INTRODUCTION

54 Inflammatory bowel diseases, such as ulcerative colitis (UC) and Crohn's disease (CD), are
55 chronic, relapsing inflammatory disorders of the gastrointestinal tract affecting approximately
56 1% of the adult population of Western countries. While the pathogenesis of inflammation
57 associated with IBD is still not well-defined, it is widely accepted that a combination of
58 genetic, environmental, and immunological factors are involved, which drive an
59 inappropriate mucosal inflammatory response (17). With this in mind, current therapeutic
60 options employ anti-inflammatory drugs, including glucocorticoids, immunosuppressants,
61 aminosalicylates, and biologics to inhibit mucosal immune responses and production of
62 proinflammatory cytokines (6). While each of these treatment approaches can be of benefit,
63 they also have significant drawbacks in terms of the occurrence of side effects, lack of
64 efficacy, and high cost (42). Thus, more effective, and safer, drugs to treat colitis are much
65 needed.

66 Epithelial cells lining the colonic lumen play a key role in IBD pathogenesis (28, 36). One of
67 the primary physiological roles of the epithelium is to act as an innate barrier against the
68 uptake of luminal toxins and pathogens. There are several components to this barrier,
69 including the physical barrier posed by the epithelium itself, along with numerous secreted
70 factors, such as mucus and cytokines. A hallmark feature of IBD is dysregulation of epithelial
71 barrier function with associated increases in permeability and induction of cytokine release
72 (2, 30). Many endogenous and exogenous components of the luminal contents have been
73 shown to have the capacity to promote epithelial cytokine release, including bacterial toxins
74 and cell wall components, viral RNA, and bile acids, all of which are altered in the setting of
75 gut inflammation (8, 26, 27). Thus, given its central role in the development of colitis, the
76 epithelium is currently receiving a great deal of interest as a target for the development of
77 new treatments (28, 42).

78 Ursodeoxycholic acid (UDCA) is a naturally-occurring secondary bile acid, produced in the
79 colon by bacterial metabolism of the primary bile acid chenodeoxycholic acid (CDCA).
80 UDCA is considered to be unique among bile acids as it has long been recognized to have
81 broad-ranging protective actions. Indeed, UDCA is often referred to as the “therapeutic” bile
82 acid as it has been used for centuries in Traditional Chinese Medicine, as a component of
83 bear bile, to treat diverse maladies, such as failing eyesight, intestinal malaise, impotency,
84 and fever (10). More recently, in Western medicine, UDCA has been used to treat liver
85 inflammation and cholestasis (24, 47), and currently it is also under investigation for a
86 number of conditions, including neurological, ocular, cardiovascular, and metabolic disorders
87 (45). Importantly, unless it is used at high doses (9), UDCA is a safe drug with few side
88 effects. While its mechanisms of action are not well-defined, it is believed that the therapeutic
89 properties of UDCA are largely due to its anti-inflammatory and cytoprotective actions (5,
90 45). The biological actions of UDCA have been mostly studied in the liver, where it has been
91 shown to exert immunomodulatory and anti-apoptotic actions, and to prevent cytokine release
92 (7, 33, 34, 37). In the current study, we hypothesised that by virtue of its anti-inflammatory
93 and cytoprotective properties, UDCA is a represents a promising target for development of
94 new treatments for diseases associated with intestinal inflammation. However, when
95 considering UDCA as a potential therapeutic for intestinal disease, it is also important to
96 consider that *in vivo*, it is extensively metabolised by the colonic microbiome and the effects
97 that this has on its therapeutic activity are not known. Thus, in the current study we used *in*
98 *vitro* and *in vivo* models to investigate the anti-inflammatory effects of UDCA in the colon
99 and the potential consequences of bacterial metabolism on its therapeutic actions.

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101

102 **MATERIALS AND METHODS:**

103 **Ethical Approval:** All experiments carried out on mice conformed to the Animal Research:
104 Reporting of *In Vivo* Experiments (ARRIVE) guidelines and were approved by the RCSI
105 Research Ethics Committee (REC739) and by the Irish Department of Health and Children
106 (B100/4159).

107 **Animal Studies:** All experiments carried out on mice conformed to the Animal Research:
108 Reporting of *In Vivo* Experiments (ARRIVE) guidelines and were approved by the RCSI
109 Research Ethics Committee (REC739) and by the Irish Department of Health and Children
110 (B100/4159). Male C57Bl/6 mice were used between 10 – 12 weeks of age. Colitis was
111 induced in mice by addition of 2.5 % DSS (MP Biomedicals, Solon, OH) to their drinking
112 water for 5 days. Disease activity index (DAI) was used as a measure of disease progression
113 and was calculated by the addition of scores designated to body weight, faecal blood and
114 stool consistency/diarrhoea, as previously described (39). Starting 24 hrs before
115 administration of DSS, and once daily thereafter, animals received by intraperitoneal
116 injection, either endotoxin-free PBS as vehicle control, Na⁺-UDCA (30 or 100 mg/kg), Na⁺-
117 6-MUDCA or Na⁺-LCA (30 mg/kg) dissolved in PBS. Mice were sacrificed on day 6, the
118 length of their colons was recorded, caecal contents were kept for analysis, and colonic tissue
119 was processed for H&E staining, or for analysis of cytokine expression. For histological
120 scoring, approximately 1 cm sections of colonic tissue were fixed in 10% paraformaldehyde
121 (pH 7.4; PBS buffered) and embedded in paraffin. Sections (4 µm) were cut and stained with
122 H&E. All sections were examined in a blinded fashion independently by 2 observers and
123 histologic scoring was carried out, as previously described (39). Blood was collected at time
124 of sacrifice by cardiac puncture. Serum was obtained by centrifugation (2,000 x g for 10
125 minutes, 4°C), aliquoted, and stored at -80°C until use. Serum creatinine and ALT were

126 measured using the RXL Dimension Autoanalyser platform (Siemens Healthcare
127 Diagnostics, Munich, Germany).

128 **Cytokine Measurements:** T₈₄ or HT29C119A cells were cultured on 96-well plates until they
129 reached approximately 80% confluence. Cells were serum-starved for 1hr prior to stimulation
130 with polyinosinic:polycytidylic acid (poly I:C) (25 µg/ml) or TNF-α (10 ng/ml) in the
131 presence or absence of UDCA or LCA (24 hr, 37°C). Mouse colons were homogenised in
132 liquid N₂ on dry ice, re-suspended by vortexing in lysis buffer (1% Nonidet P-40, 150 m
133 NaCl, 50 mmol/L Tris Base, 1 x Complete mini EDTA free protease inhibitor tablet, 0.1
134 mg/1mL PMSF, 1 mmol/L Na₃VO₄) in a m/v ratio of 1:5, lysed (45 minutes on ice),
135 sonicated (3 x 10s pulses), centrifuged (15,294 x g, 20 mins, 4°C) and supernatants were
136 retained for analysis. For measurements of TNF-α, IL-1β, IL-6, IFN-γ, IL-12p70, and GM-
137 CSF cell culture supernatants or colonic lysates were then added to a pre-coated V-Plex
138 Multi-array and Multi-sport Human Cytokine Assay plates (Catalogue #: K15007B-1) and
139 assayed as per the manufacturer's protocol (Meso Scale Diagnostics; Rockville, MD).
140 Measurements of IL-8 release from T₈₄ cells were carried out by ELISA (Beckton Dickinson,
141 San Diego, CA).

142 **Caecal bile acid analysis:** Caecal contents were collected from treated and control animals
143 and stored in isopropanol at -20°C. Caecal bile acid levels were measured by HPLC-ES-
144 MS/MS, as previously described (38).

145 **Acid Phosphatase Assay:** T₈₄ cells grown to confluency on 96-well plates were serum-
146 starved for 1hr prior to treatment with LCA. Cells were then washed in warm PBS, incubated
147 in sodium acetate buffer (0.1M C₂H₃NaO₂, pH 5.5, 0.1% Triton x-100) protected from light
148 at 37°C for 30 mins, following which absorbance was recorded at 404 nm.

149 **Statistical Analysis:** Results are expressed as mean \pm SEM for a series of n experiments.
150 Data were assumed to be normally distributed and statistical analyses were carried out using
151 GraphPad Instat software (GraphPad, San Diego, CA). Paired t-test were used for
152 comparisons of paired treatments between 2 groups, unpaired t-tests for comparisons of
153 unpaired treatments between 2 groups, and one way ANOVA using Tukey multiple
154 comparisons test for treatments of 3 groups or more. p values ≤ 0.05 were considered to be
155 significant.

156

157 **RESULTS**

158 **UDCA inhibits pro-inflammatory cytokine release from colonic epithelial cells:** First, we
159 investigated the effects of UDCA on release of pro-inflammatory cytokines from T₈₄ colonic
160 epithelial cells. For these studies, we used the TLR-3 agonist, poly I:C (25 μ g/ml), as a
161 stimulus and cytokines released into the bathing media were analysed using validated
162 multiplex arrays. We found that Poly I:C induced secretion of TNF- α from T₈₄ cells and that
163 UDCA significantly attenuated this response in a concentration-dependent manner, with a
164 maximal effect occurring at 200 μ M (Figure 1A). UDCA (200 μ M) also attenuated Poly I:C-
165 induced secretion of IL-1 β , and IL-6 (Figures 1B and C). In contrast, UDCA did not alter
166 Poly I:C-stimulated IFN- γ release (Figure 1D), or that of IL-12p70 and GM-CSF (data not
167 shown).

168 **UDCA exerts protective effects in the DSS model of mucosal inflammation:** Next, we
169 went on to examine the effects of UDCA in the DSS mouse model of mucosal inflammation.
170 The DSS model is considered to be a particularly good model for studying mucosal
171 inflammation occurring as a consequence of disrupted epithelial barrier function (31, 48).
172 Inclusion of 2.5% DSS in the drinking water of C57/BL6 mice led to a reduction in body

173 weight and increased DAI over the 5 day experimental period. Both effects were significantly
174 attenuated by daily treatment with UDCA (30 mg/kg) (Figures 2A-B). UDCA at a higher
175 dose of 100 mg/kg (Day 5 DAI = 5.8 ± 0.5) did not confer additional protection when
176 compared to its effects at 30 mg/kg (Day 5 DAI = 6.8 ± 0.9 ; n = 6). Mice treated with DSS
177 also had significantly shorter colons (60.8 ± 2.1 mm) and lack of faecal pellet formation
178 compared to controls (87.2 ± 2.1 mm, n = 6 – 12, $p \leq 0.001$), whereas treatment with UDCA
179 (30 mg/kg) prevented shortening of the colon (69.0 ± 1.5 mm, n = 6 – 12, $p \leq 0.05$) and
180 restored faecal pellet formation (Figures 2 C). Histological studies revealed that UDCA
181 reduced inflammatory cell infiltration and prevented epithelial damage, leading to a reduction
182 in overall inflammation score (Figure 2D-E). As shown in Figure 3, UDCA also tended to
183 reduce levels of TNF- α , IL-1 β , and IL-6, although none of these effects achieved statistical
184 significance. Similar to its effects in T₈₄ cells, UDCA did not attenuate IFN- γ levels and, in
185 fact, tended to enhance DSS-induced release of this cytokine.

186 **6-MUDCA is not protective against DSS-induced colonic inflammation:** In humans,
187 UDCA is known to be metabolised to LCA in the colon and GC-MS analysis of the caecal
188 contents revealed that this is also the case in mice (Figure 4A). Thus, we hypothesised that
189 bacterial metabolism of UDCA likely limits its therapeutic effects. To test this, we employed
190 a 6-methylated derivative of UDCA, 6 α -methyl-UDCA (6-MUDCA), which cannot be
191 metabolised by bacteria to LCA or other metabolites (32). We have previously shown 6-
192 MUDCA not to be metabolised to LCA in mice, but to retain the biological activity of UDCA
193 *in vitro* (16). Here, we confirmed that 6-MUDCA also retains the activity of UDCA in
194 preventing poly I:C-induced TNF- α release from T₈₄ cell monolayers (Figure 4B). 6-
195 MUDCA was also active in HT29C119A cells, reducing Poly I:C (25 μ g/ml)-induced TNF- α
196 release from 378 ± 108 pg/ml in controls to 236 ± 59 pg/ml (n = 3; $p \leq 0.01$), indicating its
197 effects are not cell line-specific. However, despite its capacity to prevent colonic epithelial

198 cytokine secretion *in vitro*, in contrast to UDCA, 6-MUDCA was not protective against DSS-
199 induced mucosal inflammation *in vivo*, as assessed by DAI measurements (Figure 4C).
200 Similarly, 6-MUDCA did not prevent weight loss or colon shortening in response to DSS
201 treatment. Body weight was reduced to $94.8 \pm 0.5\%$ of controls in response to DSS-treatment,
202 compared to $90.0 \pm 2.1\%$ in 6-MUDCA-treated mice, whereas colon length in DSS-treated
203 mice was 59.3 ± 1.5 mm compared to 57.7 ± 1.5 mm in those co-treated with 6-MUDCA.
204 This lack of efficacy of 6-MUDCA was contrary to our original hypothesis, and suggest that
205 bacterial metabolism of UDCA is necessary for it to exert its protective effects *in vivo*.

206 **LCA inhibits pro-inflammatory cytokine release from colonic epithelial cells:** Since
207 metabolism of UDCA appears to be required for it to exert protective actions, we went on to
208 investigate the effects of its major colonic metabolite, LCA, in regulating colonic
209 inflammatory responses. First, we examined LCA effects on cytokine release from colonic
210 epithelial cells *in vitro*. T₈₄ cells were treated with poly I:C, either in the absence or presence
211 of LCA (0.1 – 10 μ M) and TNF- α secretion into the bathing medium was measured.
212 Interestingly, we found that LCA treatment was considerably more effective than UDCA,
213 practically abolishing poly I:C-induced TNF- α release (Figure 5A and c.f. Figure 1A).
214 Furthermore, the effects of LCA were not specific to TLR3 activation by Poly I:C, since the
215 bile acid also inhibited IL-8 cytokine secretion in response to another pro-inflammatory
216 stimulus, TNF- α (Figure 5B). Use of the acid phosphatase activity assay, as a direct index of
217 the number of cells present, revealed only a slight reduction associated with this effect of the
218 bile acid (Figure 5C). To further assess potential LCA toxicity on colonic epithelial cells, we
219 examined its effects on transepithelial resistance (TER), a sensitive index of epithelial
220 monolayer integrity. After 24 hrs treatment, the TER of LCA (10 μ M)-treated T₈₄ cells was
221 $94 \pm 2.6\%$ (n = 5) of that in controls, indicating that, at concentrations which abolish cytokine
222 secretion, LCA does not alter monolayer integrity.

223 **LCA is protective against DSS-induced colonic inflammation and cytokine release:** We
224 next examined the effects of LCA on DSS-induced colonic inflammation *in vivo*. Daily
225 treatment with LCA (30 mg/kg; IP) significantly increased caecal LCA levels from 6.1 ± 0.5
226 to 15.7 ± 3.1 μM in controls and from 2.0 ± 0.3 to 11.5 ± 2.1 μM in DSS-treated mice ($n = 5$,
227 $p \leq 0.05$). We noted that treatment with LCA alone induced a significant loss of body weight
228 by day 5 to 89.3 ± 1.0 % of that before LCA treatment (Figure 6A), consequently causing a
229 slight, non-significant, increase in DAI (Figure 6B). Interestingly, LCA almost completely
230 prevented the onset of inflammation, as measured by DAI, which in DSS-treated animals was
231 11.2 ± 0.9 compared to 5.2 ± 0.6 in LCA-treated mice ($n = 5$, $p \leq 0.001$) (Figure 6B). LCA
232 alone caused a slight shortening of the colon but prevented that caused by DSS treatment and
233 restored the appearance of normal stool pellets (Figures 6C). Furthermore, LCA completely
234 reversed DSS-induced changes in mucosal histology and increases in inflammation score
235 (Figure 6D-E). An analysis of the effects of LCA on levels of proinflammatory cytokines
236 revealed that it was even more effective than UDCA in reducing mucosal levels of TNF- α ,
237 IL-6, and IL-1 β in DSS-treated mice (Figure 7). Interestingly, in contrast to UDCA,
238 administration of LCA also inhibited Poly I:C-induced increases in IFN- γ . Mice treated with
239 LCA actions were not associated with any apparent signs of systemic toxicity, as determined
240 by measurements of serum creatinine and ALT. Serum creatinine levels were 35.7 ± 1.2 , 29.0
241 ± 2.0 and 31.3 ± 1.8 mM/L in control, DSS, and DSS + LCA-treated mice, respectively ($n =$
242 3), while ALT levels were determined to be < 6 U/L in all treatment groups.

243

244

245 DISCUSSION

246 By virtue of its potent anti-inflammatory and cytoprotective properties, UDCA is recognised
247 as a drug with great therapeutic potential (45), and our current studies add to a growing body
248 of evidence that suggest it may also be useful in treatment of intestinal inflammation. Our
249 studies also show that the protective effects of UDCA are likely to be due, at least in part, to
250 inhibition of epithelial cytokine production and point to an important role for bacterial
251 metabolism in determining its efficacy *in vivo*.

252 An early step in intestinal inflammatory responses is the production of cytokines from the
253 epithelium in response to various luminal factors, such as bacteria and their toxins and
254 metabolites. Viruses are also present and their importance in IBD pathogenesis has recently
255 been highlighted (27). Viruses promote cytokine secretion through the release of double-
256 stranded RNA which activates epithelial Toll-like receptors (TLRs), in particular TLR3 (1,
257 11), and here we found that such responses are inhibited by UDCA treatment. These findings
258 are particularly interesting in the context of recently published data, where the effects of the
259 conjugated derivative of UDCA, tauro-UDCA (TUDCA) were investigated in the DSS model
260 (21). Although, significantly higher doses were required, similar to UDCA, TUDCA
261 prevented the development of mucosal inflammation, an effect that was closely associated
262 with inhibition of epithelial apoptosis. Also similar to our own studies, UDCA was found to
263 prevent colonic inflammation in TNBS-treated rats, a model of intestinal inflammation
264 distinct to that used in the current studies (25). Thus, UDCA has the capacity to prevent both
265 the elevated cytokine levels and increased epithelial permeability associated with intestinal
266 inflammation, suggesting it should be of therapeutic benefit in patients with IBD.

267 However, when considering the use of UDCA for treatment of colonic disease, it important to
268 consider the potential impact of the colonic microbiota on its actions. Bile acids entering the

269 colon undergo rapid metabolism by resident bacteria by deconjugation, dehydroxylation and
270 epimerisation and therefore, the fate of UDCA in the colon is determined by the relative
271 expression of bacterial hydrolases, dehydratases, and epimerases (20, 22). How UDCA
272 administration changes the makeup of the colonic bile acid pool is not well-defined but
273 studies in humans show that after UDCA treatment, LCA becomes the most prominent
274 colonic bile acid (44). This is supported by our current studies which showed extensive
275 metabolism of UDCA to LCA in the cecum of normal mice. It was also interesting to note
276 that in DSS-treated mice, despite the fact that it prevented inflammation, levels of UDCA in
277 the colon did not increase appreciably after administration of the bile acid, while those of
278 LCA increased approximately 4-fold. Also notable in these studies was the effect of DSS
279 treatment in reducing cecal levels of UDCA and LCA. These data are in line with a previous
280 study demonstrating fecal LCA levels to be decreased in DSS-treated mice (3), and a more
281 recent study demonstrating that levels of both UDCA and LCA are reduced in this model of
282 colonic inflammation. Furthermore, such changes were found to be associated with
283 significant alterations in the colonic microbiota and were partially restored by UDCA
284 treatment (43). Further studies to more precisely determine how changes in the microbiota
285 and related alterations in the colonic bile acid signature contribute to the onset of
286 inflammation and how UDCA administration influences such processes warrants further
287 investigation.

288 LCA is the most lipophilic of the secondary colonic bile acids and is classically considered
289 to be relatively toxic, particularly in the liver (15). Increased levels of hepatic LCA, which
290 occur in conditions of cholestasis, are thought to contribute to liver damage through induction
291 of apoptotic cell death. Indeed, several studies have demonstrated that supraphysiological
292 levels of LCA, cause oxidative stress, DNA damage and induce apoptosis in both hepatocytes
293 and colonic epithelial cells (4). Thus, since UDCA is normally metabolised to LCA in the

294 colon, we hypothesised that this may be a factor that limits its therapeutic actions. To test this
295 hypothesis we used 6-MUDCA, a non-metabolizable derivative of UDCA, which we have
296 previously shown to not be metabolised to LCA either in mouse colon or by the human fecal
297 microbiota (16, 32). To our surprise we found that, even though, similar to UDCA, it inhibits
298 epithelial cytokine production *in vitro*, 6-MUDCA did not confer protection in the DSS
299 model. These findings were contrary to our hypothesis and suggest that, rather than limiting
300 its therapeutic actions, bacterial metabolism of UDCA is actually required for it to fully exert
301 its protective effects.

302 While most previous studies have focussed on the cytotoxic actions of LCA at high
303 concentrations, few have investigated whether it might also have more physiological roles to
304 play. Interestingly, one recent study showed that administration of LCA to mice by enema
305 can prevent colonic epithelial apoptosis, and therefore presumably promote barrier function
306 (18). In the current studies, we found that even at concentrations as low as 10 μ M, which
307 approximates its normal physiological range in the colon (13), LCA was even more effective
308 than UDCA in preventing TNF- α release from colonic epithelial cells *in vitro*. Even more
309 remarkably, we found that when administered to mice, LCA was also more effective than
310 UDCA in preventing DSS-induced inflammation. Further analysis showed that cytokine
311 release from mucosal tissues was practically abolished in LCA-treated mice, compared to the
312 partial inhibition observed with UDCA treatment. Notably, while UDCA tended to increase
313 mucosal levels of IFN- γ in DSS-treated mice, LCA inhibited accumulation of this cytokine.
314 While we were concerned that the effects of LCA might be due to toxicity, this does not
315 appear to be the case, as indicated by a lack of effect of the bile acid on TER across epithelial
316 monolayers and only a modest effect on cell number at concentrations that abolish cytokine
317 release. Furthermore, no overt toxicity was apparent in histological sections of colonic tissue
318 from LCA-treated mice, nor were serum levels of creatinine or ALT altered by the bile acid.

319 However, it was notable that LCA treatment significantly reduced body weight over the
320 course of the experiment. Given the lack of apparent local or systemic toxicity, we speculate
321 that this could either be due to reduced food intake in the LCA-treated mice, or alternatively,
322 might reflect effects of the bile acid on energy expenditure and fat metabolism. This latter
323 hypothesis seems is possible since previous studies have shown that bile acids prevent weight
324 gain in mice on a high fat diet (46), and that this effect is mimicked by the TGR5-selective
325 agonist, INT-777 (19, 41). TGR5 is now accepted to play an important role in regulating
326 metabolism (23), suggesting that LCA, as a natural agonist of the receptor, could be an
327 endogenous regulator of metabolism, energy expenditure and body weight. Separating such
328 dual actions on metabolism and inflammation is an important issue to consider when
329 developing bile acids, or synthetic agonists, as therapeutics for IBD. However, it is notable
330 that studies by Harach and co-workers indicate that agonists of TGR5 influence metabolism
331 only when they are present in the systemic circulation, suggesting that colonic or rectal
332 delivery of such drugs may be the optimal approach for their use in treating colitis, while
333 minimising effects on weight (14).

334 Although UDCA shows excellent potential for therapeutic development in treating intestinal
335 inflammation, there is still much work to be done to elucidate mechanisms underlying its
336 effects. While our current studies suggest that its metabolism to LCA may be important, it is
337 also possible that other metabolites may be involved. For example, 7-keto-LCA, formed by
338 the action of 7 β -hydroxysteroid dehydrogenase, is the major metabolic intermediate of
339 UDCA and LCA and its actions on colonic epithelial physiology are not yet known.
340 Similarly, how sulfation of UDCA and LCA alter their physiological/pathophysiological
341 actions remains to be determined. It is also important to develop our understanding of the role
342 of the microbiota in modulating bile acid actions on colonic epithelial barrier function. This is
343 particularly important in the setting of inflammation, where the microbiome is known to be

344 significantly altered (8). Such alterations would undoubtedly influence metabolism of UDCA,
345 the generation of its metabolites, and consequently, its therapeutic actions. Finally, the
346 molecular pathways underlying the anti-inflammatory effects of UDCA and its metabolites
347 and their differential effects on epithelial cytokine secretion remain to be fully elucidated. In
348 this regard, several bile acid receptors are expressed in the colonic epithelium, including
349 TGR5 and the nuclear receptors, farnesoid x receptor, pregnane x receptor, and vitamin D
350 receptor, each of which has been shown to protect against colonic inflammation in animal
351 models (12, 29, 35, 40). Although structurally similar, UDCA and LCA have very different
352 actions at these receptors, likely underlying different responses to the bile acids. Future work
353 should aim to elucidate how expression of these receptors is altered in conditions of colonic
354 inflammation and how this impacts the effects of UDCA and its metabolites on epithelial
355 function.

356 In conclusion, our studies support the hypothesis that UDCA may be useful as a new therapy
357 for alleviating or preventing chronic intestinal inflammation but that bacterial metabolism of
358 the bile acid is necessary for its full therapeutic benefit to be apparent. We also demonstrate a
359 new anti-inflammatory role for the primary UDCA metabolite, LCA, in the colon, which
360 suggests it may be an important mediator of UDCA effects. Further studies are necessary to
361 more completely understand how the colonic microbiome and bile acids interact in order to
362 regulate epithelial barrier function in health and disease.

363

364 **FIGURE LEGENDS**

365 **Figure 1. UDCA attenuates proinflammatory cytokine release from colonic epithelial**
366 **cells.** T₈₄ cells grown on 96-well plates were serum-starved for 1hr prior to stimulation with
367 poly I:C (25 µg/ml) in the presence or absence of UDCA. After 24 hrs, supernatants were
368 collected and analysed for **A)** TNF-α (n = 6), **B)** IL-6, **C)** IL-1β, and **D)** IFN-γ (n = 4). *p <
369 0.05, **p < 0.01, ***p < 0.001 compared to control cells; #p < 0.05, ##p < 0.01 compared to
370 cells treated with poly I:C alone.

371 **Figure 2. UDCA exerts protective effects in the DSS model of mucosal inflammation.**
372 Starting 24 hrs prior to administration of DSS (2.5% in the drinking water), and daily
373 thereafter, separate groups of male C57BL6 mice received either endotoxin-free PBS or Na⁺-
374 UDCA (30 mg/kg or 100 mg/kg, dissolved in PBS) by IP injection. **A)** Disease activity index
375 (DAI) and **B)** body weight were assessed daily to monitor disease progression (n = 6 - 12
376 throughout). **C)** Mice were sacrificed on day 6 and their colons were removed and measured.
377 **D)** Sections of colon from control, DSS-treated, UDCA-treated, and DSS+UDCA-treated
378 C57BL6 mice were taken and processed for H&E staining. Sections were visualised by light
379 microscopy under 10x magnification. **E)** Inflammation score was assessed as described in
380 Materials and Methods. *** p < 0.001 compared to controls (no DSS treatment); # p < 0.05,
381 ## p < 0.01, ### p < 0.001 compared to DSS-treated mice.

382 **Figure 3. UDCA modulates expression of pro-inflammatory cytokines in the DSS model**
383 **of mucosal inflammation.** Sections of colon from control, DSS-treated, UDCA-treated, and
384 DSS+UDCA-treated C57BL6 mice were homogenised in lysis buffer and were analysed by
385 MSD assay for **A)** TNF-α, **B)** IL-6, **C)** IL-1β, and **D)** IFN-γ. n = 6 – 12; **p < 0.01, ***p <
386 0.001 compared to controls (no DSS treatment). n.s. = not significant.

387 **Figure 4. A metabolically stable analogue of UDCA, 6-MUDCA, is not protective**
388 **against DSS-induced colonic inflammation:** **A)** Caecal contents were collected from treated
389 and control mice and bile acid levels were measured by HPLC-ES-MS/MS. **B)** T₈₄ cells were
390 stimulated with poly I:C (25 µg/ml) in the presence or absence of UDCA or 6-methyl-UDCA
391 (200 µM; bilateral). After 24 hrs, supernatants were collected and analysed for TNF-α. Data
392 are expressed as fold change with respect to cells treated with poly I:C alone (n = 5; ***p <
393 0.001). **C)** Starting 24hrs prior to administration of DSS (2.5% in the drinking water), and
394 daily thereafter, separate groups of male C57BL6 mice received either endotoxin-free PBS or
395 Na⁺-6-MUDCA (30 mg/kg) by IP injection. DAI was assessed daily to monitor disease
396 progression. (n = 3 - 9).

397 **Figure 5. LCA exerts anti-inflammatory effects *in vitro*.** **A)** T₈₄ cells were stimulated with
398 poly I:C (25 µg/ml) in the presence or absence of LCA (1 nM - 10 µM). After 24 hrs,
399 supernatants were collected and analysed for TNF-α. Data are expressed as fold change with
400 respect to cells treated with poly I:C alone (n = 7; ***p < 0.001). **B)** T₈₄ cells were treated
401 with TNF-α (10 ng/ml) and LCA (10 µM) alone or in combination. After 24 hrs apical media
402 were collected and analysed for IL-8 levels by ELISA. Data are expressed as fold change
403 with respect to cells treated with TNF-α alone (n = 4; ***p < 0.001). **C)** T₈₄ cells grown on
404 96 well plates were serum starved for 1 hr prior to treatment with LCA (1 nM to 1 mM) for
405 24 hrs (n = 4), after which acid phosphatase activity was measured (**p < 0.01, ***p < 0.001
406 compared to untreated cells).

407 **Figure 6. LCA exerts protective effects in the DSS model of mucosal inflammation.**
408 Starting 24 hrs prior to administration of DSS in the drinking water, and daily thereafter,
409 separate groups of male C57BL6 mice received either endotoxin-free PBS or Na⁺-LCA (30
410 mg/kg) by IP injection. **A)** Body weight and **B)** disease activity index (DAI) were assessed

411 daily to monitor disease progression (n = 5). **C)** Mice were sacrificed on day 6 and their
412 colons were removed and measured (image is representative of n = 5). **D)** Sections of colon
413 from control, DSS-treated, LCA (30 mg/kg)-treated, and DSS+LCA-treated C57BL6 mice
414 were taken and processed for H&E staining. Sections were visualised by light microscopy
415 under 10x magnification. **E)** Inflammation score was assessed as described in Materials and
416 Methods (n = 3 – 5). *p < 0.05, **p < 0.01, ***p < 0.001 compared to controls (no DSS
417 treatment); #p < 0.05, ##p < 0.01, compared to DSS-treated mice

418 **Figure 7. LCA modulates the expression of pro-inflammatory cytokines in murine**
419 **colon.** Sections of colon from control, DSS-treated, LCA (30 mg/kg)-treated, and DSS+LCA-
420 treated C57BL6 mice were homogenised in lysis buffer and were analysed for **A)** TNF- α , **B)**
421 IL-6, **C)** IL-1 β , and **D)** IFN- γ . n = 3 – 10; **p < 0.01, ***p < 0.001 compared to controls (no
422 DSS treatment).

423

424

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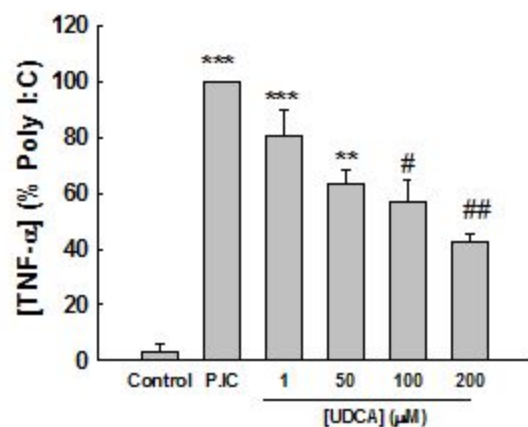
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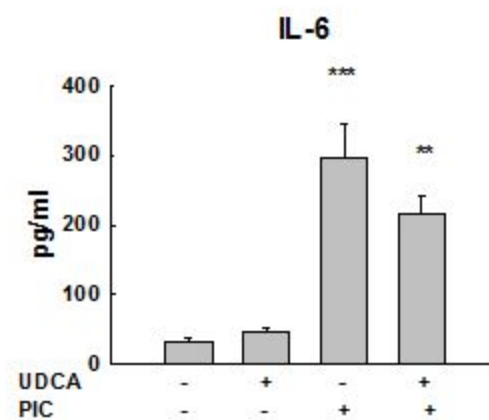
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Figure 1

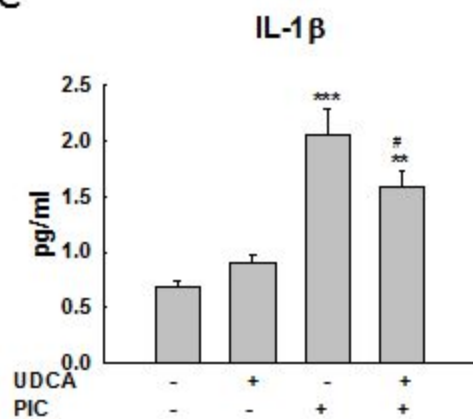
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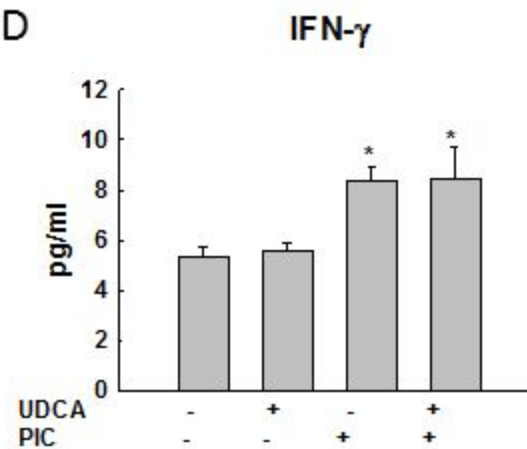


Figure 2

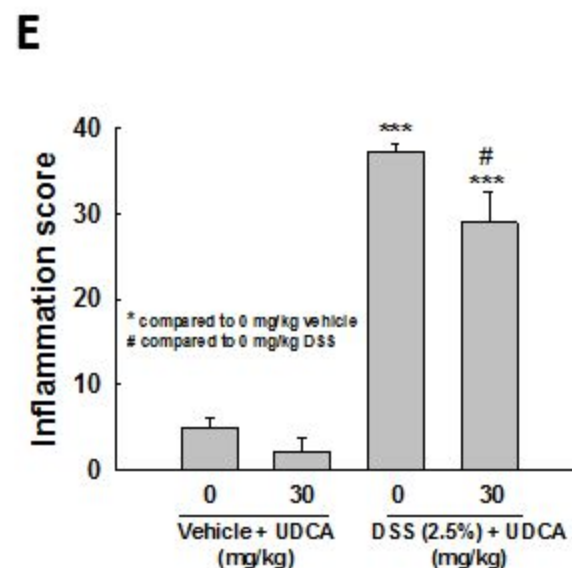
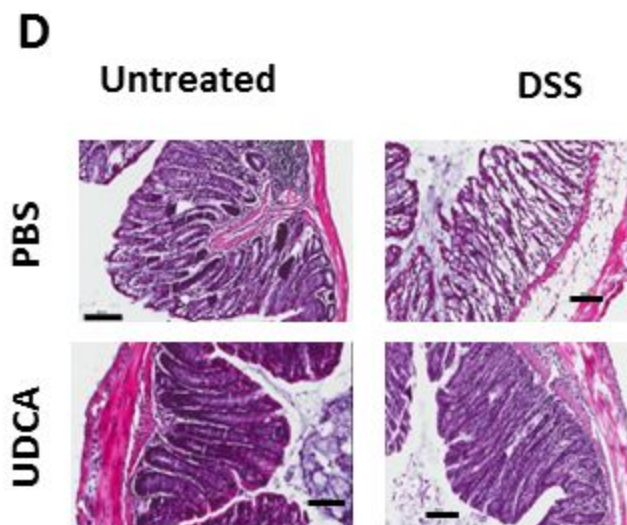
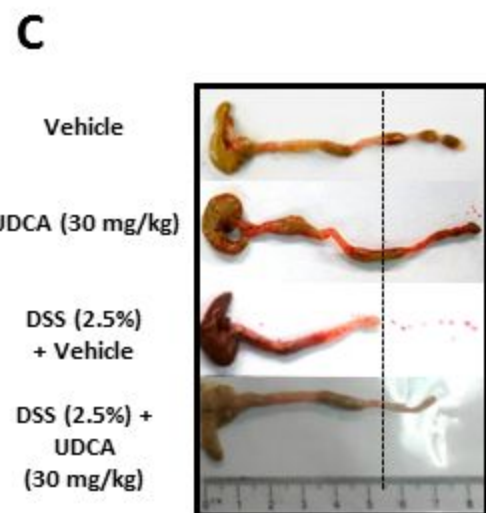
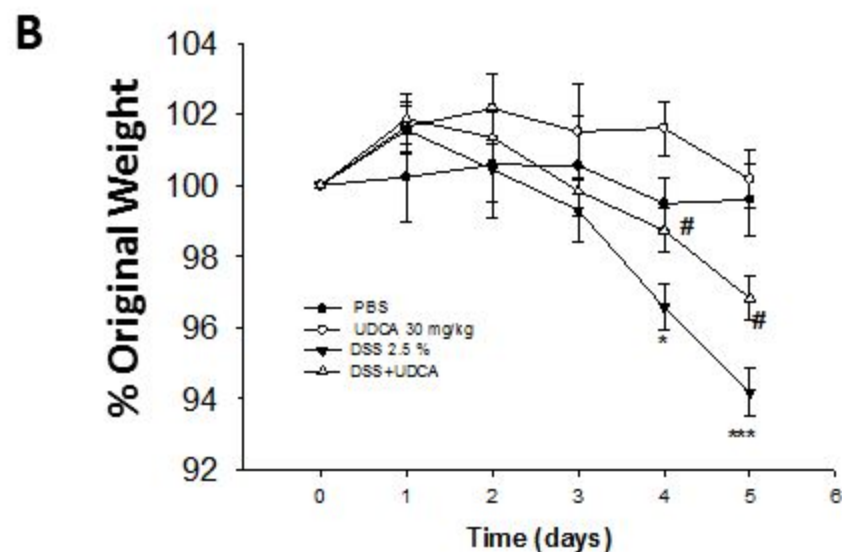
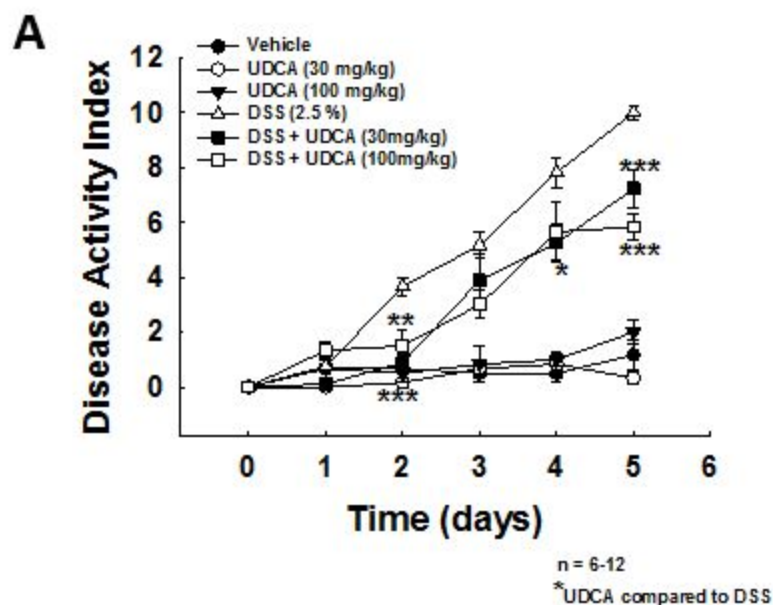
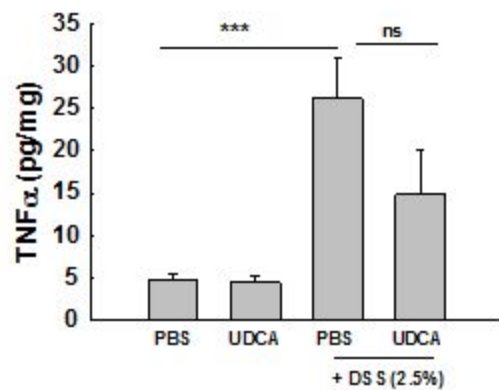
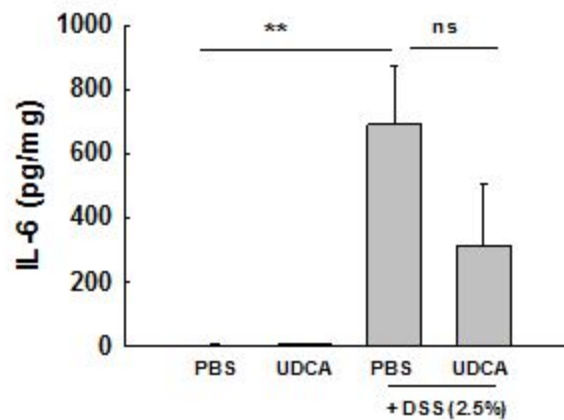


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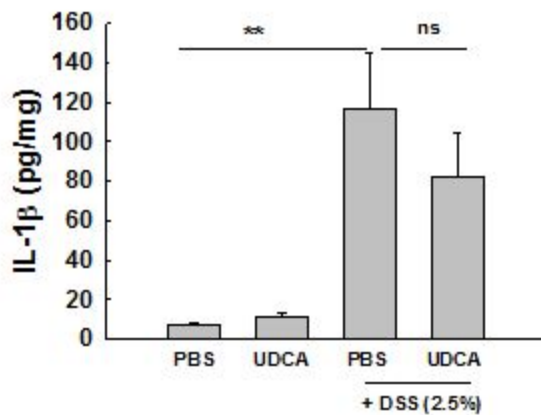
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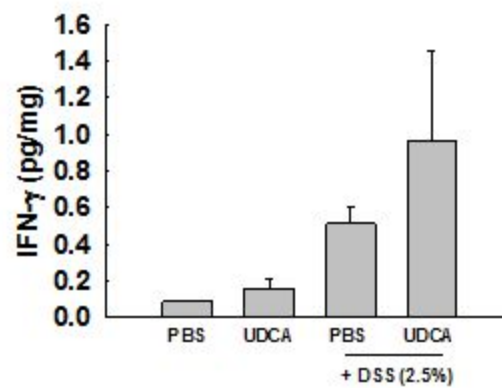
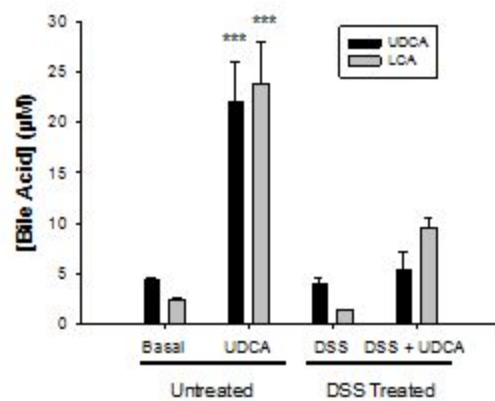
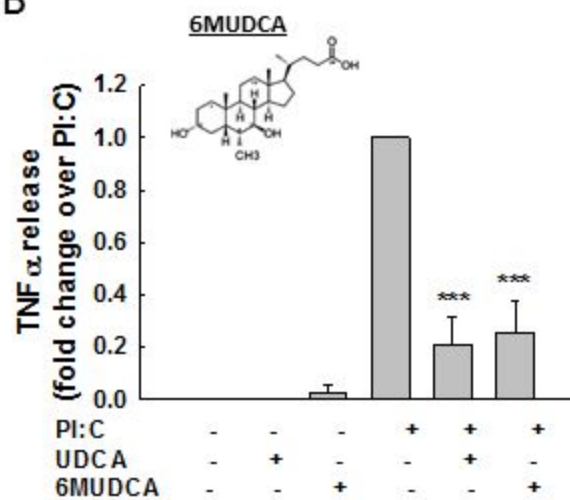


Figure 4

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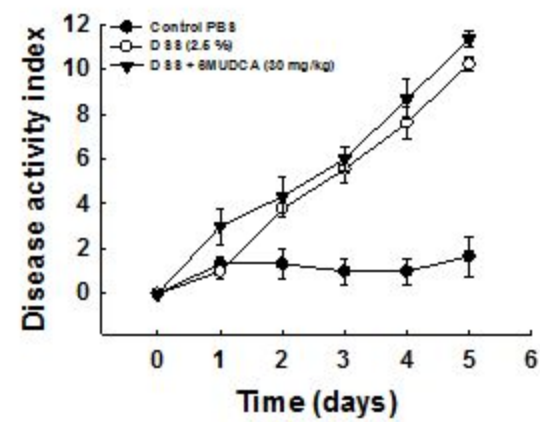
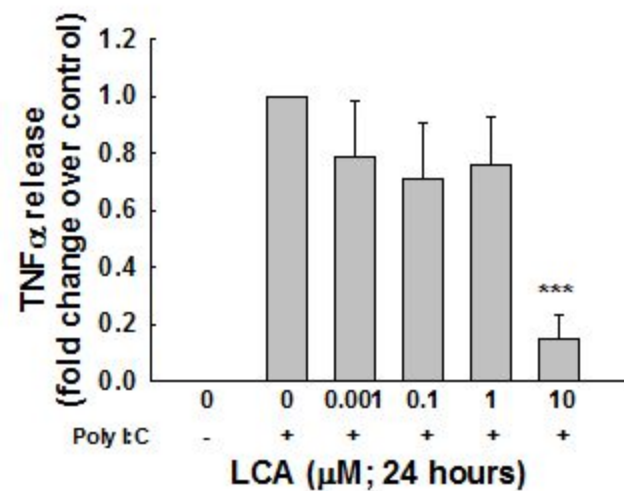
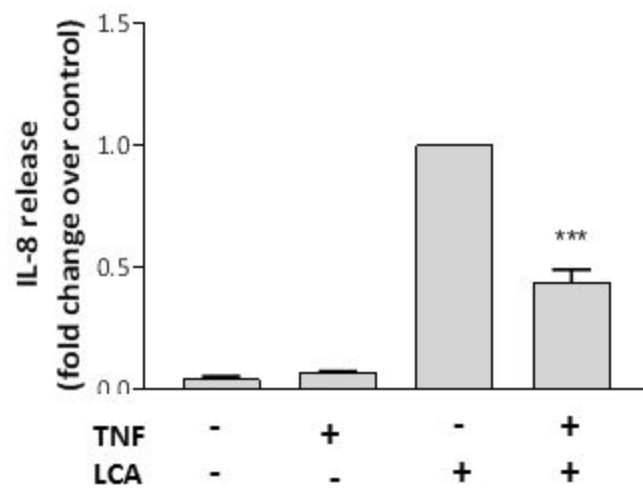


Figure 5

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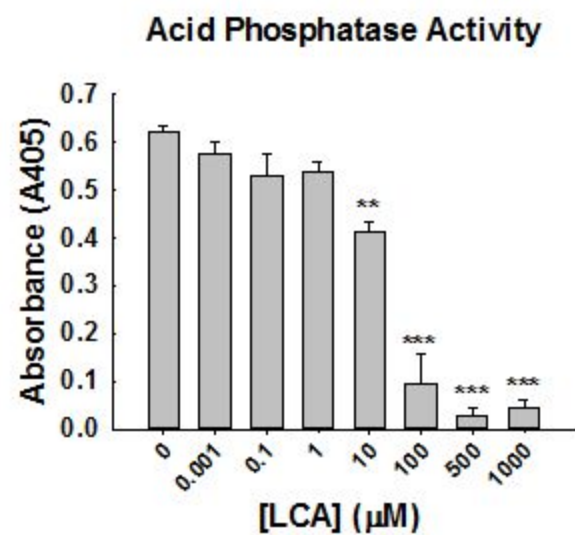
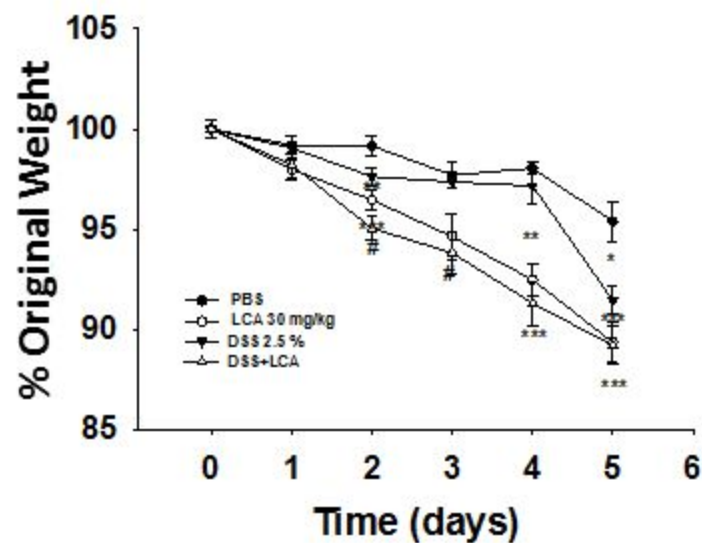
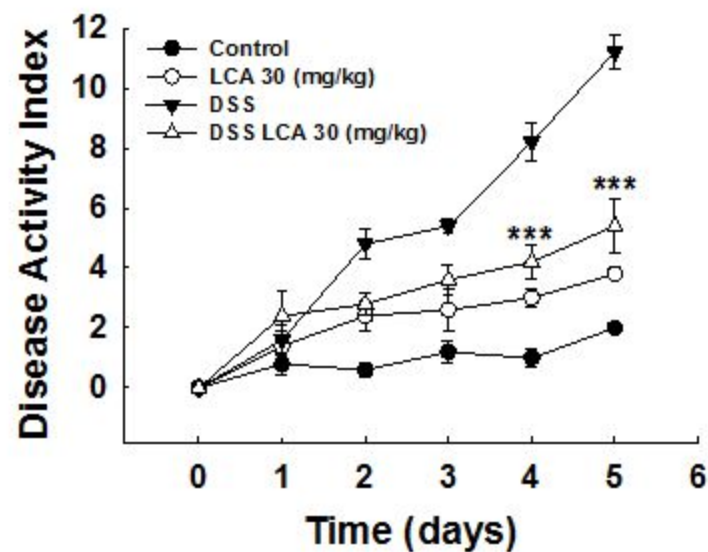


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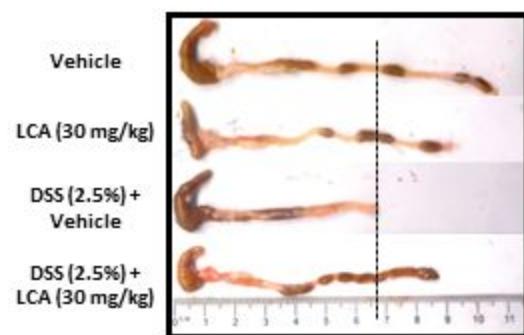
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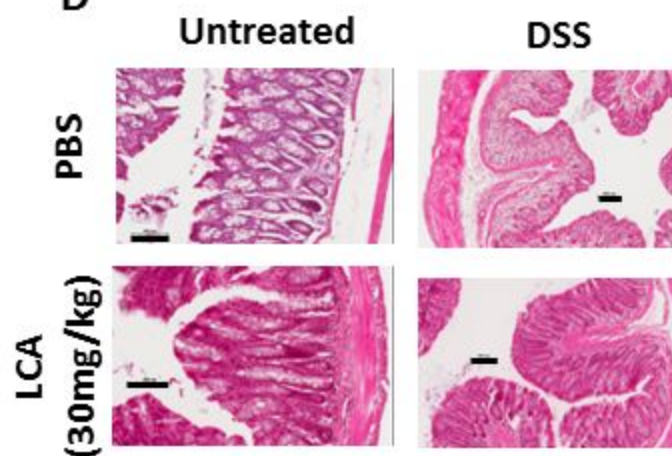
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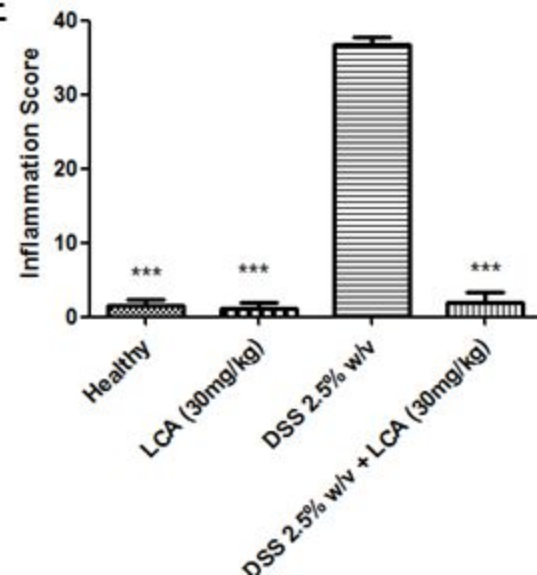


Figure 7

