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

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Running Title: UDCA and LCA prevent colonic inflammation

ABSTRACT

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

NEW AND NOTEWORTHY

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

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS:

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

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

Cytokine Measurements: T_{84} or HT29Cl19A cells were cultured on 96-well plates until they reached approximately 80% confluence. Cells were serum-starved for 1hr prior to stimulation with polyinosinic:polycytidylic acid (poly I:C) (25 μg/ml) or TNF-α (10 ng/ml) in the presence or absence of UDCA or LCA (24 hr, 37°C). Mouse colons were homogenised in liquid N_2 on dry ice, re-suspended by vortexing in lysis buffer (1% Nonidet P-40, 150 m NaCl, 50 mmol/L Tris Base, 1 x Complete mini EDTA free protease inhibitor tablet, 0.1 mg/1mL PMSF, 1 mmol/L Na_3VO_4) in a m/v ratio of 1:5, lysed (45 minutes on ice), sonicated (3 x 10s pulses), centrifuged (15,294 x g, 20 mins, 4°C) and supernatants were retained for analysis. For measurements of TNF-α, IL-1β, IL-6, IFN-γ, IL-12p70, and GM-CSF cell culture supernatants or colonic lysates were then added to a pre-coated V-Plex Multi-array and Multi-sport Human Cytokine Assay plates (Catalogue #: K15007B-1) and assayed as per the manufacturer's protocol (Meso Scale Diagnostics; Rockville, MD). Measurements of IL-8 release from T_{84} cells were carried out by ELISA (Beckton Dickinson, San Diego, CA).

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

Acid Phosphatase Assay: T_{84} cells grown to confluency on 96-well plates were serum-starved for 1hr prior to treatment with LCA. Cells were then washed in warm PBS, incubated in sodium acetate buffer (0.1M $C_2H_3NaO_2$, pH 5.5, 0.1% Triton x-100) protected from light at 37°C for 30 mins, following which absorbance was recorded at 404 nm.

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

RESULTS

UDCA inhibits pro-inflammatory cytokine release from colonic epithelial cells: First, we investigated the effects of UDCA on release of pro-inflammatory cytokines from T_{84} colonic epithelial cells. For these studies, we used the TLR-3 agonist, poly I:C (25 μg/ml), as a stimulus and cytokines released into the bathing media were analysed using validated multiplex arrays. We found that Poly I:C induced secretion of TNF-α from T_{84} cells and that UDCA significantly attenuated this response in a concentration-dependent manner, with a maximal effect occurring at 200 μM (Figure 1A). UDCA (200 μM) also attenuated Poly I:C-induced secretion of IL-1β, and IL-6 (Figures 1B and C). In contrast, UDCA did not alter Poly I:C-stimulated IFN-γ release (Figure 1D), or that of IL-12p70 and GM-CSF (data not shown).

UDCA exerts protective effects in the DSS model of mucosal inflammation: Next, we went on to examine the effects of UDCA in the DSS mouse model of mucosal inflammation. The DSS model is considered to be a particularly good model for studying mucosal inflammation occurring as a consequence of disrupted epithelial barrier function (31, 48). Inclusion of 2.5% DSS in the drinking water of C57/BL6 mice led to a reduction in body weight and increased DAI over the 5 day experimental period. Both effects were significantly attenuated by daily treatment with UDCA (30 mg/kg) (Figures 2A-B). UDCA at a higher dose of 100 mg/kg (Day 5 DAI = 5.8 ± 0.5) did not confer additional protection when compared to its effects at 30 mg/kg (Day 5 DAI = 6.8 ± 0.9; n = 6). Mice treated with DSS also had significantly shorter colons (60.8 ± 2.1 mm) and lack of faecal pellet formation compared to controls (87.2 ± 2.1 mm, n = 6 – 12, $p \leq 0.001$), whereas treatment with UDCA (30 mg/kg) prevented shortening of the colon (69.0 ± 1.5 mm, n = 6 – 12, $p \leq 0.05$) and restored faecal pellet formation (Figures 2 C). Histological studies revealed that UDCA reduced inflammatory cell infiltration and prevented epithelial damage, leading to a reduction in overall inflammation score (Figure 2D-E). As shown in Figure 3, UDCA also tended to reduce levels of TNF-α, IL-1β, and IL-6, although none of these effects achieved statistical significance. Similar to its effects in T_{84} cells, UDCA did not attenuate IFN-γ levels and, in fact, tended to enhance DSS-induced release of this cytokine.

6-MUDCA is not protective against DSS-induced colonic inflammation: In humans, UDCA is known to be metabolised to LCA in the colon and GC-MS analysis of the caecal contents revealed that this is also the case in mice (Figure 4A). Thus, we hypothesised that bacterial metabolism of UDCA likely limits its therapeutic effects. To test this, we employed a 6-methylated derivative of UDCA, 6α-methyl-UDCA (6-MUDCA), which cannot be metabolised by bacteria to LCA or other metabolites (32). We have previously shown 6-MUDCA not to be metabolised to LCA in mice, but to retain the biological activity of UDCA *in vitro* (16). Here, we confirmed that 6-MUDCA also retains the activity of UDCA in preventing poly I:C-induced TNF-α release from T_{84} cell monolayers (Figure 4B). 6-MUDCA was also active in HT29Cl19A cells, reducing Poly I:C (25 µg/ml)-induced TNF-α release from 378 ± 108 pg/ml in controls to 236 ± 59 pg/ml (n = 3; $p \leq 0.01$), indicating its effects are not cell line-specific. However, despite its capacity to prevent colonic epithelial cytokine secretion in vitro, in contrast to UDCA, 6-MUDCA was not protective against DSS-induced mucosal inflammation in vivo, as assessed by DAI measurements (Figure 4C). Similarly, 6-MUDCA did not prevent weight loss or colon shortening in response to DSS treatment. Body weight was reduced to 94.8 ± 0.5% of controls in response to DSS-treatment, compared to 90.0 ± 2.1% in 6-MUDCA-treated mice, whereas colon length in DSS-treated mice was 59.3 ± 1.5 mm compared to 57.7 ± 1.5 mm in those co-treated with 6-MUDCA. This lack of efficacy of 6-MUDCA was contrary to our original hypothesis, and suggest that bacterial metabolism of UDCA is necessary for it to exert its protective effects *in vivo.*

LCA inhibits pro-inflammatory cytokine release from colonic epithelial cells: Since metabolism of UDCA appears to be required for it to exert protective actions, we went on to investigate the effects of its major colonic metabolite, LCA, in regulating colonic inflammatory responses. First, we examined LCA effects on cytokine release from colonic epithelial cells *in vitro*. T_{84} cells were treated with poly I:C, either in the absence or presence of LCA (0.1 – 10 μM) and TNF-α secretion into the bathing medium was measured. Interestingly, we found that LCA treatment was considerably more effective than UDCA, practically abolishing poly I:C-induced TNF-α release (Figure 5A and c.f. Figure 1A). Furthermore, the effects of LCA were not specific to TLR3 activation by Poly I:C, since the bile acid also inhibited IL-8 cytokine secretion in response to another pro-inflammatory stimulus, TNF-α (Figure 5B). Use of the acid phosphatase activity assay, as a direct index of the number of cells present, revealed only a slight reduction associated with this effect of the bile acid (Figure 5C). To further assess potential LCA toxicity on colonic epithelial cells, we examined its effects on transepithelial resistance (TER), a sensitive index of epithelial monolayer integrity. After 24 hrs treatment, the TER of LCA (10 μM)-treated T_{84} cells was 94 ± 2.6% (n = 5) of that in controls, indicating that, at concentrations which abolish cytokine secretion, LCA does not alter monolayer integrity.

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

DISCUSSION

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

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

However, when considering the use of UDCA for treatment of colonic disease, it important to consider the potential impact of the colonic microbiota on its actions. Bile acids entering the colon undergo rapid metabolism by resident bacteria by deconjugation, dehydroxylation and epimerisation and therefore, the fate of UDCA in the colon is determined by the relative expression of bacterial hydrolases, dehydratases, and epimerases (20, 22). How UDCA administration changes the makeup of the colonic bile acid pool is not well-defined but studies in humans show that after UDCA treatment, LCA becomes the most prominent colonic bile acid (44). This is supported by our current studies which showed extensive metabolism of UDCA to LCA in the cecum of normal mice. It was also interesting to note that in DSS-treated mice, despite the fact that it prevented inflammation, levels of UDCA in the colon did not increase appreciably after administration of the bile acid, while those of LCA increased approximately 4-fold. Also notable in these studies was the effect of DSS treatment in reducing cecal levels of UDCA and LCA. These data are in line with a previous study demonstrating fecal LCA levels to be decreased in DSS-treated mice (3), and a more recent study demonstrating that levels of both UDCA and LCA are reduced in this model of colonic inflammation. Furthermore, such changes were found to be associated with significant alterations in the colonic microbiota and were partially restored by UDCA treatment (43). Further studies to more precisely determine how changes in the microbiota and related alterations in the colonic bile acid signature contribute to the onset of inflammation and how UDCA administration influences such processes warrants further investigation.

LCA is the most lipophillic of the secondary colonic bile acids and is classically considered to be relatively toxic, particularly in the liver (15). Increased levels of hepatic LCA, which occur in conditions of cholestasis, are thought to contribute to liver damage though induction of apoptotic cell death. Indeed, several studies have demonstrated that supraphysiological levels of LCA, cause oxidative stress, DNA damage and induce apoptosis in both hepatocytes and colonic epithelial cells (4). Thus, since UDCA is normally metabolised to LCA in the colon, we hypothesised that this may be a factor that limits its therapeutic actions. To test this hypothesis we used 6-MUDCA, a non-metabolizable derivative of UDCA, which we have previously shown to not be metabolised to LCA either in mouse colon or by the human fecal microbiota (16, 32). To our surprise we found that, even though, similar to UDCA, it inhibits epithelial cytokine production *in vitro*, 6-MUDCA did not confer protection in the DSS model. These findings were contrary to our hypothesis and suggest that, rather than limiting its therapeutic actions, bacterial metabolism of UDCA is actually required for it to fully exert its protective effects.

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

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

Although UDCA shows excellent potential for therapeutic development in treating intestinal inflammation, there is still much work to be done to elucidate mechanisms underlying its effects. While our current studies suggest that its metabolism to LCA may be important, it is also possible that other metabolites may be involved. For example, 7-keto-LCA, formed by the action of 7β-hydroxysteroid dehydrogenase, is the major metabolic intermediate of UDCA and LCA and its actions on colonic epithelial physiology are not yet known. Similarly, how sulfation of UDCA and LCA alter their physiological/pathophysiological actions remains to be determined. It is also important to develop our understanding of the role of the microbiota in modulating bile acid actions on colonic epithelial barrier function. This is particularly important in the setting of inflammation, where the microbiome is known to be significantly altered (8). Such alterations would undoubtedly influence metabolism of UDCA, the generation of its metabolites, and consequently, its therapeutic actions. Finally, the molecular pathways underlying the anti-inflammatory effects of UDCA and its metabolites and their differential effects on epithelial cytokine secretion remain to be fully elucidated. In this regard, several bile acid receptors are expressed in the colonic epithelium, including TGR5 and the nuclear receptors, farnesoid x receptor, pregnane x receptor, and vitamin D receptor, each of which has been shown to protect against colonic inflammation in animal models (12, 29, 35, 40). Although structurally similar, UDCA and LCA have very different actions at these receptors, likely underlying different responses to the bile acids. Future work should aim to elucidate how expression of these receptors is altered in conditions of colonic inflammation and how this impacts the effects of UDCA and its metabolites on epithelial function.

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

FIGURE LEGENDS

Figure 1. UDCA attenuates proinflammatory cytokine release from colonic epithelial cells. T_{84} cells grown on 96-well plates were serum-starved for 1hr prior to stimulation with poly I:C (25 μg/ml) in the presence or absence of UDCA. After 24 hrs, supernatants were collected and analysed for **A)** TNF-α (n = 6), **B)** IL-6, **C)** IL-1β, and **D)** IFN-γ (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control cells; $^{\#}p < 005$, $^{\#\#}p < 0.01$ compared to cells treated with poly I:C alone.

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

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

Figure 4. A metabolically stable analogue of UDCA, 6-MUDCA, is not protective against DSS-induced colonic inflammation*:* A) Caecal contents were collected from treated and control mice and bile acid levels were measured by HPLC-ES-MS/MS. **B)** T_{84} cells were stimulated with poly I:C (25 μg/ml) in the presence or absence of UDCA or 6-methyl-UDCA (200 μM; bilateral). After 24 hrs, supernatants were collected and analysed for TNF-α. Data are expressed as fold change with respect to cells treated with poly I:C alone (n = 5; ***p < 0.001). **C)** Starting 24hrs prior to administration of DSS (2.5% in the drinking water), and daily thereafter, separate groups of male C57BL6 mice received either endotoxin-free PBS or Na^+-6-MUDCA (30 mg/kg) by IP injection. DAI was assessed daily to monitor disease progression. (n = 3 - 9).

Figure 5. LCA exerts anti-inflammatory effects *in vitro*. A) T_{84} cells were stimulated with poly I:C (25 μg/ml) in the presence or absence of LCA (1 nM - 10 μM). After 24 hrs, supernatants were collected and analysed for TNF-α. Data are expressed as fold change with respect to cells treated with poly I:C alone (n = 7; ***p < 0.001). **B)** T_{84} cells were treated with TNF-α (10 ng/ml) and LCA (10 μM) alone or in combination. After 24 hrs apical media were collected and analysed for IL-8 levels bu ELISA. Data are expressed as fold change with respect to cells treated with TNF-α alone (n = 4; ***p < 0.001). **C)** T_{84} cells grown on 96 well plates were serum starved for 1 hr prior to treatment with LCA (1 nM to 1 mM) for 24 hrs (n = 4), after which acid phosphatase activity was measured (**p < 0.01, ***p < 0.001 compared to untreated cells).

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

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

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

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Figure 1

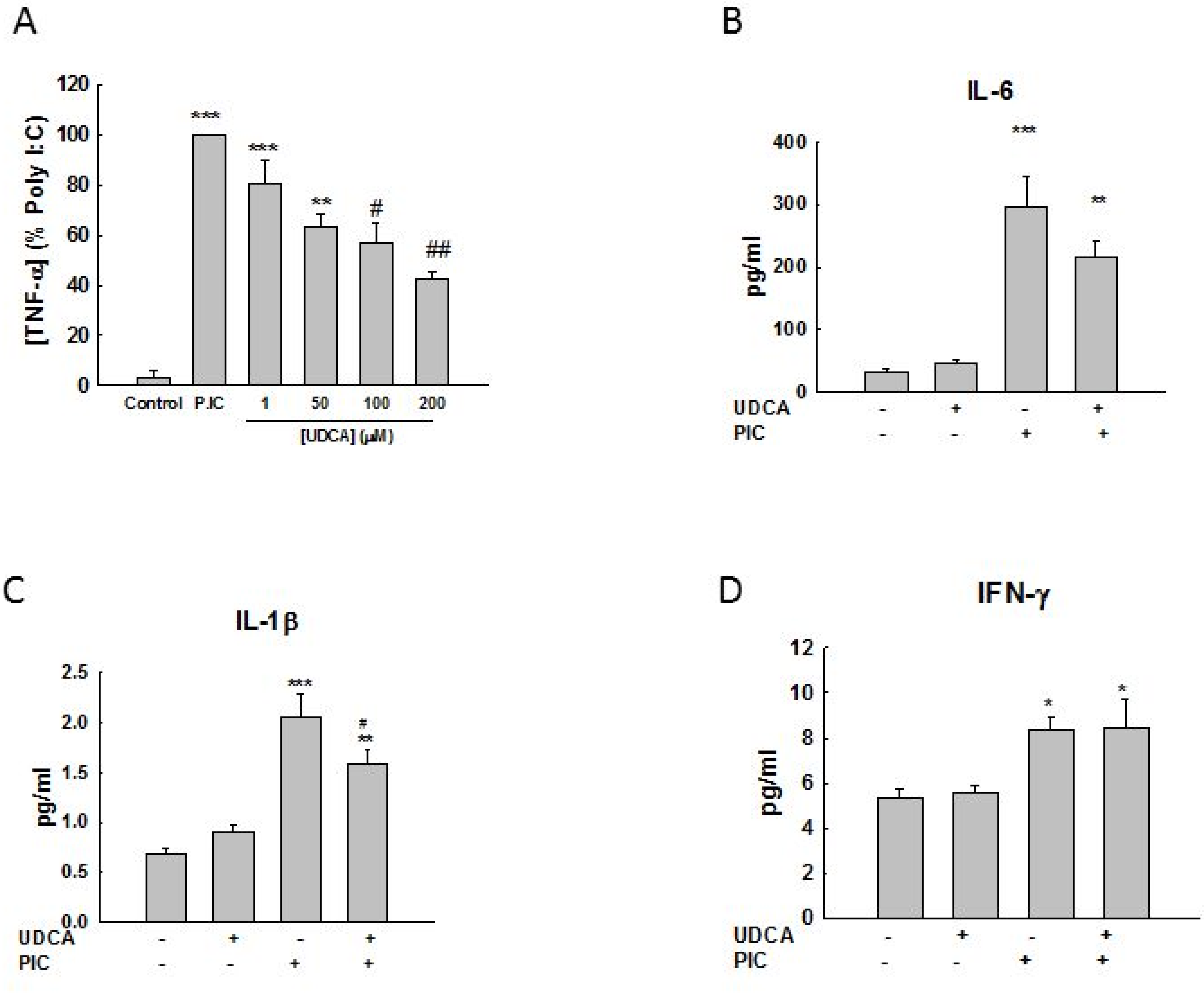

A
120
100
80
60
40
20
0
[TNF-α] (% Poly I:C)
***
***
**
#
##
Control
P.IC
1
50
100
200
[UDCA] (μM)
B
IL-6
400
300
200
100
0
pg/ml
***
**
UDCA - + - +
PIC - - + +
C
IL-1β
2.5
2.0
1.5
1.0
0.5
0.0
pg/ml
***
#
**
UDCA - + - +
PIC - - + +
D
IFN-γ
12
10
8
6
4
2
0
pg/ml
*
*
UDCA - + - +
PIC - - + +

Figure 2

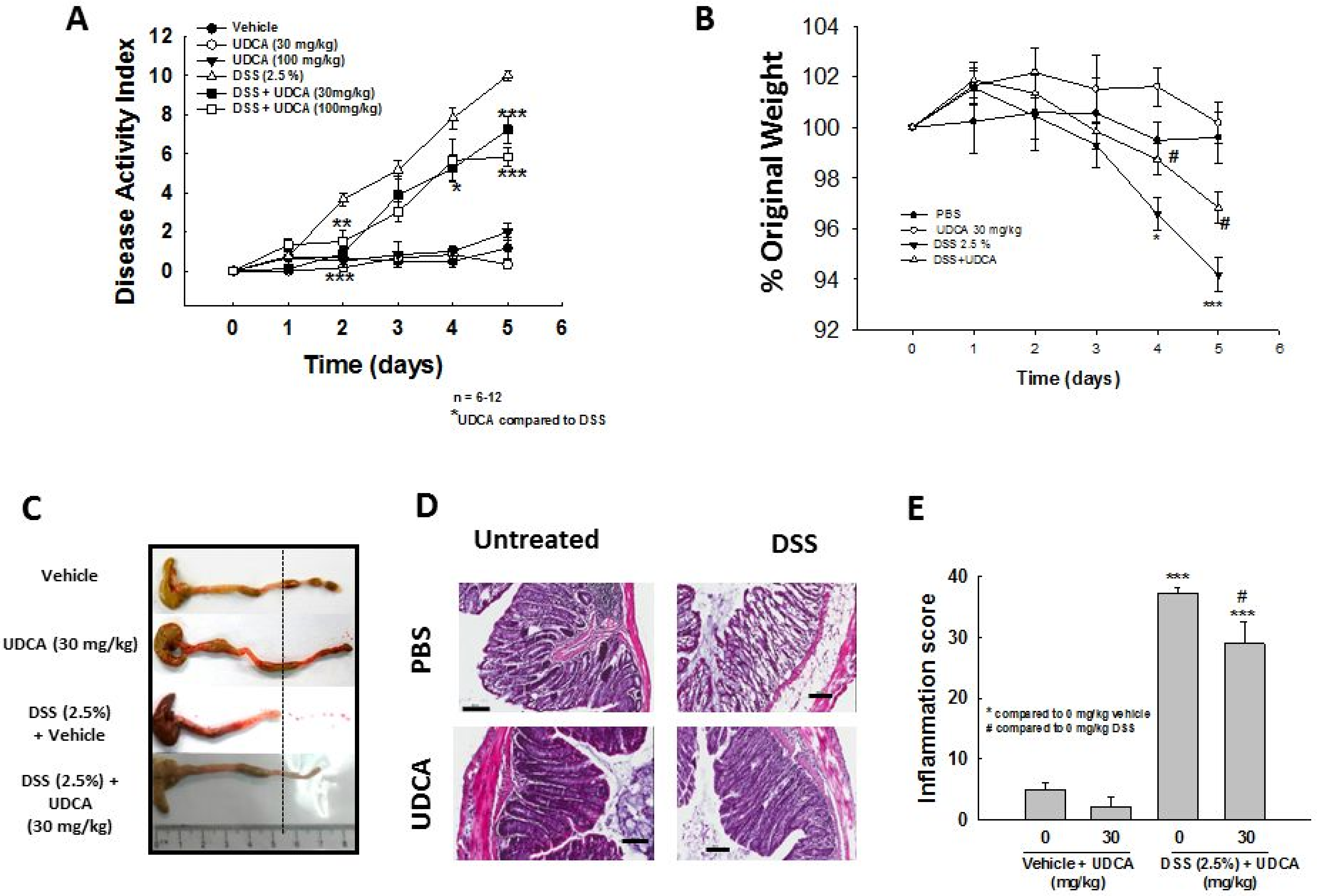

A
Disease Activity Index
12
10
8
6
4
2
0
Vehicle
UDCA (30 mg/kg)
UDCA (100 mg/kg)
DSS (2.5 %)
DSS + UDCA (30mg/kg)
DSS + UDCA (100mg/kg)
***
***
*
**
***
0
1
2
3
4
5
6
Time (days)
n = 6-12
*UDCA compared to DSS
B
% Original Weight
104
102
100
98
96
94
92
PBS
UDCA 30 mg/kg
DSS 2.5 %
DSS+UDCA
#
#
*
***
0
1
2
3
4
5
6
Time (days)
C
Vehicle
UDCA (30 mg/kg)
DSS (2.5%) + Vehicle
DSS (2.5%) + UDCA (30 mg/kg)
D
Untreated
DSS
PBS
UDCA
E
Inflammation score
40
30
20
10
0
***
#
***
* compared to 0 mg/kg vehicle
# compared to 0 mg/kg DSS
0
30
0
30
Vehicle + UDCA (mg/kg)
DSS (2.5%) + UDCA (mg/kg)

Figure 3

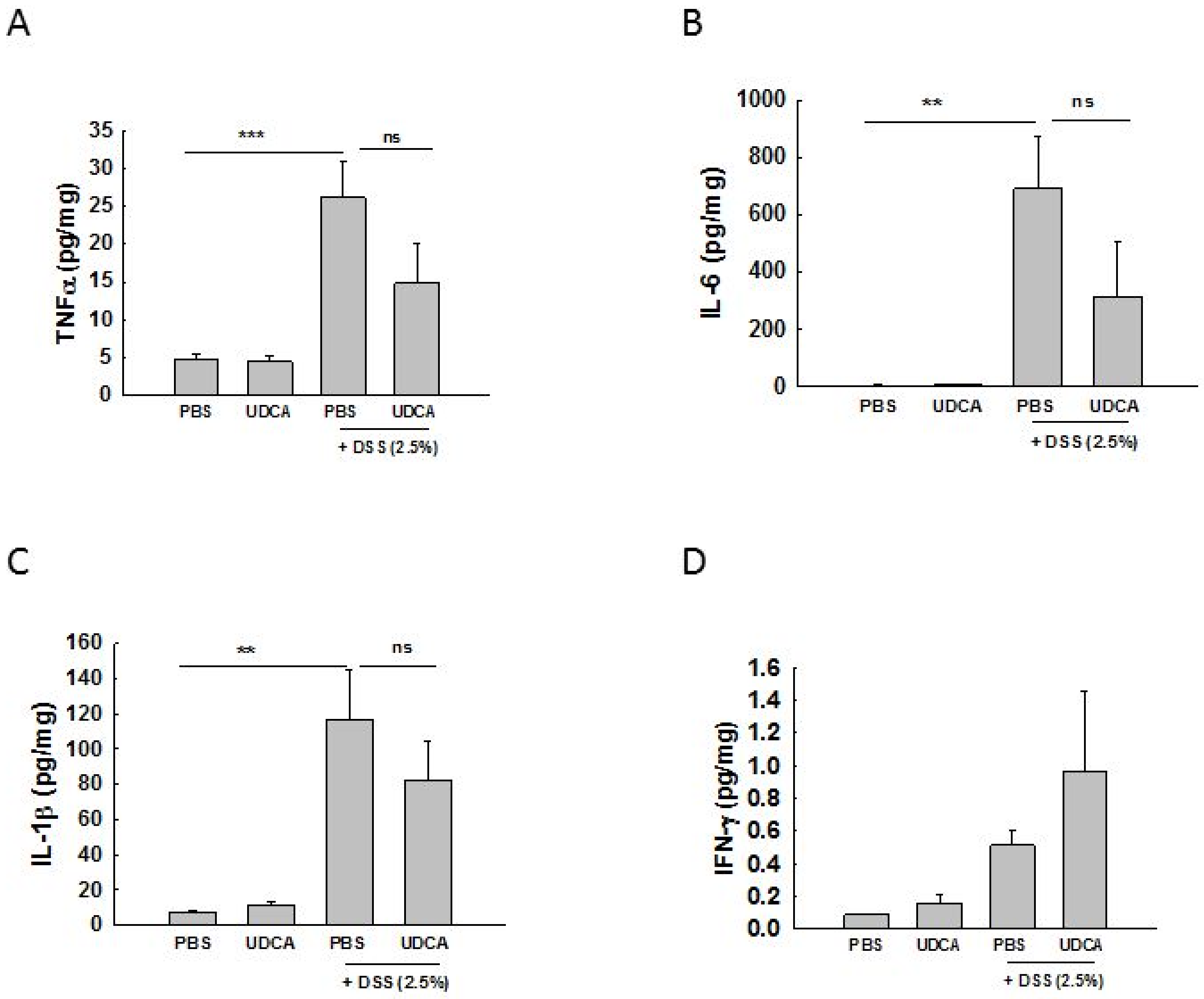

A
TNFα (pg/mg)
0
5
10
15
20
25
30
35
***
ns
PBS
UDCA
PBS
UDCA
+ DSS (2.5%)
B
IL-6 (pg/mg)
0
200
400
600
800
1000
**
ns
PBS
UDCA
PBS
UDCA
+ DSS (2.5%)
C
IL-1β (pg/mg)
0
20
40
60
80
100
120
140
160
**
ns
PBS
UDCA
PBS
UDCA
+ DSS (2.5%)
D
IFN-γ (pg/mg)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
PBS
UDCA
PBS
UDCA
+ DSS (2.5%)

Figure 4

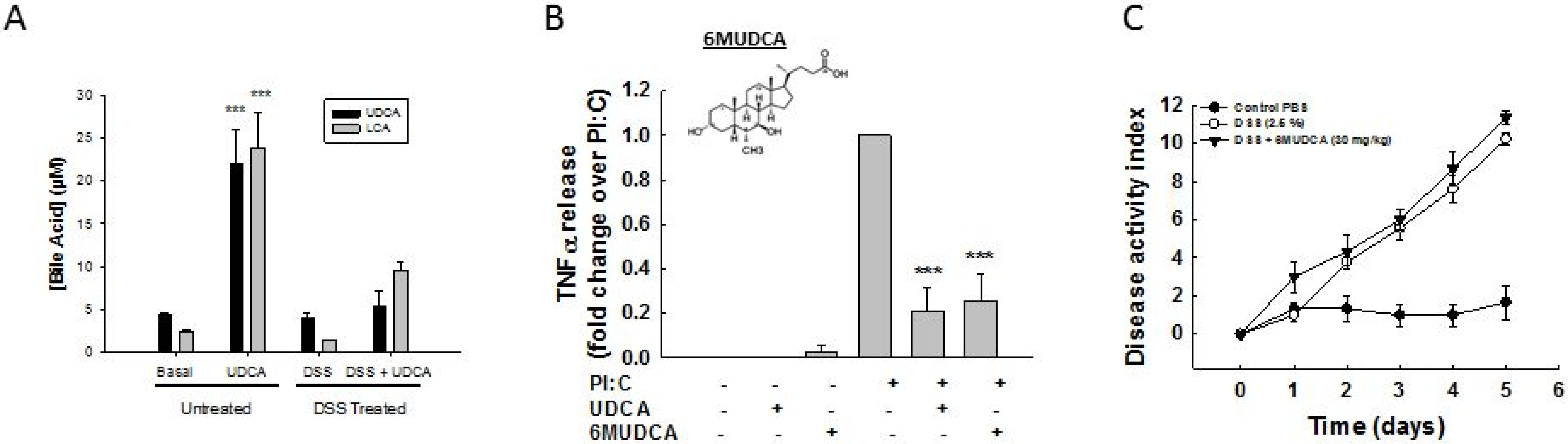

A
[Bile Acid] (μM)
0
5
10
15
20
25
30
UDCA
LCA
***
***
Basal
UDCA
DSS
DSS + UDCA
Untreated
DSS Treated
B
6MUDCA
HO
H
H
H
OH
CH3
O
OH
TNFα release
(fold change over PI:C)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
***
***
PI:C - - - + + +
UDCA - + - - + -
6MUDCA - - + - - +
C
Disease activity index
0
2
4
6
8
10
12
Control PBS
DSS (2.5 %)
DSS + 6MUDCA (30 mg/kg)
0
1
2
3
4
5
6
Time (days)

Figure 5

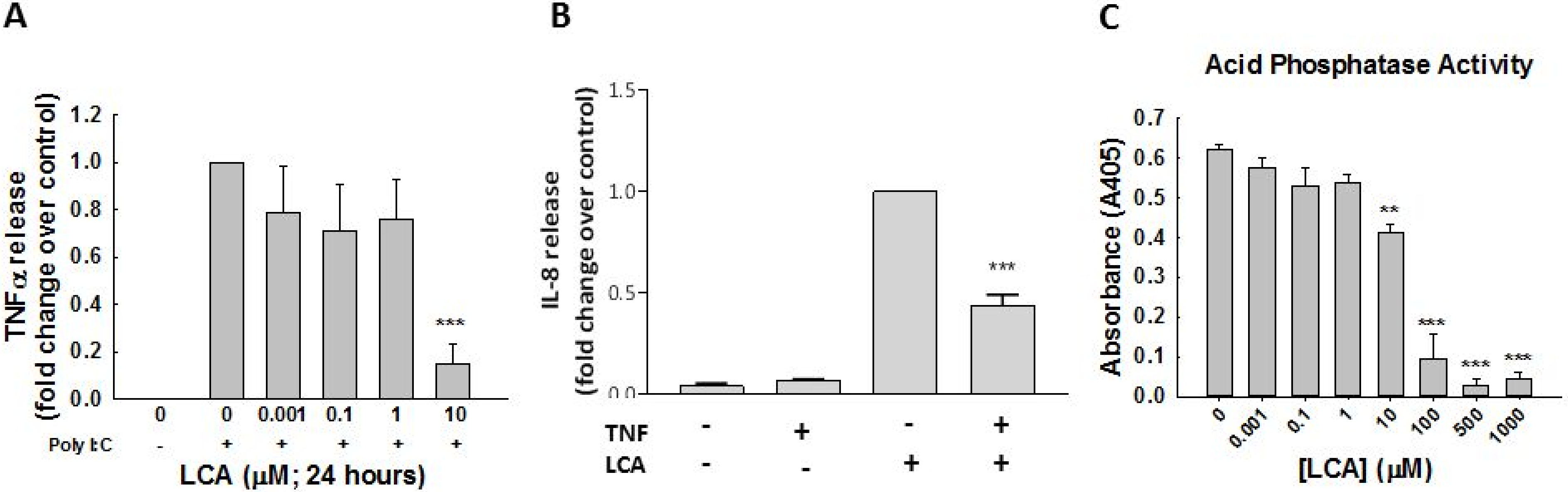

A
TNFα release
(fold change over control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
0
0
0.001
0.1
1
10
Poly I:C
-
+
+
+
+
+
LCA (μM; 24 hours)
B
IL-8 release
(fold change over control)
1.5
1.0
0.5
0.0
***
TNF
-
+
-
+
LCA
-
-
+
+
C
Acid Phosphatase Activity
Absorbance (A405)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
***
***
***
0
0.001
0.1
1
10
100
500
1000
[LCA] (μM)

Figure 6

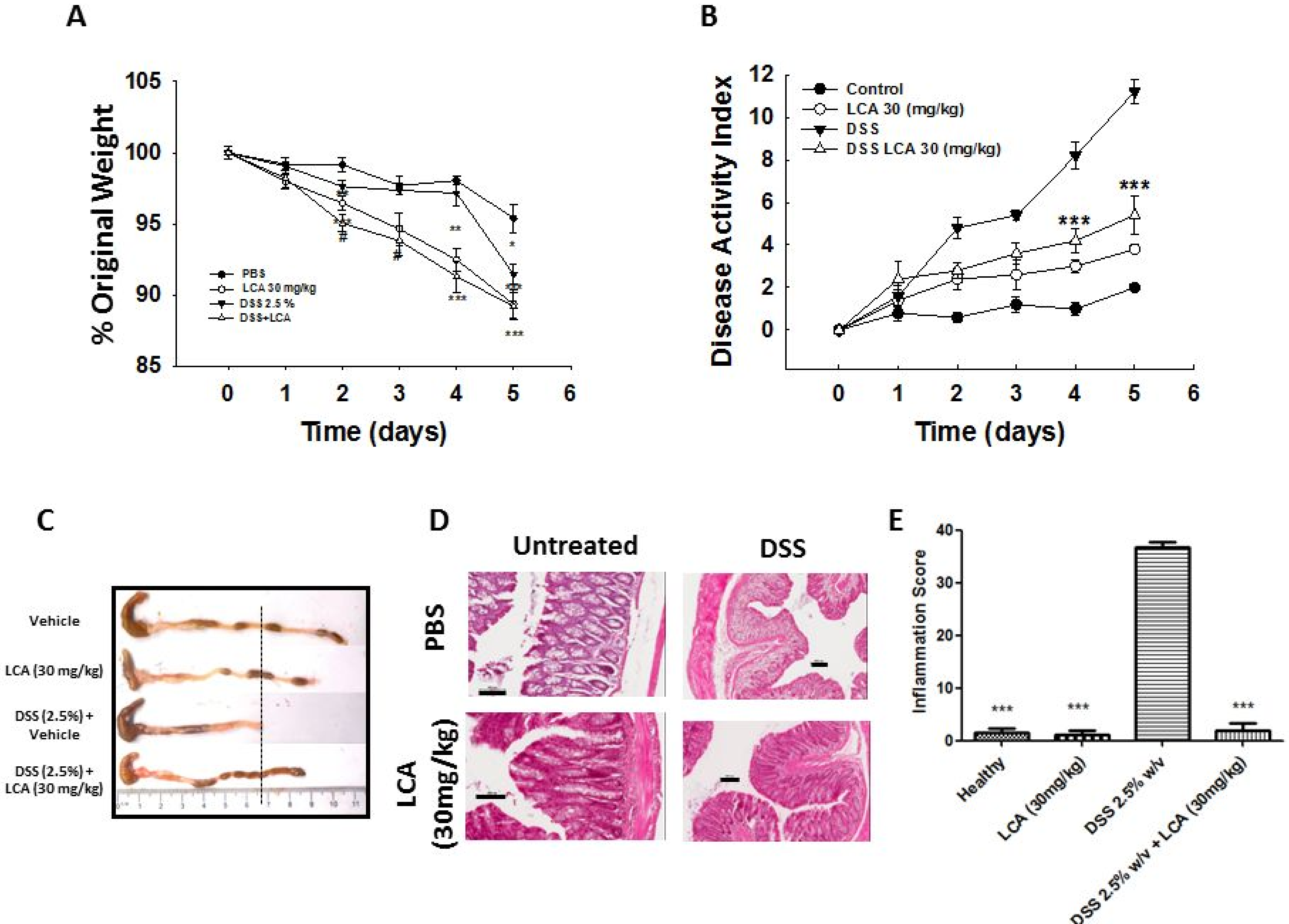
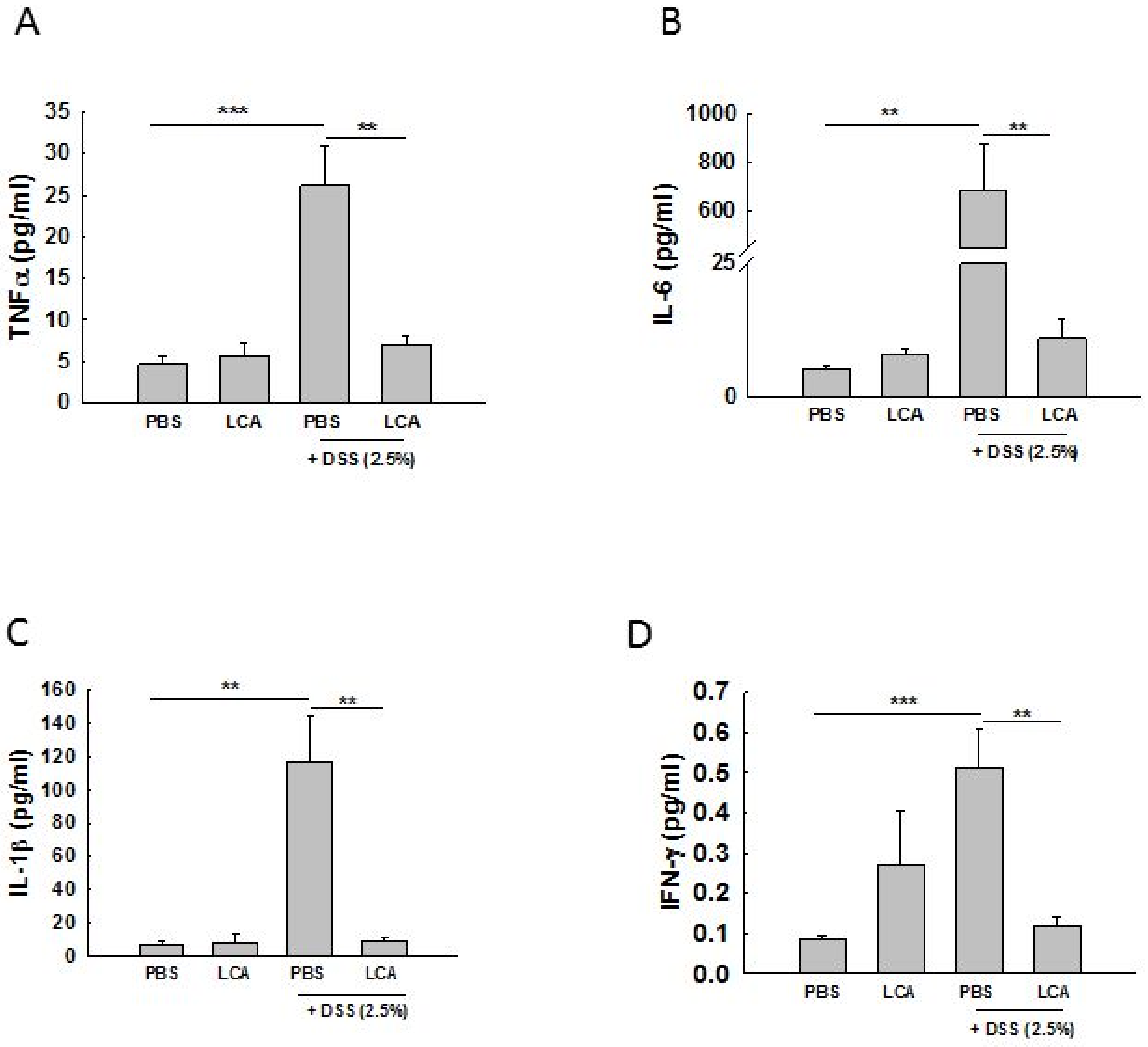

A
% Original Weight
105
100
95
90
85
0
1
2
3
4
5
6
Time (days)
PBS
LCA 30 mg/kg
DSS 2.5 %
DSS+LCA
***
#
#
**
*
***
***
B
Disease Activity Index
12
10
8
6
4
2
0
Control
LCA 30 (mg/kg)
DSS
DSS LCA 30 (mg/kg)
***
***
0
1
2
3
4
5
6
Time (days)
C
Vehicle
LCA (30 mg/kg)
DSS (2.5%) +
Vehicle
DSS (2.5%) +
LCA (30 mg/kg)
D
Untreated
DSS
PBS
LCA
(30mg/kg)
E
Inflammation Score
40
30
20
10
0
***
***
***
Healthy
LCA (30mg/kg)
DSS 2.5% w/v
DSS 2.5% w/v + LCA (30mg/kg)

Figure 7


A
TNFα (pg/ml)
35
30
25
20
15
10
5
0
***
**
PBS
LCA
PBS
LCA
+ DSS (2.5%)
B
IL-6 (pg/ml)
1000
800
600
25
0
**
**
PBS
LCA
PBS
LCA
+ DSS (2.5%)
C
IL-1β (pg/ml)
160
140
120
100
80
60
40
20
0
**
**
PBS
LCA
PBS
LCA
+ DSS (2.5%)
D
IFN-γ (pg/ml)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
**
PBS
LCA
PBS
LCA
+ DSS (2.5%)