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Ursodeoxycholic Acid Inhibits TNFα-Induced IL-8 Release From Monocytes

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Running Title: **Modulation of IL-8 release from monocytes by UDCA**

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Abstract

Monocytes are critical to the pathogenesis of inflammatory bowel disease (IBD) as they infiltrate the mucosa and release cytokines that drive the inflammatory response. Ursodeoxycholic acid (UDCA), a naturally-occurring bile acid with anti-inflammatory actions, has been proposed as a potential new therapy for IBD. However, its effects on monocyte function are not yet known. Primary monocytes from healthy volunteers or cultured U937 monocytes were treated with either the pro-inflammatory cytokine, TNFα (5 ng/mL), or the bacterial endotoxin, lipopolysaccharide (LPS; 1 μg/ml) for 24 hrs, in the absence or presence of UDCA (25 - 100 μM). IL-8 release into the supernatant was measured by ELISA. mRNA levels were quantified by qPCR and changes in cell signalling proteins were determined by western blotting. Toxicity was assessed by measuring lactate dehydrogenase (LDH) release. UDCA treatment significantly attenuated TNFα-, but not LPS-driven, release of IL-8 from both primary and cultured monocytes. UDCA inhibition of TNFα-driven responses was associated with reduced IL-8 mRNA expression. Both TNFα and LPS stimulated NFκB activation in monocytes, while IL-8 release in response to both cytokines was attenuated by an NFκB inhibitor, BMS-345541. Interestingly, UDCA inhibited TNFα-, but not LPS-stimulated, NFκB activation. Finally, TNFα, but not LPS, induced phosphorylation of TNF receptor associated factor (TRAF2), while UDCA co-treatment attenuated this response. We conclude that UDCA specifically inhibits TNFα-induced IL-8 release from monocytes by inhibiting TRAF2 activation. Since such actions would serve to dampen mucosal immune responses in vivo, our data and support the therapeutic potential of UDCA for IBD.

New and Noteworthy

The secondary bile acid, ursodeoxycholic acid (UDCA), specifically inhibits TNF-α-induced release of the proinflammatory cytokine, IL-8, from monocytes. The effects of the bile acid appear to be mediated by inhibition of TRAF-2-mediated NFκB activation and subsequent downregulation of IL-8 mRNA expression. Such actions of UDCA would serve to dampen mucosal immune responses in vivo, suggesting it may provide an alternative approach to the current use of biologics for prevention of TNF-α-induced inflammation in IBD patients.

Introduction

Inflammatory Bowel Diseases (IBD), comprised of Ulcerative Colitis (UC) and Crohn's Disease (CD), are common chronic inflammatory disorders of the intestinal tract. While the pathogenesis of IBD is still not fully understood, it is now widely accepted to involve genetic, environmental, and immunological factors that ultimately lead to dysregulation of innate barrier function (3). Current treatments for IBD employ salicylates, immunosupressants, corticosteroids, and biologics in order to first induce, and then maintain remission (5). However, while such treatments are effective in some patients, they are often limited by their lack of efficacy, cost, and occurrence of side effects. Despite intense research activity in the area, few new treatments for IBD have emerged over the past decades and new approaches are constantly being sought.

In recent years there has been increased interest in the potential for targeting innate intestinal barrier function for treatment of IBD. Innate barrier function is critical for normal intestinal health as it maintains separation of the body from the luminal contents, excluding the entry of harmful substances, such as bacteria and their toxins, to the mucosa. The innate barrier consists of the physical barrier posed by the epithelial layer, secreted factors, such as mucus, immunoglobulins, defensins, and cytokines, and myeloid immune cell populations present within the mucosa (24, 47).

Many aspects of epithelial barrier are altered in conditions of IBD (20, 31), and that dysregulated innate immune function is a primary contributor to the progression of the disease is beyond doubt. Both UC and CD are characterised by the accumulation of inflammatory macrophages and dendritic cells within the mucosa. When the epithelial barrier is breached, these cells act as the first line of defence performing important roles, including phagocytosis, production of pro- and anti-inflammatory mediators and cytokines, antigen presentation, and ultimately recruitment of the adaptive immune response (24). Key to initiating and driving these events is the infiltration of monocytes, from which macrophages and dendritic cells are derived, from the circulation to the mucosa. Under the influence of chemokines produced by the epithelium, circulating monocytes increase their surface expression of the gut homing receptor, CCR9 (27), thereby targeting them to

the mucosa and upregulating the expression of pro-inflammatory cytokines (15, 21, 42). In turn, such pro-inflammatory mediators (e.g., interleukin-8; IL-8) induce the influx of neutrophils and eosinophils, thereby further driving the inflammatory response (41, 46). The importance of monocytes in the development of IBD is evidenced by studies showing that their recruitment to the mucosa is necessary for the progression of inflammatory disease in animal models and that treatments which reduce monocyte numbers, such as apheresis, are effective in alleviating intestinal inflammation in patients (38, 44). Thus, given their central role in the initiation and maintenance of inflammatory responses, monocytes present an excellent target for the development of new therapies for IBD.

Originally identified in bear bile, 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). While quantitative and qualitative changes in the colonic bile acid pool are associated with the pathogenesis of IBD (10, 11, 25), UDCA is unique among the family of bile acids as it has long been appreciated to have therapeutic actions. As a component of bear bile, it has been used for centuries in Traditional Chinese Medicine to treat a range of ailments, and more recently in Western medicine to treat liver inflammation and cholestasis (12, 32). UDCA is considered to be a very safe drug and unless used at high doses has few side effects. In addition to its FDA-approved uses for liver disorders, UDCA is currently under investigation for a range of extraintestinal conditions, including neurological, ocular, and cardiovascular diseases (45). The distinct mechanisms underlying the wide-ranging therapeutic effects of UDCA are still poorly defined but they are believed to the anti-inflammatory and cytoprotective actions of the bile acid (35, 45). The biological actions of UDCA have been mostly studied in the liver, where it has been shown to have immunomodulatory and anti-apoptotic effects, and to prevent cytokine release from hepatocytes (1, 34, 36). Interestingly, several studies have shown that UDCA also exerts protective actions in animal models of intestinal inflammation, effects which were associated with reduced levels of mucosal cytokines, nitric oxide, and prevention of epithelial apoptosis (8, 22, 23, 28).

However, while UDCA shows excellent promise for development into a new therapy for IBD, there is still little known of its actions on monocyte function and in the current

study we set out to begin to address this gap in our knowledge. Using primary monocytes isolated from humans and cultured cells *in vitro*, we assessed the effects of UDCA on cytokine secretion in response to stimulation with either the inflammatory mediator, TNFα, or the bacterial endotoxin, lipopolysaccharide (LPS). We used IL-8, as a marker of monocyte-derived cytokine release, as it has been well-established as a key player in driving IBD pathogenesis.

Methods

Chemicals: UDCA (sodium salt) was obtained from Santa Cruz Technologies and stock solutions were prepared using deionised H_2O. LPS (E.coli, K235 strain) and TNFα were obtained from Sigma Chemical Co. (Poole, Dorset, UK). BMS-345541 was obtained from Merck Millipore (Darmstadt, Germany). SB203580 and all antibodies were obtained from Cell Signalling Inc (Danvers, MA). All other reagents were of analytical grade.

Cell Culture: U937 monocytes were maintained in a humidified atmosphere, containing 5% CO_2 at 37°C. The cells were cultured in 75 cm^3 polystyrene flasks in RPMI medium supplemented with 10% heat inactivated fetal bovine serum, 50 U/mL penicillin, 55 mg/mL streptomycin, and 2 mM L-glutamine. While in culture, cells were fed every 2-3 days and passaged every 4 days. Cell passages between 11 and 22 were used for experimentation.

Isolation of Primary Monocytes: Monocytes were isolated from whole blood of healthy volunteers by density gradient centrifugation using Lymphoprep (Axis-Shield PoC As, Norway). Lymphoprep was under-layered with whole blood and centrifuged at 800 *x g* for 10 mins. The resulting mononuclear cell band at the blood/lymphoprep interface was aspirated and washed with Hank's balanced salt solution (HBSS). Monocytes were purified from the mononuclear cell suspension using the EasySep human CD14 selection cocktail (StemCell Technologies, Grenoble, France). The mononuclear cell pellet was resuspended in 1 mL EasySep recommended media (1 mM EDTA, 2% FCS in PBS). 100 μL of EasySep positive selection cocktail was added and the mixture was incubated at room temperature for 15 mins. Following incubation, 100 μL of EasySep magnetic nanoparticles were added and the solution was incubated for a further 10 mins. The cell suspension was brought up to 2.5 mL with recommended media and placed into an EasySep magnet for 5 mins before pouring off the supernatant fraction. Isolated monocytes were then resuspended in complete RPMI, counted and seeded at a density of 50,000 cells/well in 96 plates prior to experimentation.

qPCR: RNA was extracted from U937 monocytes using the RNeasy kit (Qiagen, Valencia, CA) and genomic DNA contamination was removed by treatment with the DNase I kit (Ambion, Austin, TX). cDNA was synthesised from extracted RNA using

the ImProm II Reverse Transcriptase kit (Promega, Madison, WI). Primers specific to IL-8 and the 18s rRNA subunit were then used with SYBR green Master Mix (Roche, Indianapolis, IN) and qPCR was performed. The primers used were as follows; 18S Forward;5' CGGCTACCACATCCAAGGAA 3',18S Reverse ; 5 'GCTGGAATTACCGCGGCT 3',IL-8 Forward ; 5' ACTGTGCCTTGGTTTCTCCTTTAT 3', IL-8 Reverse; 5' CAACACAGCTGGCAATGACAA 3'.

ELISA: U937 monocytes and isolated primary monocytes were seeded in 96 well plates were treated with either LPS (1 μg/mL) or TNFα (5 ng/mL) in the presence or absence of UDCA (25 – 100 μM) for 24 hrs. Supernatant was then collected and analysed for IL-8 release using the BD-OptEIA™ Human IL-8 ELISA kit (BD Biosciences, San Jose, CA).

Lactate Dehydrogenase (LDH) Assay: U937 monocytes were seeded in 96 well plates and treated with UDCA for 24 hrs. Following treatment, samples of the incubation media were assayed for LDH release using a commercially-available kit (TOX-7, Sigma Chemical Co., Poole, Dorset). Lysis buffer was added to cells for 1 hr prior to the experiment to serve as a positive control.

Western blotting: U937 monocytes were washed with ice-cold PBS and lysed (1% IGEPAL CA630, 150 mmol/L 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). Lysates were then centrifuged (15,294 x g; 10 min; 4ºC), and the pellet discarded. Samples were normalised for protein content and 2X gel loading buffer (Laemmli Buffer, Sigma Chemical Co., Poole, Dorset) was added. After heating to 95°C, samples were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were pre-blocked in 5% blocking buffer (Marvel Skimmed Milk, Premier Foods Group Ltd., UK) in TBST for 60 minutes at room temperature followed by incubation with primary antibody in 5% blocking buffer overnight at 4ºC. After washing (x4) in Tris buffered saline with 1% tween (TBST), membranes were incubated with HRP-conjugated secondary antibodies in 5% blocking buffer for 60 min at room temperature. After further washing (x4) in TBST, immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden).

Statistical Analysis: Results are expressed as mean ± standard error of the mean (SEM) for a series of *n* experiments. Statistical analyses were performed by paired t-tests or analysis of variance (ANOVA) with the Newman Keul's post-test (GraphPad Prism software, San Diego, CA). p values ≤ 0.05 were considered to be statistically significant.

Results

UDCA specifically attenuates TNFα-induced IL-8 expression and release from monocytes. We first examined the effects of UDCA on IL-8 release from primary monocytes isolated from healthy volunteers. Treatment of the monocytes with either TNFα (5 ng/mL) or LPS (1 μg/mL) increased IL-8 levels in the supernatant from 195 ± 82 pg/mL to 2,343 ± 282 and 26,017 ± 394 pg/ml, respectively. Furthermore, we found that co-treatment with UDCA (100 μM) significantly reduced TNFα-stimulated IL-8 release ~~from 2,343 ± 282 to 1,684 ± 243 (n = 3; p ≤ 0.01),~~ but had no effect on that induced by LPS~~-driven IL-8 release (n = 3)~~ (Figures 1 A-B).

The effect of UDCA on a well-established in vitro model of monocytes, U937 cells, was also examined. Similar to our findings in primary monocytes, both TNFα and LPS induced increases in IL-8 release, although LPS was not as potent as it was in primary monocytes. ~~IL-8 release from TNFα- and LPS-stimulated cells increased from 755 ± 234 pg/mL to 4356 ± 818 and 6135 ± 2112 pg/mL, respectively. Similar~~Furthermore, similar to its effects in primary monocytes, UDCA significantly attenuated TNFα-stimulated IL-8 release~~. UDCA was most effective at the highest concentration tested, 100 μM, reducing TNFα-driven IL-8 release from 4356 ± 818 to 1880 ± 374 pg/ml (n = 5; p ≤ 0.001)~~ (Figure 2A). However, also similar to our findings in primary cells, UDCA had no effect on LPS-stimulated IL-8 release (Figure 2B). All concentrations of UDCA inhibited basal secretion of IL-8 (Figure 2C) but were not toxic, as determined by measurements of LDH release (Figure 2D). Thus, given that U937 cells recapitulate our findings in primary monocytes, these cells were used in all subsequent experiments.

Next, we carried out experiments to determine if the effects of UDCA on IL-8 secretion could be due to alterations in mRNA expression. In these experiments we carried out PCR on cDNA prepared from RNA isolated from monocytes treated with TNFα or LPS, either in the absence or presence of UDCA. We found that the bile acid attenuated both basal and TNFα-induced increases in IL-8 mRNA expression, but was without effect on responses to LPS (Figure 3).

Effects of UDCA on IL-8 secretion are independent of p38 MAP kinase. Having demonstrated differential effects of UDCA on TNFα and LPS-induced IL-8 release from monocytes, we ~~next carried out experiments to~~ investigated signalling mechanisms that may be involved. Since the p38 MAPK pathway has been previously linked to driving IL-8 release from monocytes, we first focussed on investigating the potential involvement of this pathway. We found that treatment with either LPS or TNFα increased phosphorylation of p38 MAPK in U937 monocytes (Figure 4A-B). Interestingly, treatment with a specific p38 MAPK inhibitor, SB203580 (10 µM), attenuated only LPS-driven IL-8 release without altering TNFα-induced responses (Figure 4C-D). UDCA did not inhibit either TNF~~α~~ α– or LPS-induced phosphorylation of p38 MAPK and even tended to increase this response to LPS (Figure 4A-B).

UDCA specifically attenuates TNFα-induced activation of NFκB. Next, we investigated a potential role for NFκB, ~~an integral signalling protein that~~which has been shown to mediate IL-8 release in response to LPS and TNFα in other systems (13, 16), as a target for the actions of UDCA on cytokine release from monocytes. ~~Thus, we first investigated the effects of UDCA on NFκB activation in U937 cells, using phosphorylation of the p65 subunit as a marker of NFκB activation.~~ We found that both TNFα and LPS induced ~~5.7 ± 0.6 and 2.1 ± 0.4 fold increases in~~ phosphorylation of p65, a reliable marker of NFκB activation, in U937 cells~~, respectively~~. Interestingly, UDCA co-treatment specifically attenuated TNFα-induced p65 phosphorylation ~~of p65 to 2.8 ± 1.2 fold (n = 3; p ≤ 0.05)~~, while it had no effect on responses to LPS ~~(n = 4)~~ (Figure 5A-B). In further experiments, we found that inhibition of NFκB with the specific inhibitor, BMS-345541 (10 µM), abolished TNFα-induced IL-8 release, and significantly reduced LPS-induced responses (Figure 5C-D).

UDCA inhibits TNFα-induced phosphorylation of TRAF2. To further elucidate how UDCA might exert its differential effects on TNFα– and LPS-induced NFκB activation, we next examined TRAF2 activation. TRAF2 is an adaptor protein that,

upon TNFα stimulation, is recruited to the TNFR and becomes phosphorylated, ultimately leading to activation of downstream effectors, including NFκB (9). As expected, we found that TNFα treatment induced a rapid phosphorylation of TRAF2, whereas LPS had no effect (Figure 6A). Furthermore, when cells were co-treated with UDCA, phosphorylation of TRAF2 in response TNFα was significantly inhibited (Figure 6B).

Discussion

A growing body of evidence suggests that UDCA may be useful in treatment of intestinal inflammation. In particular, it has been repeatedly shown that UDCA, or its taurine-conjugate, tauroursodexoycholic acid (TUDCA), is protective in animal models of intestinal inflammation and that such actions may be attributed to prevention of epithelial apoptosis and inhibition of mucosal cytokine accumulation (23, 28). Our current studies suggest that inhibition of mucosal monocyte function may also have a role to play in mediating the anti-inflammatory actions of the bile acid.

TNFα and IL-8 are well-established as 2 of the primary cytokines driving mucosal inflammation in IBD (18). Inflammatory cells within the mucosa have been previously identified as the main source of IL-8 in IBD (2, 7), while TNFα and LPS are both known to potently stimulate release of the cytokine from monocytes. In turn, monocyte-derived IL-8 acts as a potent chemoattractant to other innate myeloid cells, such as neutrophils, leading to increased proinflammatory cytokine production and perpetuation of inflammation. This vicious cycle of inflammation underlies the mucosal damage that occurs in IBD and strategies that dampen its activity should be of use in disease treatment. Here, we found that UDCA inhibits TNFα induction of IL-8 secretion from monocytes. The effects of UDCA were apparent at the level of IL-8 mRNA expression, suggesting inhibition of cytokine expression at the level of gene transcription is likely involved. Furthermore, we found that the effects of UDCA on IL-8 release from monocytes were specific to TNFα, since the bile acid did not alter secretion of the cytokine in response to the bacterial endotoxin, LPS.

Further investigation into the signaling mechanisms involved suggested that the differential effects of UDCA on TNFα and LPS-induced IL-8 production is likely due to the distinct mechanisms by which these agonists act on monocytes. LPS exerts its actions through binding to TLR4 and its co-receptor, CD14 (17, 37), whereas TNFα acts through TNFRs. While both receptors activate both NFκB and p38 MAPK in monocytes, these signaling pathways appear to have distinct roles in mediating IL-8 secretion in response to the different agonists. We found that a selective p38 MAPK inhibitor, SB203580, significantly attenuated LPS-induced IL-8 secretion, without altering that in response to TNFα. In contrast, inhibition of NFκB with BMS-345541

reduced IL-8 production in response to LPS, while abolishing responses to TNFα. Together, these data suggest that while LPS-induced IL-8 release from monocytes is mediated by pathways involving both p38 MAPK and NFκB, responses to TNFα do not involve p38 MAPK but are more dependent on NFκB. Interestingly, we also found that while treatment with UDCA prevented activation of NFκB in response to TNFα, as measured by phosphorylation of the NFκB p65 subunit, it was without effect on LPS-induced NFκB activation. Thus, our data suggests that the specificity of UDCA for inhibiting TNFα-induced IL-8 secretion likely stems from inhibition of an upstream component that is distinct to the TNFR/NFκB signaling pathway. With this in mind, we investigated the possibility that TRAF-2 may be a target of UDCA action in monocytes. TRAF-2 is a signaling protein that is recruited to activated TNFR and which is a critical regulator of downstream signaling, including NFκB activation (9). In our current studies, we found that TNFα, but not LPS, treatment of monocytes induced phosphorylation of TRAF-2 and, in support of our hypothesis, UDCA significantly inhibited this effect.

While the current study is the first to demonstrate an inhibitory effect of UDCA on TNFα-induced cytokine release from monocytes, previous studies have shown its lack of effects on LPS-induced cytokine production (4, 14). Indeed, previous studies have also reported differential actions of UDCA compared to other colonic bile acids on multiple aspects of monocyte function. For example, while DCA and CDCA inhibit cytokine release and the pro-coagulant activity of LPS-stimulated monocytes, UDCA is without effect (6, 14). Such differential actions imply strict structure-activity requirements for the actions of bile acids on different aspects of monocyte function. This likely reflects different potencies of different bile acids at bile acid receptors. For example, in contrast to the actions of UDCA shown here, agonists of the cell surface bile acid receptor, TGR5, have been shown to inhibit LPS-induced cytokine release from monocytes (26). This, along with previous reports of the low potency of UDCA at TGR5 (19), suggests it is unlikely that this receptor mediates effects of the bile acid reported in the current studies, A role for FXR also seems unlikely since it has been previously shown not to be expressed in U937 cells (29), a finding confirmed by our own preliminary studies (data not shown) Other possibilities for how UDCA could induce responses in monocytes include activation of the pregnane x receptor

or the glucocorticoid receptor, both of which have been reported to be activated by UDCA in other systems (39, 40, 48) and both of which have been shown to be protective in animal models of intestinal inflammation (33, 43). This question of how UDCA triggers anti-inflammatory responses in monocytes is the subject of ongoing work in our research group.

In summary, the results described in this paper illustrate for the first time, the ability of UDCA to specifically inhibit TNFα-induced IL-8 release from monocytes. Given the central role that these cytokines play in the pathogenesis of IBD, one would expect that such actions of the bile acid would dampen mucosal inflammation in vivo. Indeed, proof of principal for the efficacy of TNF-α blockade in treatment of IBD comes from the successful use of biologics, such as, infliximab, adalimumab and etanercept (30). Thus, by virtue of its capacity to inhibit the TNF-α pathway, along with its previously reported anti-apoptotic and anti-inflammatory actions in the colon, UDCA could afford an alternative approach for treatment of IBD. Furthermore, the excellent safety profile of UDCA over decades of use in treatment of liver diseases makes it a particularly attractive candidate for further development. Clinical trials of the efficacy of UDCA in inducing and/or maintaining remission in IBD patients are warranted.

FIGURE LEGENDS

Figure 1. Effects of UDCA on TNFα- and LPS-induced IL-8 release from primary human monocytes. Monocytes were isolated from whole blood and seeded at a density of 50,000 cells/well in 96 well plates. **A)** IL-8 release was measured from cells co-treated with UDCA (100 µM) in the presence of TNFα (5 ng/mL) for 18 hrs (n = 3). **B)** IL-8 release was measured from cells co-treated with UDCA (100 µM) in the presence of LPS (1 µg/mL) for 18 hrs (n = 3). Data represent the mean ± SEM for a series of *n* experiments. Statistical analyses were performed using ANOVA followed by the Student Newman Keul's post-test (** $p \leq 0.01$, *** $p \leq 0.001$).

Figure 2. UDCA attenuates TNFα-, but not LPS-induced, IL-8 release from cultured U937 monocytes. U937 monocytes were seeded at a density of 50,000 cells/well in 96 well plates. **A)** IL-8 release was measured from cells co-treated with UDCA (25-100 µM) in the presence of TNFα (5 ng/mL) for 24 hrs (n = 5). **B)** IL-8 release was measured from cells co-treated with UDCA (25-100 µM) in the presence of LPS (1 µg/mL) for 24 hrs (n = 7). **C)** IL-8 release was measured from cells treated with UDCA alone (25 – 100 µM) for 24 hrs (n = 5). **D)** Cells were treated with UDCA (25 – 200 µM) for 24 hrs and LDH release was measured by a commercially-available kit (n = 3). "Lysed cells", treated with lysis buffer for 45 mins, were included as a positive control. Data represent the mean ± SEM for a series of *n* experiments. Statistical analyses were performed by ANOVA followed by the Student Newman Keul's post-test (*$p < 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Figure 3. UDCA attenuates TNFα-, but not LPS-induced, IL-8 mRNA expression in monocytes. U937 monocytes were seeded at a density of 500,000 cells/well in 24 well plates. Cells were treated for 24 hrs, pelleted by centrifugation, RNA was extracted and cDNA was synthesised. qPCR was performed to measure IL-8 mRNA levels. 18s RNA was used as an internal control. **A)** Cells were treated with UDCA (100 µM) for 24 hrs (n = 4). **B)** Cells were co-treated with TNFα (5 ng/mL) in the presence of UDCA (100 µM) for 24 hrs (n = 4). **C)** Cells were co-treated with LPS (1

μg/mL) in the presence of UDCA (100 μM) for 24 hrs (n = 4). Data represent the mean ± SEM for a series of *n* experiments. Statistical analyses were performed using either Student's t-test or ANOVA followed by the Student Newman Keul's post-test (* $p \leq 0.05$, ** $p \leq 0.01$).

Figure 4. UDCA does not target p38 MAPK to attenuate IL-8 release. U937 monocytes were seeded at a density of 500,000 cells/well in 24 well plates. Cells were co-treated with UDCA (100 μM), in the presence of **A)** TNFα (5 ng/mL) or **B)** LPS (1 μg/mL) for the time points indicated. Cells were then lysed and western blot analysis was performed. All samples were normalised to total levels of p38 MAPK. Data represent the mean ± SEM for a series of 3 experiments. U937 monocytes were seeded at a density of 50,000 cells/well in 96 well plates and pre-treated for 1 hr with the specific p38 MAPK inhibitor, SB203580 (10 μM), and co-treated with either **C)** TNFα (5 ng/mL) or **D)** LPS (1 μg/mL) for 24 hrs. Samples of the incubation media were then taken and assayed for IL-8 by ELISA. Data represent the mean ± SEM for a series of 4 experiments. Statistical analyses were performed using ANOVA followed by the Student Newman Keul's post-test (* $p \leq 0.05$,** $p \leq 0.01$, *** $p \leq 0.001$).

Figure 5. UDCA attenuates TNFα-induced activation of NFκB. U937 monocytes were seeded at a density of 500,000 cells/well in 24 well plates. Cells were treated for 1 hr, lysed, and western blot analysis was performed in order to assess changes in phosphorylation of p65. All samples were normalised to β-actin. **A)** Cells were treated with TNFα (5 ng/mL) in the presence of UDCA (100 μM) for 1 hr (n = 3). **B)** Cells were treated with LPS (1 μg/mL) in the presence of UDCA (100 μM) for 1 hr (n = 4). U937 monocytes, seeded at a density of 50,000 cells/well in 96 well plates were pretreated with the specific NFκB inhibitor, BMS-345541 (10 μM) for 1 hr prior to treating with **C)** TNFα (5 ng/mL; n = 4) or **D)** LPS (1 μg/mL; n = 5) for 24 hrs. Samples of the incubation media were then taken and assayed for IL-8 by ELISA. Data represent the mean ± SEM for a series of *n* experiments. Statistical analyses

were performed using ANOVA followed by the Student Newman Keul's post-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Figure 6. UDCA attenuates TNFα-induced phosphorylation of TRAF2. U937 monocytes were seeded at a density of 500,000 cells/well in 24 well plates. **A)** Cells were treated with LPS (1 μg/mL) or TNFα (5 ng/mL) for 1 hr. Cells were then lysed and western blot analysis was performed (n = 3). **B)** Cells were co-treated with UDCA (100 μM) in the presence of TNFα (5 ng/mL) for 1 hr. Cells were then lysed and western blot analysis was performed. All values were normalised to β-actin. Data represent the mean ± SEM for a series of 7 experiments. Statistical analysis was performed using ANOVA followed by the Student Newman Keul's post-test (** $p \leq 0.001$; *** $p \leq 0.001$).

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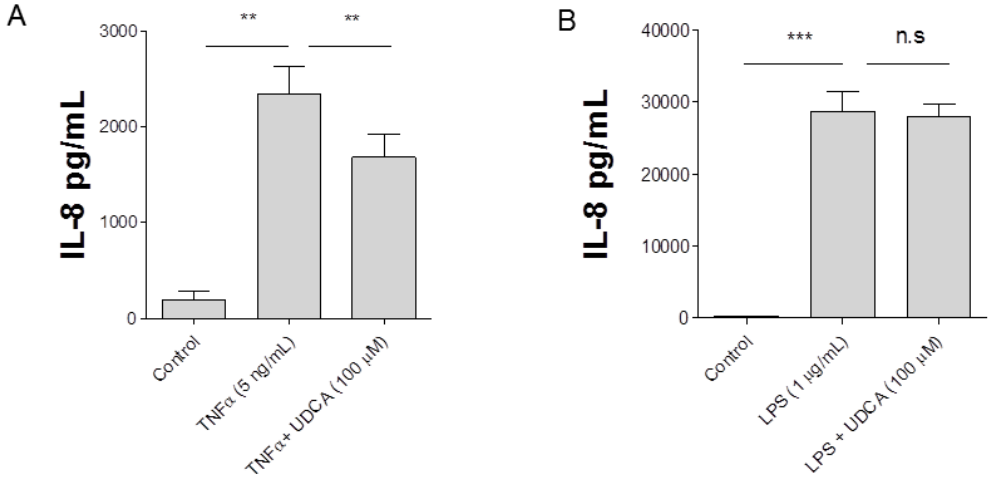
Figure 1


A
IL-8 pg/mL
3000
2000
1000
0
**
**
Control
TNFα (5 ng/mL)
TNFα+ UDCA (100 μM)
B
IL-8 pg/mL
40000
30000
20000
10000
0
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n.s
Control
LPS (1 μg/mL)
LPS + UDCA (100 μM)

Figure 2

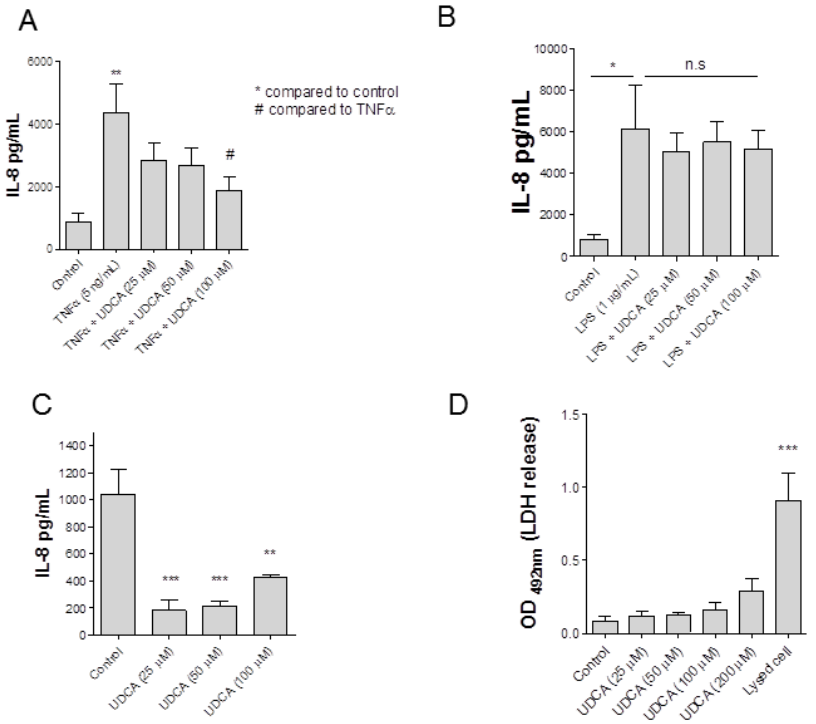

A
IL-8 pg/mL
6000
4000
2000
0
**
#
* compared to control
# compared to TNFα
Control
TNFα (5 ng/mL)
TNFα + UDCA (25 μM)
TNFα + UDCA (50 μM)
TNFα + UDCA (100 μM)
B
IL-8 pg/mL
10000
8000
6000
4000
2000
0
*
n.s
Control
LPS (1 μg/mL)
LPS + UDCA (25 μM)
LPS + UDCA (50 μM)
LPS + UDCA (100 μM)
C
IL-8 pg/mL
1400
1200
1000
800
600
400
200
0
***
***
**
Control
UDCA (25 μM)
UDCA (50 μM)
UDCA (100 μM)
D
OD 492nm (LDH release)
1.5
1.0
0.5
0.0
***
Control
UDCA (25 μM)
UDCA (50 μM)
UDCA (100 μM)
UDCA (200 μM)
Lysed cell

Figure 3

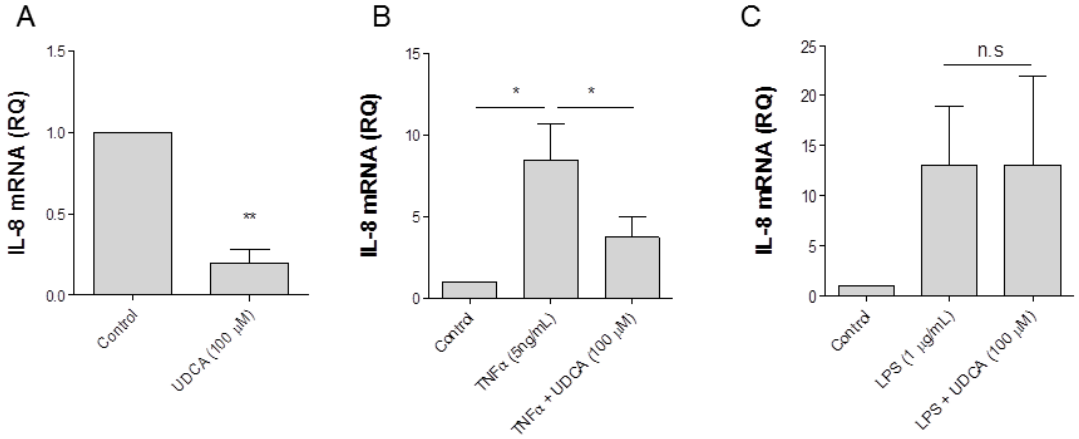

A
IL-8 mRNA (RQ)
1.5
1.0
0.5
0.0
**
Control
UDCA (100 μM)
B
IL-8 mRNA (RQ)
15
10
5
0
*
*
Control
TNFα (5ng/mL)
TNFα + UDCA (100 μM)
C
IL-8 mRNA (RQ)
25
20
15
10
5
0
n.s
Control
LPS (1 μg/mL)
LPS + UDCA (100 μM)

Figure 4

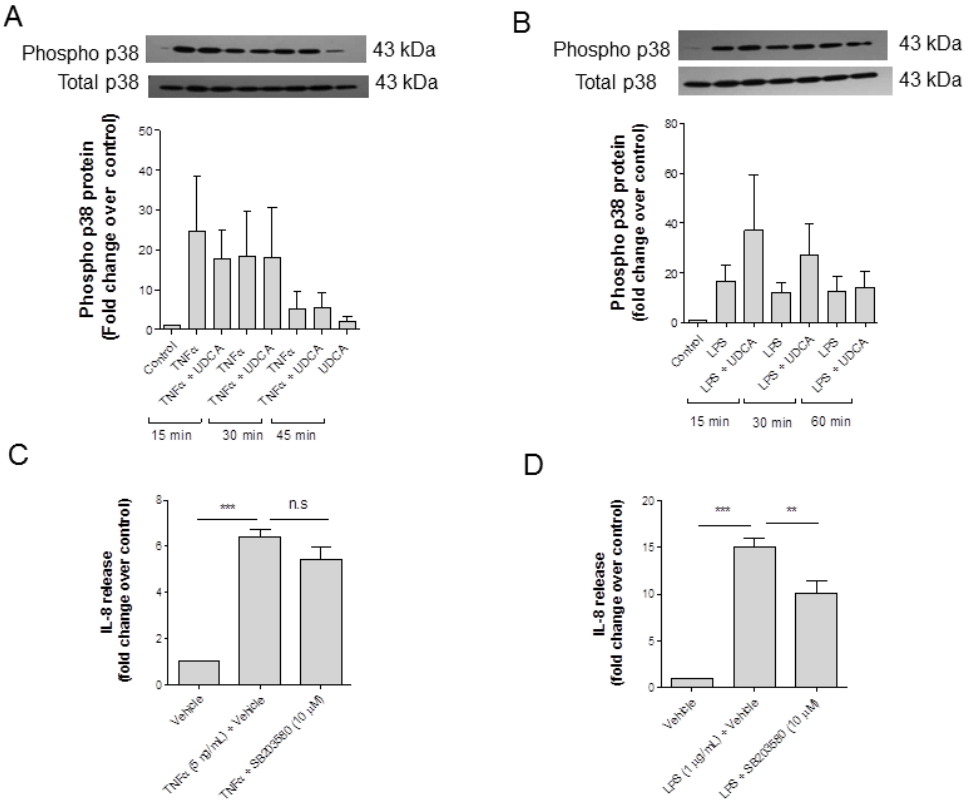

A
Phospho p38
43 kDa
Total p38
43 kDa
Phospho p38 protein
(Fold change over control)
Control
TNFα
TNFα + UDCA
TNFα
TNFα + UDCA
TNFα
TNFα + UDCA
UDCA
15 min
30 min
45 min
B
Phospho p38
43 kDa
Total p38
43 kDa
Phospho p38 protein
(fold change over control)
Control
LPS
LPS + UDCA
LPS
LPS + UDCA
LPS
LPS + UDCA
15 min
30 min
60 min
C
IL-8 release
(fold change over control)
***
n.s
Vehicle
TNFα (5 ng/mL) + Vehicle
TNFα + SB203580 (10 μM)
D
IL-8 release
(fold change over control)
***
**
Vehicle
LPS (1 μg/mL) + Vehicle
LPS + SB203580 (10 μM)

Figure 5

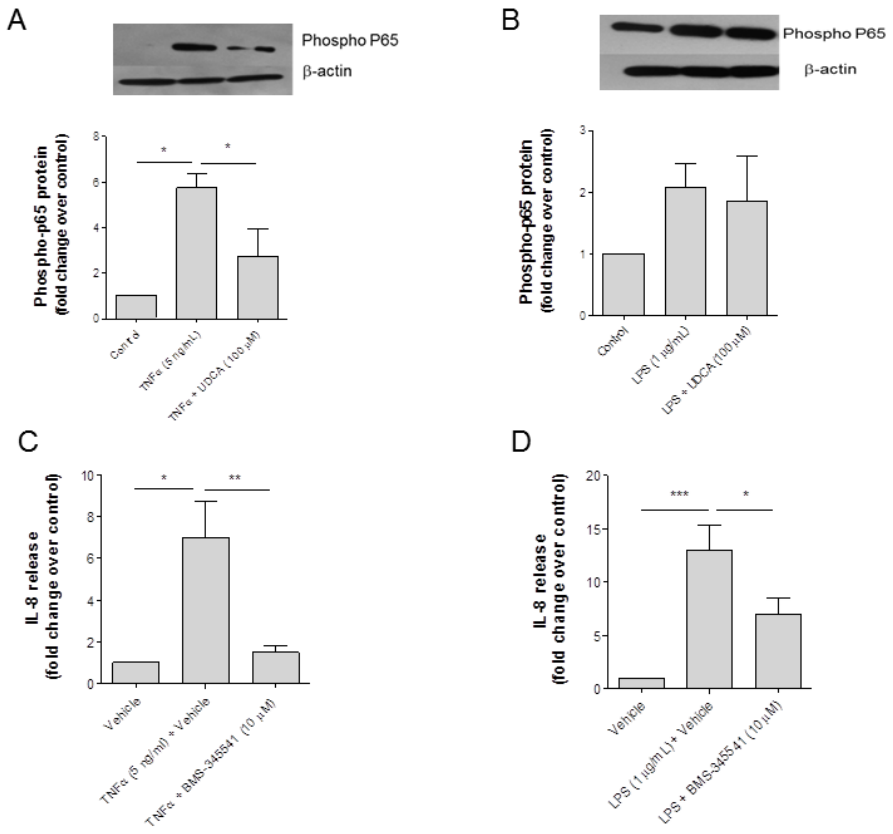
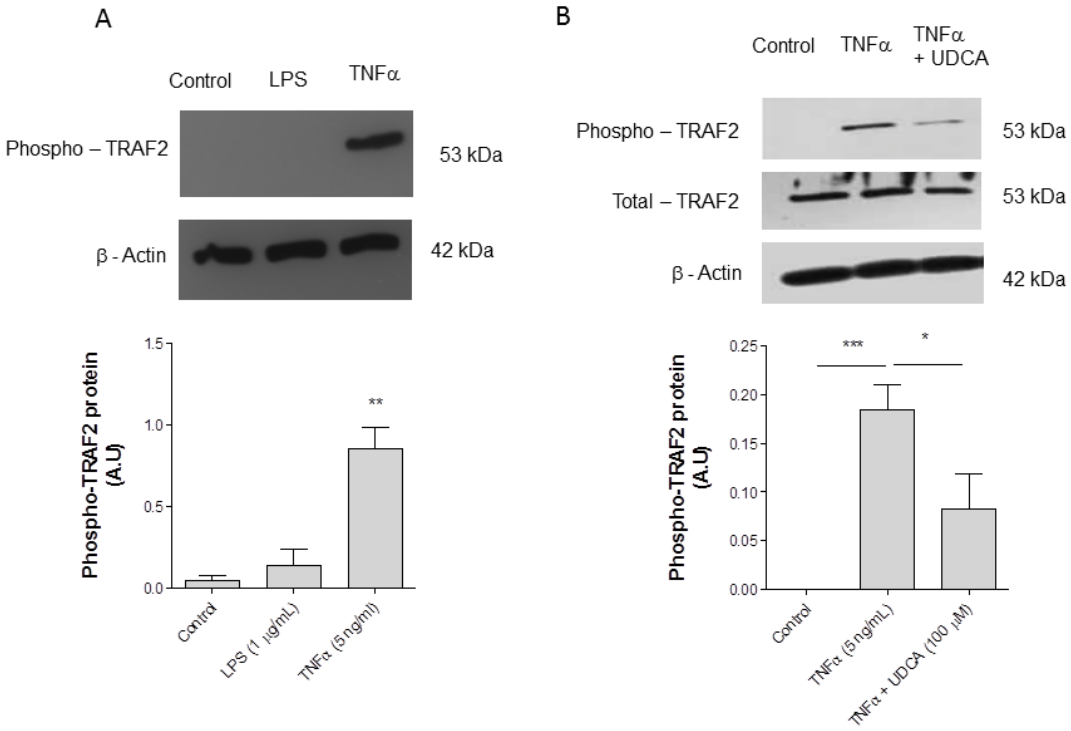

A
Phospho P65
β-actin
Phospho-p65 protein
(fold change over control)
Control
TNFα (5 ng/mL)
TNFα + UDCA (100 μM)
B
Phospho P65
β-actin
Phospho-p65 protein
(fold change over control)
Control
LPS (1 μg/mL)
LPS + UDCA (100 μM)
C
IL-8 release
(fold change over control)
Vehicle
TNFα (5 ng/ml) + Vehicle
TNFα + BMS-345541 (10 μM)
D
IL-8 release
(fold change over control)
Vehicle
LPS (1 μg/mL) + Vehicle
LPS + BMS-345541 (10 μM)

Figure 6


A
Control
LPS
TNFα
Phospho – TRAF2
53 kDa
β - Actin
42 kDa
Phospho-TRAF2 protein (A.U)
1.5
1.0
0.5
0.0
**
Control
LPS (1 µg/mL)
TNFα (5 ng/ml)
B
Control
TNFα
TNFα + UDCA
Phospho – TRAF2
53 kDa
Total – TRAF2
53 kDa
β - Actin
42 kDa
Phospho-TRAF2 protein (A.U)
0.25
0.20
0.15
0.10
0.05
0.00
***
*
Control
TNFα (5 ng/mL)
TNFα + UDCA (100 µM)