Gene silencing of TNF-alpha in a murine model of acute colitis using a modified cyclodextrin delivery system


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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic relapsing inflammation of the gastrointestinal tract. The cytokine TNF-alpha (TNF-α) plays a pivotal role in mediating this inflammatory response. RNA interference (RNAi) holds great promise for the specific and selective silencing of aberrantly expressed genes, such as TNF-α in IBD. The aim of this study was to investigate the efficacy of an amphiphilic cationic cyclodextrin (CD) vector for effective TNF-α siRNA delivery to macrophage cells and to mice with induced acute-colitis. The stability of CD.siRNA was examined by gel electrophoresis in biorelevant media reflecting colonic fluids. RAW264.7 cells were transfected with CD.TNF-α siRNA, stimulated with lipopolysaccharide (LPS) and TNF-α and IL-6 responses were measured by PCR and ELISA. Female C57BL/6 mice were exposed to dextran sodium sulphate (DSS) and treated by intrarectal administration with either CD.siRNA TNF-α or a control solution. In vitro, siRNA in CD nanocomplexes remained intact and stable in both fed and fasted simulated colonic fluids. RAW264.7 cells transfected with CD.TNF-α siRNA and stimulated with LPS displayed a significant reduction in both gene and protein levels of TNF-α and IL-6. CD.TNF-α siRNA-treated mice revealed a mild amelioration in clinical signs of colitis, but significant reductions in total colon weight and colonic mRNA expression of TNF-α and IL-6 compared to DSS-control mice were detected. This data indicates the clinical potential of a local CD-based TNF-α siRNA delivery system for the treatment of IBD.

1. Introduction

Crohn’s disease and ulcerative colitis (UC) are two chronic immune-mediated forms of IBD [1]. Current theories postulate that the condition results from a dysregulated, aberrant intestinal immune response to indigenous bacterial microorganisms in individuals with a genetic susceptibility [2–4]. IBD is a chronic remittent relapsing condition with a diversity of clinical manifestations. Most IBD treatments are based on anti-inflammatory and immune-suppressive drugs. However, while these are effective in acute inflammation there is a high relapse rate in IBD patients due to poor tolerability and high toxicity or diminishing efficacy over time [5]. The location and extent of disease differs significantly, with Crohn's disease presenting a transmural inflammation along the gastrointestinal tract, compared to a superficial, mucosal inflammation in the colon of UC patients. Crohn’s disease is generally associated with a T helper (Th) cell type 1 (Th1)/Th17 phenotype with exaggerated production of IL-12, IFN-γ, IL-17 accompanied by high production of TNF-α and IL-1β, in contrast to UC which is associated with an atypical Th2 cytokine profile of high IL-10, IL-5 and IL-13 [6,7]. It is acknowledged that TNF-α plays a central role in IBD pathobiology and anti-TNF-α therapy has been shown to be efficacious in the treatment of both conditions [8,9]. Recent advances in the molecular pathogenesis of IBD have caused a shift towards novel biological approaches for the treatment of this disease, including therapeutic gene silencing by RNA interference.

Small interfering RNA (siRNA), are double stranded segments of RNA ~20–25 nucleotides in length which are part of the cellular RNAi machinery that represses mRNA gene expression in a sequence-specific manner [10]. The use of siRNA against pro-inflammatory cytokines such as TNF-α is a potential treatment for IBD. However, the application of siRNA therapy in humans is limited by delivery and stability issues. Following systemic administration, siRNA has a short half-life in plasma, due to degradation by nucleases [11]. In contrast to systemic administration, delivery of siRNA to the gastrointestinal tract has several advantages, one being the large population of target cells available for uptake of the siRNA. In addition, several diseases can be treated locally
including IBD, intestinal cancer or familial adenomatous polyposis [12]. Recently, novel oral delivery systems have been used to successfully deliver TNF-α siRNA to the inflamed colon in experimental IBD models [13–15], as proof-of-concept for oral delivery of siRNA.

As mentioned previously, stability is a major obstacle to the use of siRNA as a gene therapy. Thus, optimal delivery vectors are required to improve siRNA stability and efficacy. Recently, modified cyclodextrins (CDs), which are naturally occurring macrocyclic oligosaccharides, have been used as non-viral gene delivery vectors [16,17]. CDs exhibit low immunogenicity and toxicity coupled with high biocompatibility in biological environments. In addition, CDs can be chemically modified with functional groups such as cationic groups, PEG chains, lipid groups and targeting ligands to enable siRNA complexation and enhance transfection and delivery [18–20]. We have shown that cationic amphiphilic CDs can effectively deliver pDNA and siRNA into different cell types, including intestinal epithelial cells and neuronal cells, resulting in successful transfection and gene silencing [21–23]. Other modifications of the CD core with targeting ligands including galactosylated and anisamide groups, facilitated cell-specific transfection of hepatocytes and prostate cancer cells, respectively [19,20]. In this work the application of our modified CDs as vectors for siRNA delivery was extended to an experimental model of acute-colitis. The aims of this study were to verify the stability of CD.siRNA complexes in simulated intestinal fluids, to examine the effect of CD.TNF-α siRNA on LPS-induced cytokine expression in macrophages in vitro and finally to assess the therapeutic efficacy of intrarectally administered CD.TNF-α siRNA to mice with dextran sulphate (DSS)-induced acute colitis.

2. Material and methods

2.1. Reagents and siRNA

All reagents were purchased from Sigma-Aldrich unless otherwise stated. LPS-UK Ultrapure from E.coli was purchased from InvivoGen (San Diego, USA). Mouse TNF-α and non-silencing (N/S) siRNA were custom made by Sigma-Aldrich as previously reported [13] with the following sequence; (sense 5’-3’) GACAACACACUGUGUGGCTT, (anti-sense 5’-3’) GCACCACAGUGUGGUGUGCTT. Non-silencing (N/S) siRNA (sense 5’-3’) UUCUCCGAACUGUGACUCCUTT, (anti-sense 5’-3’) ACCUCACAGUUCUGGAGAATT. Lipofectamine 2000 (LF2000) from Invitrogen was obtained from Biosciences (Dublin, Ireland) and in vivo-jetPolyethyleneimine (PEI) from Source Bioscience LifeSciences (Nottingham, UK).

2.2. Preparation of CD.siRNA and PELsiRNA delivery systems

The structure of the CD used in this study is given in Fig. 1. The CD was synthesised as previously described [18]. Briefly, β-cyclodextrin was functionalised by bromo groups on the primary face at the 6-position and on the secondary face by alkyne groups at the 2-position. Copper(I)-catalysed azide-alkyne cycloaddition (commonly called “click”) chemistry was used to add propyl chains bearing a terminal Boc-protected primary amine to the secondary face. The primary face was thioalkylated with dodecane thiol, followed by cleavage of the Boc groups to give SC12-CD-click-propylamine. In vitro studies: To prepare the complexes, CD was dissolved in chloroform to a concentration of 1 mg/mL. The solvent was removed under a gentle stream of nitrogen leaving a film of CD, this was hydrated with water (CD concentration 1 mg/mL) under sterile conditions, followed by sonication at room temperature (RT) for 1 h for size reduction. A solution of siRNA was mixed with the CD solution in equal volumes, and complexed for 20 min at RT (final CD concentration 0.5 mg/mL). A mass ratio (MR) of CD to siRNA (μg CD: μg siRNA) of 10 was previously reported to be efficacious in vitro (equivalent to nitrogen/phosphate, N/P ratio of ~6.9) [18,24]. For in vivo studies: CD was prepared as described above using 5% glucose. TNF-α and N/S siRNA solutions were prepared in 5% glucose and added to CD to achieve a MR of 10. Complexes were allowed to “mix” for 20 min at RT. The CD.siRNA was concentrated to 50 μg siRNA/100 μl by ultrafiltration using the Vivaspin 500 (3 K MWCO) (Sartorius Stedim Biotech, Gottingen, Germany). In the PEI studies, 50 μg of TNF-α siRNA was complexed with in vivo-jetPEI® (N/P of 7) followed by complexation for 15 min at RT as per manufacturer’s instructions. Size and charge of PELsiRNA complexes were tested in 5% glucose using the Malvern zetasizer as previously described [18].

2.3. Stability of CD.siRNA in simulated colonic fluids

Gel electrophoresis was used to determine the binding of siRNA by CD in simulated colonic fluids. CD siRNA were prepared at various MR; 1, 2.5, 5, 10 and 20, followed by incubation in either fasted (fa) state pH 7.8 (bile acids 150 μM) or fed (fe) pH 6.0 (bile acids 600 μM) state simulated colonic fluids (SSCoF) [25] for 24 h at 37 °C. In other experiments, the stability of CD.siRNA was examined in FeSSCoF media containing α-amylase from Aspergillus oryzae at a concentration of 10 U/mL. Following incubation, samples were mixed with loading media and deionised water to a final volume of 30 μl (containing 0.5 μg siRNA). Samples were run on a 1% agarose gel. Electrophoresis was carried out at 90 V for 40 min and a 1× Tris-borate-EDTA media containing safeview nucleic acid stain (NBS Bio, UK). Bands corresponding to the DNA ladder (100 b.p.) and unbound siRNA were visualised by U.V. using the Biorad Quantity One 1-D analytical software (Version 4.6.9) and ChemiDoc MP Gel imaging device.

2.4. Quantification of TNF-α and IL-6 secretion and mRNA expression in transfected RAW264.7 cells stimulated with LPS

The inhibitory effect of TNF-α siRNA on the cytokine secretion and expression of TNF-α and IL-6 by RAW264.7 cells in the presence of
GENE DELIVERY

1. DSS + CD.N/S, gr. 2-DSS + Naked TNF-α

2.6. Induction of colitis and TNF-α

Cells were used as controls. siRNA was used at a concentration of 100 nM/mL as previously reported [26]. LF2000 was complexed with siRNA according to manufacturer’s instructions. After transfection, RAW264.7 cells were left to rest overnight in full media before being challenged with LPS (10 ng/mL, chosen from the optimisation study) in SF media for 6 h. Cells were collected in lysis media containing β-mercaptoethanol for subsequent total RNA extraction (RNA mini-prep kit from Agilent Technologies) and RT-PCR analysis of TNF-α and IL-6 expression. Cell culture supernatants were analysed for TNF-α and IL-6 secretion by ELISA (Mouse, platinum ELISA, Ebioscience, Vienna, Austria).

2.5. Toxicity and cell viability

Cell viability was determined by MTT assay as previously reported [18]. Briefly, RAW264.7 cells were seeded into a 96 well plate at a density of 30,000 cells/well in 100 μL DMEM media plus 10% FBS. After 24 h, the cells were treated for 4 or 15 h, with the following complexes: CD.sirnA (TNF-α or N/S) at MR10, CD alone, naked TNF-α siRNA and LF2000. TNF-α prepared in SF OPTIMEM. Untreated cells were used as controls. siRNA was used at a concentration of 50 mM/mL.

2.6. Induction of colitis and TNF-α siRNA treatment

Female C57BL6/JolaHsd mice, weighing 18–20 g (Harlan), received 3% DSS as previously described [27]. Animals were housed at a temperature of 21 °C, 50% humidity and 12:12 h light-dark cycles and fed a standard pellet diet and tap water ad libitum. Mice were acclimatised for ~2 weeks before starting the study. All animal procedures were performed according to national ethical guidelines following approval by the University College Cork Animal Experimentation Ethics Committee. Mice were randomised with 4 to 10 mice per group and received 3% (w/v) DSS (47 kDa; Tdb Consultancy, Uppsala, Sweden) dissolved in tap water for a period of 5 days. Fresh DSS was prepared every day. The clinical symptoms recorded were body weight, stool consistency (0–3) and fur texture and posture (0–2), which were used to generate a daily disease activity index (DDAI).

Mice were treated by intraoral administration of test solution (100 μL) as follows; in PEI-study: group (gr) 1-DSS + PEI, gr. 2-DSS + PEI.TNF-α and gr. 3-healthy; and in CD.TNF-α study: gr. 1-DSS + CD.N/S, gr. 2-DSS + Naked TNF-α, gr. 3-DSS + CD.TNF-α, gr. 4-Healthy.CD.N/S, gr. 5-healthy (untreated, UT). Mice were sedated under isoflurane anaesthesia for intraoral administration of solutions. This route of delivery has been previously reported for its effectiveness for siRNA uptake in the colon [28]. Solutions were administered to mice on day 2 and day 4 post initial DSS treatment. Mice were sacrificed on day 5 post-DSS with the entire colon dissected. The colons were opened longitudinally, rinsed in PBS, measured and weighed. A 0.5 cm section of distal and proximal colon was collected and stored in RNA later solution and kept at 2–8 °C overnight. The following day samples were placed in the -80 °C until RNA extraction.

2.7. RNA extraction and real-time polymerase chain reaction

Dissected tissues were homogenised using MagNalyser green beads (Roche diagnostics, Mannheim, Germany). RNA was isolated from homogenised distal and proximal colon tissues and from RAW264.7 cells using the absolutely RNA mini-prep kit from Agilent Technologies according to the manufacturer’s instructions. A TURBO DNA-free kit (Applied Biosystems) was used to further purify the extracted RNA. RNA quantity and purity was assessed spectrophotometrically (Nanodrop ND-1000, Labtech International, Ringmer, East Sussex, U.K.). RNA was reverse-transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Woolston, UK). Real-time PCR was performed in triplicate, using pre-designed Applied Biosystems TaqMan® Gene Expression Assays for mouse TNF-α, IL-6 (FAM labelled) and β-actin (VIC labelled) and Applied Biosystems TaqMan® Universal PCR Master Mix in a 7300 Real-Time PCR System (Applied biosystems, Carlsbad, California, USA). In all experiments β-actin was used as a housekeeping gene. The expression of each gene within a sample was normalised against β-actin mRNA expression and expression relative to the control sample using the formula 2- \( \Delta \Delta C_{T} \), in which \( \Delta \Delta C_{T} = (C_{T} \text{ mRNA}_{-\alpha \text{TNF}} - C_{T} \beta \text{-actin mRNA})_{\text{sample}} - (C_{T} \text{ mRNA}_{-\alpha \text{TNF}} - C_{T} \beta \text{-actin mRNA})_{\text{control sample}} \) [29].

2.8. Statistical and data analysis

Statistical analysis of the mean difference between multiple groups was determined by one-way Anova, followed by either Bonferroni’s (in vivo data) or Tukey (in vitro data) post-tests where appropriate. A P-value of <0.05 is considered statistically significant. All statistical analyses were performed using GraphPad (Version 5.00 for Windows, San Diego, CA, USA).

3. Results and discussion

3.1. In vitro studies

3.1.1. CD.sirnA stability in simulated colonic fluids

The physicochemical properties of the CD.sirnA nanocomplexes used in this study have previously been characterised with a size of ~240 nm at MR10, polydispersity index of 0.235 and a zeta-potential of +42 mV [18]. In this study, we aimed to predict the lower gastrointestinal in vivo stability by incubating the CD.sirnA complexes in simulated colonic fluids for 24 h [25] and assessing stability by gel electrophoresis.

Fasted and fed SSCoF differ with respect to pH and bile salt concentration. Following incubation in FaSSCoF, the gel electrophoresis studies indicate that siRNA remains complexed to the CD from MR 5 to 20 (Fig. 2A). Similarly, in the case of FeSSCoF, optimal complexation of siRNA by the CD was observed at MR5, 10 and 20 (Fig. 2B). Heparin displacement confirms the stability of siRNA after complexation with CD and incubation in FeSSCoF (Fig. 2C) with quantitative analysis of peak density of each fluorescent band illustrated in Fig. 2D.

It has previously been reported that CDs are susceptible to degration by bacterial and fungal enzymes present in the colon that can hydrolyse α(1,4)-glycosidic bonds present in β-CDS [30]. The stability of siRNA when complexed with CDs in the presence of α-amylase (10 U/mL) from A. oryzae, a common opportunistic micro-organism detected among IBD patients [31,32], was assessed in FeSSCoF. Results show that siRNA remained bound to the CD at MR 5 to 10 (Fig. 2E) indicating that degradation of the CD by α-amylase and subsequent release of siRNA does not occur.

3.1.2. CD.TNF-α siRNA reduces LPS-induced TNF-α and IL-6 production in RAW264.7 cells

In preliminary experiments, we observed that CD.TNF-α siRNA silenced TNF-α gene expression in unstimulated RAW264.7 cells in a similar manner to LF2000.TNF-α (data not shown). To extend this work, the ability of CD.TNF-α siRNA to reduce cytokine expression under inflammatory conditions was investigated. For this purpose, RAW264.7 cells were activated with LPS, a ligand for membrane
The expression and production of TNF-α gene as IL-6 [33]. Interestingly, TLR4 has been shown to be up-regulated in both Crohn’s disease and UC [34].

A concentration of 10 ng/mL of LPS was identified as optimal to increase both TNF-α and IL-6 gene expression (~16 and 37 fold, respectively) (Fig. 3A–B) and secretion levels (18.5 ± 1.7 ng/mL and 168.2 ± 22.0 pg/mL, respectively) in RAW264.7 cells (Fig. 3C–D). Following the optimisation of the LPS-induced response in RAW264.7 cells, we investigated whether silencing of TNF-α by siRNA inhibited LPS-induced cytokine production. RAW264.7 cells transfected with CD.TNF-α siRNA (MR20) for 24 h followed by LPS-treatment (10 ng/mL) for 6 h, displayed a ~21 fold drop in TNF-α protein secretion compared to CD.N/S siRNA (N = 3, *P < 0.05 compared to LPS only-treated cells, PC).

similarly, significantly lower amounts of TNF-α protein were secreted from RAW264.7 cells transfected with CD.TNF-α siRNA (MR10) (7.1 ± 0.2 and MR20, 4.3 ± 0.4 ng/mL, respectively) versus LPS-only treated cells (positive control (PC) cells) (Fig. 4A, *P < 0.05, N = 3–4). Similarly, 10 ng/mL LPS (10 ng/mL) for 6 h, displayed a ~21 fold drop in TNF-α gene expression versus LPS-only treated cells (positive control (PC) cells) (Fig. 4A, *P < 0.05, N = 3–4). Similarly, significantly lower amounts of TNF-α protein were secreted from RAW264.7 cells transfected with CD.TNF-α siRNA (MR10) (7.1 ± 0.2 and MR20, 4.3 ± 0.4 ng/mL, respectively) versus LPS-only treated RAW264.7 cells (18.3 ± 0.9 pg/mL) (Fig. 4C, *P < 0.05, N = 3–4). The responses seen with the CD.TNF-α siRNA were similar to those observed with the commercially available gene delivery vector LF2000.TNF-α (Fig. 4A and C).

Studies have shown that targeting the TNF-α signaling pathway in macrophages with siRNA can affect other pro-inflammatory genes such as IL-6 [35]. As shown in Fig. 4B, RAW264.7 cells transfected with CD.TNF-α siRNA (MR20) had lower IL-6 expression (~7.0 fold reduction) under LPS-conditions versus control cells (Fig. 4B, *P < 0.05). Similarly, IL-6 protein production was also reduced in RAW264.7 cells transfected with CD.TNF-α siRNA at MR10 and 20 (Fig. 4D, *P < 0.05). No significant changes were observed in the expression and production of TNF-α/IL-6 in CD.N/S transfected cells compared to LPS-only activated cells (Fig. 4). In summary, these results demonstrate that CD.TNF-α siRNA can significantly reduce LPS-induced TNF-α and IL-6 levels in murine macrophages.
and 100.4 ± 10.1%, respectively. In contrast, following treatment of RAW264.7 cells with LF2000.TNF-α siRNA viability was reduced to 40 ± 6.5% after 15 h, this level of toxicity is consistent with previous reports in other cell lines [18,22].

3.2. In vivo studies

3.2.1. PEI.TNF-α study

Initially we used the murine acute colitis model to investigate the response to treatment with PEI.siRNA. PEI.TNF-α siRNA was administered intrarectally to mice on day 2 and 4 post-DSS start. Table 1 summarises changes in % body weight, DDAI scores, colon weight, length and PCR expression of TNF-α and IL-6 in mice treated with DSS + PEI and DSS + PEI.TNF-α siRNA compared to healthy mice. At day 5 of the study, DSS + PEI treated mice showed a significant reduction in % relative body weight, higher DDAI scores, significantly shorter colons and increased total colon weight compared to healthy mice (N = 3–10, *P < 0.05). These results are in agreement with signs of colitis previously reported in this model [36,37]. PCR analysis of colonic TNF-α and IL-6 expression supported an inflammatory response, as gene expression was significantly increased in DSS + PEI mice versus healthy (*P < 0.05, N = 3–10). Mice treated with DSS + PEI.TNF-α displayed no significant improvement in % relative body weight, DDAI scores, colon length or colon weight (P > 0.05) and no significant knockdown of colonic TNF-α or IL-6 mRNA expression versus DSS only-treated mice. These results suggest a lack of TNF-α siRNA delivery by PEI. In a previous study [14], orally administered TNF-α siRNA complexed with PEI to LPS-treated mice was reported to successfully reduce macrophage and colonic TNF-α production. However in that study [14], the PEI vector was branched (BPEI) in structure, with an N/P ratio of 30 (size ~380 nm, charge −8 mV) and was encapsulated into polylactide nanoparticles. In contrast, in this current study, we used linear PEI (LPEI) at a lower N/P ratio of 7 (size ~420 nm, charge +24 mV). BPEI is composed of primary, secondary and tertiary amines whereas LPEI has exclusively only secondary amines. These differences in charge between BPEI and LPEI are important factors in the higher level of uptake and gene transfection efficiency into cells by BPEI compared with LPEI at a lower N/P ratio [38]. Thus, these differences in formulation may explain the different results obtained.

3.2.2. CD.TNF-α study

Having shown good stability of the CD.siRNA delivery system in colonic fluids and a high level of silencing of TNF-α expression/secretion in vitro, the ability of the CD vector to deliver therapeutic TNF-α siRNA to DSS-treated mice was investigated. For comparison, the same experimental methods as in the DSS + PEI study were used. Changes in % body weight, colon weight and length of DSS-treated (CD.N/S, Naked TNF-α, CD.TNF-α) and healthy (healthy + CD.N/S and healthy) mice groups are shown in Fig. 5A–C. The clinical and macroscopic responses in DSS + CD.N/S treated mice are similar to DSS + PEI treated mice with

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (%)</th>
<th>DDAI</th>
<th>Length (cm)</th>
<th>Colon weight (mg/cm)</th>
<th>Colonic TNF-α (2ddct)</th>
<th>Colonic IL-6 (2ddct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0.0 ± 1.3 *</td>
<td>0.5 ± 1.0*</td>
<td>6.2 ± 0.3 *</td>
<td>27.1 ± 2.7 *</td>
<td>2.7 × 10^{-4} *</td>
<td>7.4 × 10^{-5} *</td>
</tr>
<tr>
<td>DSS</td>
<td>−6.6 ± 1.1</td>
<td>1.5 ± 0.1</td>
<td>4.6 ± 1.3</td>
<td>41.9 ± 2.5</td>
<td>3.5 × 10^{-3}</td>
<td>9.2 × 10^{-3}</td>
</tr>
<tr>
<td>+ PEI</td>
<td>−6.5 ± 1.3</td>
<td>1.2 ± 0.1</td>
<td>5.0 ± 1.1</td>
<td>38.8 ± 1.6</td>
<td>3.8 × 10^{-3}</td>
<td>6.4 × 10^{-3}</td>
</tr>
<tr>
<td>+ PEI.TNF-α</td>
<td>−6.5 ± 1.3</td>
<td>1.2 ± 0.1</td>
<td>5.0 ± 1.1</td>
<td>38.8 ± 1.6</td>
<td>3.8 × 10^{-3}</td>
<td>6.4 × 10^{-3}</td>
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</table>

* Healthy mice or mice exposed to 3% DSS for 5 days.

* Denotes per cent of body weight loss at day of termination (day 5) in relation to starting body weight at the start of DSS (day 0).

* DDAI = daily disease activity index including stool consistency and fur texture/posture.

* mRNA expression expressed as 2ddct as explained in material and methods.

* P < 0.05 healthy versus DSS + PEI.
significant loss in % relative body weight, appearance of liquid bloody stools, and poor fur texture and posture (i.e. DDAI), shorter colons and increased colon weight compared to healthy mice (*P < 0.05, Fig. 5A–C).

In contrast to the DSS + PEI.TNF-α siRNA study, a clinical improvement in body weight loss, although not significant, was observed in DSS mice treated with CD.TNF-α siRNA mice (Fig. 5A). In addition, an improvement in visible blood in faeces was observed, as only 3/10 of the DSS + CD.TNF-α siRNA treated mice versus 7/10 in the DSS + CD.N/S treated mice presented this sign of disease. No other differences between DSS-treated groups regarding DDAI scores were observed. However, total colon weight was significantly reduced ("P < 0.05) by ~5.6 mg/cm in DSS + CD.TNF-α siRNA treated mice versus DSS + CD.N/S siRNA indicating a therapeutic effect (Fig. 5B). This reduction in colon weight was attributed to a significant reduction in proximal colon weight by ~12.5 mg/cm in DSS + CD.TNF-α siRNA treated mice compared to DSS-control ("P < 0.05), while no significant reduction in distal colon weight was observed (data not shown). Mice treated with DSS + CD.N/S siRNA had significantly shorter colons (48 ± 0.1 cm, "P < 0.05) compared to healthy mice (~6 cm), this has previously been observed with this model [36,37]. No significant changes were observed in colon length between DSS + CD.TNF-α siRNA group versus DSS-control (Fig. 5C, P > 0.05).

To investigate if the clinical improvements observed in DSS + CD.TNF-α siRNA treated mice were directly related to successful delivery of siRNA by the CD formulation and the resulting gene silencing, RT-PCR analysis on distal and proximal colons from DSS-treated and healthy mice was performed. Mice were administered siRNA by the CD formulation and the resulting gene silencing using siTNF-OMe-P correlated in vitro with the efficacy predicted in vitro using a murine model of acute colitis. Following intrarectal administration of a liposome.TNF-α siRNA delivery system on day 0 and 2 of a 5 day study, Ocampo et al. [41] administered intrarectally TNF-α siRNA with 2'-O-methyl and panediapoli modifications (siTNF-OMe-P) to 5% DSS-treated mice over an 8-day period. As in the current study, they found that in vivo gene silencing using siTNF-OMe-P correlated well with knockdown of TNF-α in vivo. While different disease index parameters were recorded by Ocampo et al. they also reported reductions in total colonic tissue levels of TNF-α mRNA but to a lesser extent (~1.6 fold reduction) than observed in the current study (~3.5 fold). This group also showed a decrease in the weight-over-length colon ratio of ~40% in DSS + siTNF-OMe-P-treated mice versus the DSS-control. This is in agreement with our results for total colon weight where a significant decrease of ~50% in DSS + CD.TNF-α siRNA treated mice was detected. The CD.TNF-α delivery system used in this study was given intra-rec tally to mice, and based on these promising results we envision future studies will focus on improving this formulation for oral gene delivery. While many challenges exist to developing a formulation for oral delivery to treat IBD, reports from other investigators [13,42] have shown that robust formulation approaches are capable of overcoming these barriers and of achieving improved therapeutic outcomes.

4. Conclusions

In vitro cell culture studies using LPS indicated the potential of the CD delivery system to silence TNF-α under inflammatory conditions. The efficacy predicted in vitro was confirmed in vivo using a murine model of acute colitis. Following intrarectal administration the CD.TNF-α siRNA complex, shown to be stable in simulated colonic fluids, alleviated the clinical indicators of disease and decreased the expression not only of TNF-α but also IL-6. To our knowledge this is the first in vivo report of a CD-based siRNA delivery system with potential use for IBD treatment.

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References


Fig. 6. Colonic TNF-α and IL-6 gene expression in healthy and DSS mice treated with CD.TNF-α siRNA measured by RT-PCR. (A) TNF-α and (B) IL-6 mRNA expression in the proximal colon. (C) TNF-α and (D) IL-6 mRNA expression in the distal colon. Differences in gene expression of TNF-α/IL-6 compared to DSS + CD.N/S siRNA. Data represents the average ± SEM. Mice per group for DSS groups: CD.N/S N = 10, Naked TNF-α N = 8, CD.TNF-α N = 10, healthy groups: CD.N/S N = 4, untreated (UT) N = 7. *P < 0.05 compared to DSS + CD.N/S siRNA.