

# Functionally Enhanced siRNA Targeting TNF $\alpha$ Attenuates DSS-induced Colitis and TLR-mediated Immunostimulation in Mice

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Tumor necrosis factor (TNF $\alpha$ ) is a proinflammatory cytokine involved in the pathogenesis of inflammatory bowel disease (IBD). Although TNF $\alpha$  has been extensively targeted using systemic drugs, the use of antisense and small interfering RNA (siRNA) to drive down its expression at the site of inflammation should provide important advantages. In this study, native and chemically modified siRNA against TNF $\alpha$  was developed and characterized using a murine model of IBD. siRNA with 2'-O-methyl and propanediol modifications (siTNF-OMe-P) were resistant to nuclease degradation and provided better silencing efficacy *in vitro* as compared to unmodified siRNA. Every modification reduced nonspecific Toll-like receptor (TLR)-mediated immunomodulation in human peripheral blood mononuclear cells (PBMC) cells. Intrarectal administration of siTNF-OMe-P significantly ameliorated the clinical endpoints and histopathological severity in 5% dextran sulphate sodium (DSS)-treated mice as compared to unmodified and other chemically modified siRNAs. Differential gene expression assessed in siTNF-OMe-P-treated animals correlated with improved colon integrity and reduced TLR activation as compared to all treatment groups. All in all, this study demonstrates that propanediol and 2'-O-methyl modifications have profound functional consequences for siRNA efficacy *in vivo*. Consequently, this strategy has potential implications for therapeutic intervention in IBD and other diseases.

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## INTRODUCTION

The etiology and pathophysiological mechanisms of inflammatory bowel disease (IBD) are not completely understood. However,

the loss of tolerance to intestinal microbiota, the activation of immune cells and the production of proinflammatory cytokines such as TNF $\alpha$ , interleukin-1 $\beta$ , interleukin-6, and interleukin-8 are common features of IBD. It is widely accepted that TNF $\alpha$  plays a prominent role in this process by contributing to the recruitment of immunocompetent cells that amplify the inflammatory response in T cells, macrophages, and mucosal cells.<sup>1-5</sup> Indeed, TNF $\alpha$  levels are increased in both serum and mucosa of IBD patients.<sup>6-9</sup> Furthermore, numerous studies have also shown significantly increased TNF $\alpha$  levels in animal models of IBD (2,4,6-trinitrobenzenesulphonic acid and dextran sulphate sodium (DSS)-induced colitis).<sup>10,11</sup> TNF $\alpha$  also has deleterious effects on tight junctions, impairing barrier function, and enhancing the immune challenge by luminal antigens.<sup>12,13</sup> Mucosal cells may thus be considered putative targets of anti-inflammatory therapy in the early phases of intestinal inflammation.<sup>14</sup>

Blockade of TNF $\alpha$  has been shown to improve or prevent inflammation in both animal models<sup>11,15,16</sup> and humans.<sup>17</sup> Anti-TNF $\alpha$  antibodies, such as infliximab, and adalimumab, have proven to be efficacious against IBD in clinical trials. However, anti-TNF $\alpha$  therapies require parenteral administration at relatively high doses to achieve their therapeutic effect at specific diseased sites, thereby increasing the chance of adverse effects such as increased vulnerability of patients to intracellular pathogens,<sup>18</sup> lupoid reactions<sup>3</sup> or the generation of anti-infliximab antibodies.<sup>19</sup> A possible association of infliximab treatment with the development of lymphoma<sup>20</sup> has also been suggested. Therefore, strategies providing an organ-selective blockade or inhibition of TNF $\alpha$  effects should improve the safety of these biological agents.

Among alternative strategies to block TNF $\alpha$  locally, the use of antisense oligonucleotides and small interfering RNA (siRNA) has received some attention,<sup>15,21</sup> whereas antisense strategies addressing other molecular targets of inflammation are already undergoing clinical trials.<sup>22,23</sup> Although siRNA is considerably more interesting than antisense as a therapeutic approach, the

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inherent instability of the RNA moiety has hindered its clinical development.<sup>24</sup>

The present study describes the efficacy of chemically modified siRNA in modulating IBD symptoms mediated by TNF $\alpha$  after rectal administration of a siRNA–lipoplex complex. Propanediol and 2'-O-methyl-modified siRNA were stable and active *in vitro* and reduced nonspecific siRNA-mediated Toll-like receptor (TLR) immunomodulation in human peripheral blood mononuclear cells (PBMC) and the colon of diseased mice. These data have immediate implications for therapeutic intervention in IBD alone or in combination with novel oral delivery strategies.<sup>25</sup>

## RESULTS

### Negative modulation of TNF $\alpha$ gene expression by siRNA in primary cells

Several siRNA sequences have been extensively used to down-regulate TNF $\alpha$  expression. We used one such siRNA<sup>26</sup> to verify its efficiency *in vitro* against endogenous TNF $\alpha$  production by 4T1 mouse breast carcinoma cells. Our data show a substantial inhibition of TNF $\alpha$  mRNA and protein (data not shown) using unmodified siRNA, although this was unable to significantly reduce TNF $\alpha$  when transfected into mouse primary peritoneal macrophages (Figure 1a). Therefore, we studied the capacity of several chemically modified siRNAs (Table 1) to effectively silence TNF $\alpha$  in peritoneal macrophages. Specifically we have examined the biological properties of several modified siRNAs bearing 2'-O-methyl-RNA, locked nucleic acids (LNA), phosphorothioate (PS) linkages and propanediol modification at the 3'-end.<sup>27</sup> All these modifications have been introduced only in the sense or passenger strand. Figure 1 shows that the propanediol modification of the 3'-end and a double methylation of the 5'-end of the sense strand of siTNF was significantly more effective at silencing endogenous TNF $\alpha$  than were either unmodified siTNF or any other chemical modifications tested: LNA, PS, OMe

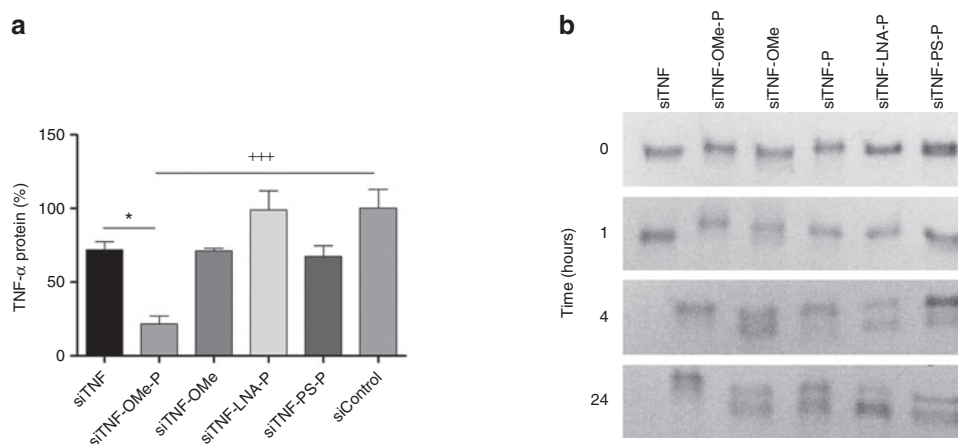
alone, or propanediol alone. Differential siRNA efficacy inside the cell is a balance between persistence and its ability to engage RNA-induced silencing complex. In order to study whether our most effective siRNA (siTNF-OMe-P) was resistant to degradation, we subjected all the siRNAs to a degradation assay in the presence of 50% fetal bovine serum (Figure 1b). All modifications improved upon the stability of unmodified siRNA against nuclease degradation as visualized by ethidium bromide staining. However, siTNF-OMe-P was significantly more resistant over time (at 24 hours, 52% of siRNA remained intact by densitometry analysis; data not shown) than all other modifications.

Therefore, we next evaluated the therapeutic efficacy of siTNF-OMe-P in different cell lines expressing endogenous and exogenous mouse TNF $\alpha$  and *in vivo* in an animal model of colitis induced by 5% DSS.

### siTNF administration and therapeutic efficacy in a DSS colitis model

siTNF-OMe-P was found to be very efficacious in both HeLa and 4T1 cells to inhibit the production of mouse TNF $\alpha$  as compared to both unmodified siTNF or siControl (Figure 2a,b). *In vivo*, experimental rectal administration of siRNA conjugated to lipofectamine 2000 was initiated in 5% DSS-treated mice (56 hours). Target gene silencing was measured in total RNA from colon extracts 16 hours postadministration by quantitative reverse transcriptase-PCR. In the experimental conditions chosen, sham control animals (siControl) showed several-fold increase in TNF $\alpha$  mRNA with respect to healthy mice upon exposure to 5% DSS (Figure 2c). By contrast, TNF $\alpha$  mRNA levels in siTNF-OMe-P-treated animals were not significantly different than healthy mice group.

We next sought to ascertain whether, in view of their improved performance *in vitro*, chemically modified siRNAs had therapeutic potential as compared to unmodified siRNA. To this end, we treated a group of mice on days 2 and 4 using various siRNAs.



**Figure 1** Antitumor necrosis factor (TNF $\alpha$ ) silencing efficiency in mouse peritoneal macrophages correlates with small interfering RNA (siRNA) stability. **(a)** Mouse peritoneal macrophages were transfected with siRNA (100nmol/l) using DOTAP. After 20 hours of transfection, cells were stimulated with lipopolysaccharide (LPS) (10 ng/ml) for 10 hours and protein levels were determined by enzyme-linked immunosorbent assay (ELISA) in cell supernatants. siTNF, unmodified siRNA against TNF- $\alpha$ ; siTNF-PS-P, siRNA anti-TNF- $\alpha$  modified with phosphorothioate and propanediol; siTNF-LNA-P, siRNA anti-TNF- $\alpha$  modified with LNA and propanediol; siTNF-OMe, siRNA anti-TNF- $\alpha$  modified with O-methyl; siTNF-OMe-P, siRNA anti-TNF $\alpha$  modified with 2'-O-methyl and propanediol; siControl, negative control siRNA modified with 2'-O-methyl. The data represent the mean  $\pm$  SEM,  $n = 3$ .  $+++P < 0.001$  versus siControl;  $*P < 0.05$  versus siTNF. ANOVA, Bonferroni *post-hoc* test. **(b)** siRNAs were incubated with 50% fetal bovine serum for the indicated amount of time (at 37°C). Thereafter, RNAs were separated using a nondenaturing, 20% polyacrylamide gel.

**Table 1** Oligonucleotide sequences of sense and antisense strands and their chemical modifications

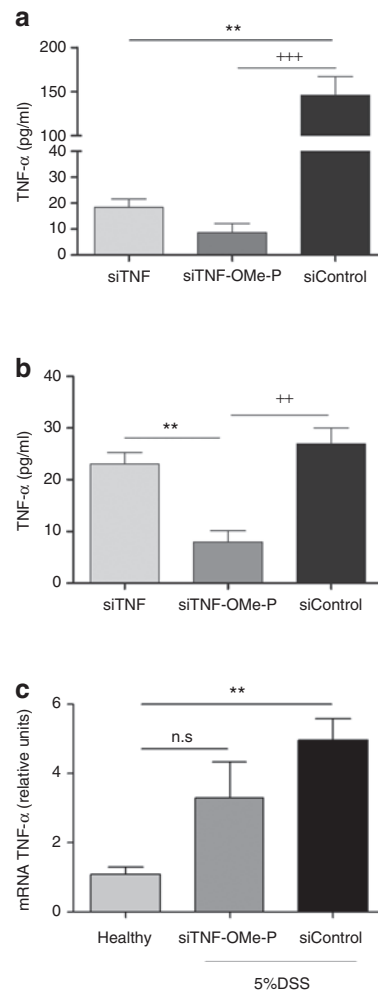
Modification	Sequence
siTNF (sense/antisense)	5'-GUGCCUAUGUCUCAGCCUC-dT-dT-3' 5'-GAGGCUGAGACAUAGGCAC-dT-dT-3'
siTNF-P (sense)	5'-GUGCCUAUGUCUCAGCCUC-dT-dT-(CH <sub>2</sub> ) <sub>3</sub> -OH-3'
siTNF-OMe-P (sense)	5'-guGCCUAUGUCUCAGCCUC-dT-dT-(CH <sub>2</sub> ) <sub>3</sub> -OH-3'
siTNF-PS-P (sense)	5'-GUGCCUAUGUCUCAGCCUC-dT*dT*(CH <sub>2</sub> ) <sub>3</sub> -OH-3'
siTNF-LNA-P (sense)	5'-GUGCCUAUGUCUCAGCCUC-T-T-(CH <sub>2</sub> ) <sub>3</sub> -OH-3'
siTNF-OMe (sense)	5'-guGCCUAUGUCUCAGCCUC-dT-dT-3'
siControl (sense/antisense)	5'-caGUCGCGUUUGCGACUGG-dT-dT-3' 5'-CCAGUCGCAAACGCGACUG-dT-dT-3'

Abbreviations: LNA, locked nucleic acid; OMe, 2'-O-methyl; PS, phosphorothioate; siTNF, unmodified siRNA against tumor necrosis factor.

Clinical endpoints of disease were evaluated 8 days after initiating the addition of 5% DSS. Animals on 5% DSS showed a weight loss over the 8-day period of analysis that was unaffected by the nature of the treatment (data not shown). However, siTNF-OMe-P treatment afforded a significantly ameliorated disease activity index (DAI; **Figure 3a**) and weight-to-length colon ratio (**Figure 3b**). Indeed, colons from mice treated with siTNF-OMe-P were less oedematous on necropsia, suggesting reduced inflammation confirmed by lower myeloperoxidase (MPO) activity (**Figure 3c**), which is a marker of neutrophil infiltration into the colon. Additionally, both the animal survival rate and the degree of hemorrhage in the caecum (**Supplementary Figure S3**) were clearly improved in animals treated with siTNF-OMe-P, demonstrating that the beneficial effects observed locally on colon inflammation correlated with significant amelioration of the physiological state of the animal. Therefore, siTNF-OMe-P treatment produces significant improvements in qualitative and quantitative clinical outcomes of colitis in treated animals, as compared to siTNF (unmodified siRNA against TNF- $\alpha$ ) and siControl-treated animals. Clinical improvement was mirrored at the histological level (**Figure 3d,e**). Both inflammatory infiltrates and crypt damage scores were ameliorated with siTNF-OMe-P treatment. Mice exposed to DSS in their drinking water exhibited crypt pathology, including crypt shortening, erosion of the epithelial layer, hyperplasia, and an inflammatory cell infiltrate in the lamina propria. These signs of disease only decreased in the colon of mice treated with siTNF-OMe-P.

### Gene array analysis in IBD mice treated with various siRNAs anti-TNF $\alpha$

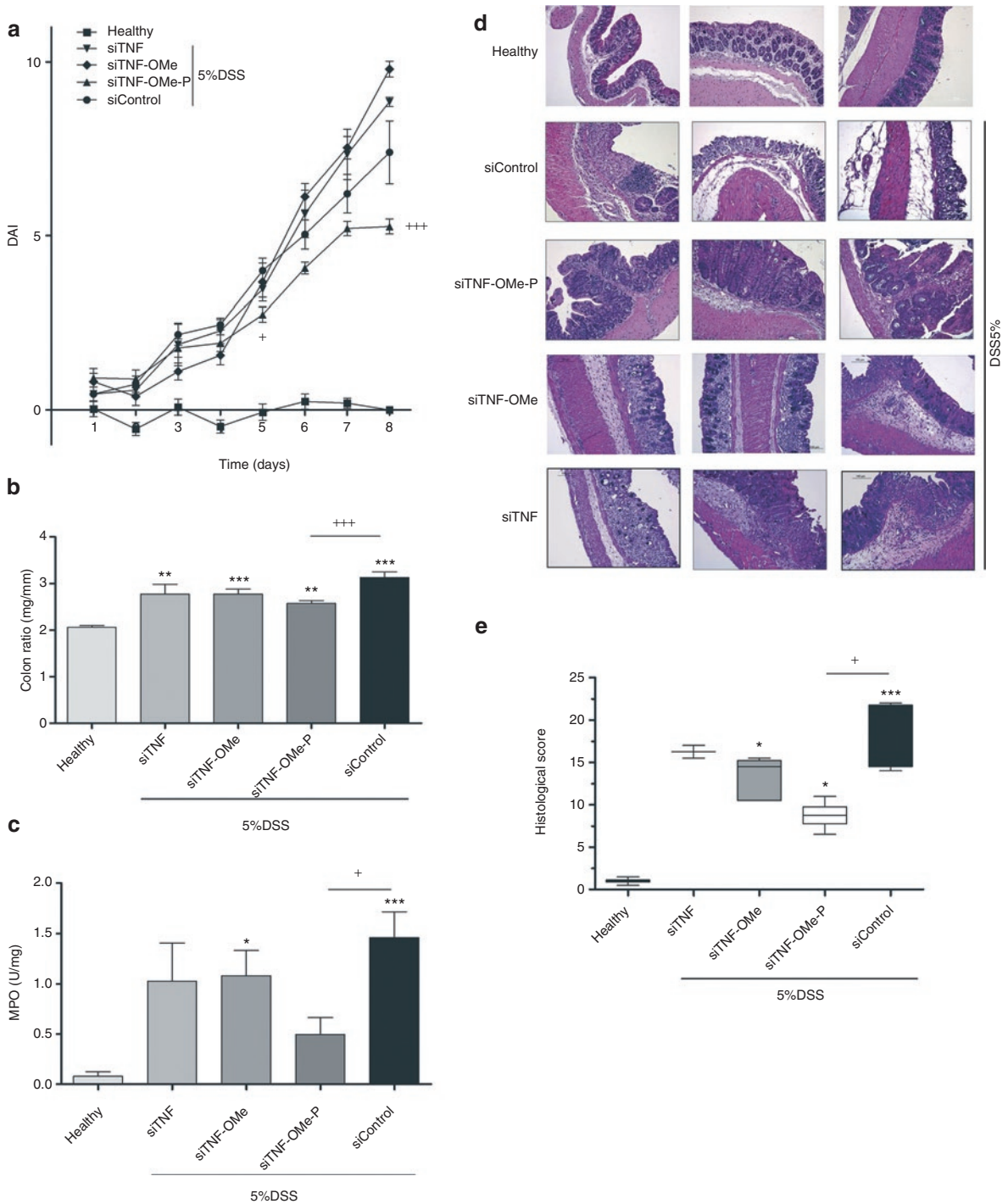
To gain insight into the molecular mechanisms underlying the therapeutic benefit provided by siTNF-OMe-P in this murine model of colitis, we analyzed the differential gene expression in colitic mice treated with various modified or unmodified TNF $\alpha$  specific siRNAs and siControl (eight mice/treatment were analyzed without pooling) and compared them to healthy animals, using a Mouse Gene ST 1.0 microarray (Affymetrix, Santa Clara, CA) consisting of 25,000 genes. This study



**Figure 2** *In vitro* and *in vivo* potency of chemically modified small interfering RNAs (siRNAs) against tumor necrosis factor (TNF $\alpha$ ). **(a)** Amount of TNF $\alpha$  produced after 24 hours of transfection of 50 nmol/l of siRNAs in HeLa cells expressing mouse TNF $\alpha$  from an exogenous expression cassette. siTNF, unmodified siRNA against TNF- $\alpha$ ; siTNF-OMe-P, siRNA anti-TNF- $\alpha$  modified with 2'-O-methyl and propanediol; siControl, negative control siRNA modified with 2'-O-methyl. The data represent the mean  $\pm$  SEM,  $n = 3$ . +++ $P < 0.001$  versus siControl; \*\* $P < 0.01$  versus siTNF. ANOVA, Bonferroni *post-hoc* test. **(b)** Amount of TNF $\alpha$  produced after 24 hours of transfection of 100 nmol/l of siRNAs in 4T1 cells. The data represent the mean  $\pm$  SEM,  $n = 3$ . ++ $P < 0.01$  versus siControl; \*\* $P < 0.01$  versus siTNF. ANOVA, Bonferroni *post-hoc* test. **(c)** TNF $\alpha$  mRNA levels in the colon of an animal model of chronic colitis 56 hours after rectal infusion of 4 nmol of siTNF-OMe-P ( $n = 4$ ) or siControl ( $n = 4$ ) conjugated with lipofectamine 2000. TNF $\alpha$  mRNA was measured by quantitative reverse transcriptase (qRT)-PCR and normalized with *Gapdh*. The data represent the mean  $\pm$  SEM with respect to healthy mice ( $n = 9$ ). Statistical analysis was performed using a non-parametric ANOVA with Kruskal-Wallis *post-hoc* test. \*\* $P < 0.01$  versus healthy mice.

identified circa 4,000 genes that were modulated significantly by the 5% DSS treatment. These genes were tagged as "pathology-related" genes. "siRNA-specific" genes were identified for each treatment group as those that were differentially expressed in each treatment group.

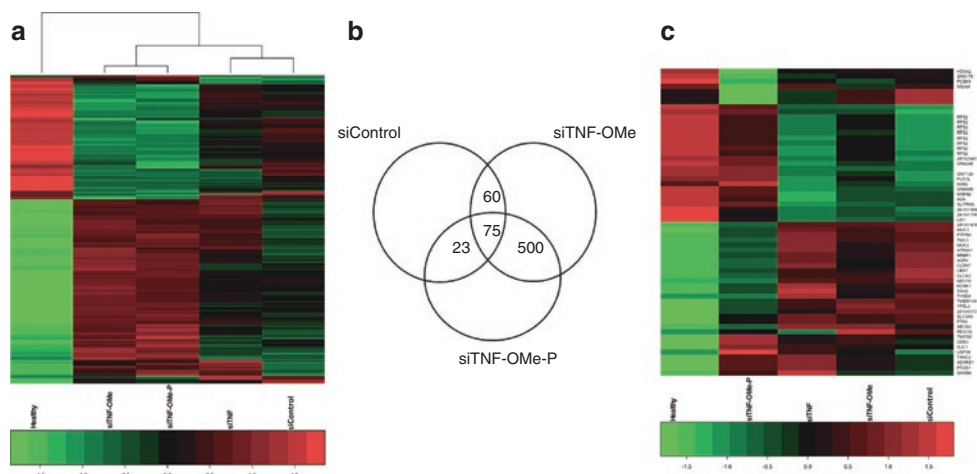
A heatmap was built using all "pathology-related" genes that allowed for a statistical similarity test per group (**Figure 4a**). This study showed that siTNF (unmodified siRNA against TNF $\alpha$ )



**Figure 3** Clinical parameters of colitis in the animals treated with small interfering RNA (siRNA) antitumor necrosis factor (TNF $\alpha$ ). Fifty C57BL/6 mice were administered 5% dextran sulphate sodium (DSS) in drinking water and subsequently treated on days 2 and 4 with 4 nmol of various siRNAs. Some animals died before the end of the study and were not included in the analysis. Animals that survived up to 8 days (healthy controls,  $n = 6$ ; siControl,  $n = 7$ ; siTNF,  $n = 3$ ; siTNF-OMe,  $n = 5$ ; and siTNF-OMe-P = 8), were killed to sample the distal colon and perform a histopathological evaluation. **(a)** disease activity index (DAI) was evaluated from unbiased determination of rectal bleeding, feces consistency, and overall appearance. **(b)**. Weight-over-length ratio of the colon. **(c)** Myeloperoxidase activity in colon extracts. **(d)** H&E stained specimens ( $\times 200$ ). **(e)** Histological score was assessed by the Dieleman method. Error bars represent the SEM of the mean. Statistical analysis was performed by ANOVA with Bonferroni *post-hoc* test. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  or \* $P < 0.05$  versus healthy mice, and +++ $P < 0.001$ , + $P < 0.05$  versus siControl.

and siControl are related in terms of their gene modulation pattern, whereas siTNF-OMe-P is closer to siTNF-OMe. Pattern-similarity assignment correlated well with the therapeutic potential observed through objective clinical parameters (Figure 3).

Differentially expressed genes in a given treatment subgroup were analyzed according to the Venn diagram shown in Figure 4b. Note that most of the genes that were significantly altered (500) exhibited similar regulation in siTNF-OMe and



**Figure 4** Differential gene expression in a 5% dextran sulphate sodium (DSS) murine colitis model after specific small interfering RNA (siRNA) treatments. **(a)** Heat map of differentially expressed genes after treatment with various siRNAs. Treatments are grouped by similarity based on their effects on gene expression. Horizontal strips represent genes and columns show treatment protocols. The absolute-fold changes of gene ratio are color coded as shown in the bar below. **(b)** Venn diagram of significantly upregulated or downregulated genes upon treatment. Intersections indicate the number of differentially expressed genes in response to specific treatments. **(c)** Clustering analysis of genes significantly altered by treatment with siTNF-OMe-P (total 60 genes). This study was conducted at the Institute of Biomedical Research of Barcelona, located in the Barcelona Science Park (PCB).

siTNF-OMe-P and were differently regulated in siControl and siTNF. Only 60 genes were exclusively affected by siTNF-OMe-P treatment (**Supplementary Table S1**). A heatmap of these genes showed strong pattern similarity with healthy controls for most genes (**Figure 4c**). Among them, several genes implicated in tissue repair, such as *cldn-7* (claudin 7) and *ssh-2*, were upregulated in the siTNF-OMe-P group as compared to controls, whereas other genes present in a healthy colon epithelium (*mucin1* and 2 and *aqp4*) were found to closely match the levels found in healthy controls. When siTNF-OMe-P treatment group was stringently compared pair wise against siControl or siTNF (**Supplementary Table S1**), several keratin genes, including keratin 4, were found to be upregulated (more than twofold induction and probability of differentially expressed higher than 0.95). *Crabp2*, upregulated by agents that induce keratinocyte differentiation such as retinoic acid was similarly upregulated, together with *Ga733* (*Tacstd2*; murine epithelial glycoprotein), a protein involved in cell-adhesion, all in all suggesting that siTNF-OMe-P treatment helps re-establish a healthy colon epithelium.

### Pathways differentially enriched in colons treated with siTNF-OMe-P

In order to gain insight into the molecular mechanism mediating the therapeutic effects evidenced for siTNF-OMe-P, we performed a gene ontology enrichment analysis comparing siTNF-OMe-P and siControl (**Table 2**). Several pathology-related pathways (apoptosis, innate immune response, cytokine production, leukocyte adhesion, lymph node development, etc.) were significantly enriched in siControl treatment but not in siTNF-OMe-P. By contrast, several pathways related to tissue repair and regeneration (cell junction, tight junction, morphogenesis of epithelium, regulation of cell differentiation, etc.), and to the normal physiology of the colon epithelium (keratin filaments, water channel activity, sodium transport, amino acid transport, etc.), were significantly enriched in siTNF-OMe-P but not in siControl.

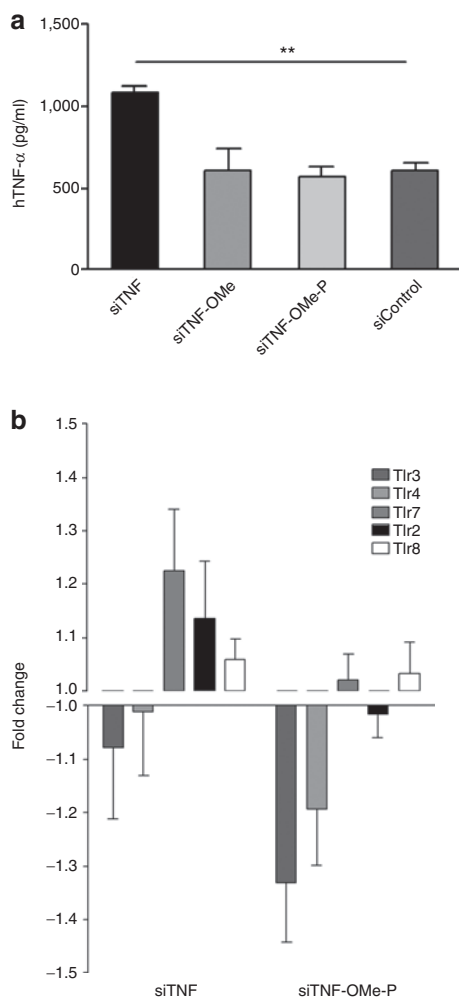
### Chemical modifications result in reduced innate immune system activation

SiRNA and other RNA and DNA oligonucleotides stimulate innate immune response through the activation of TLR7/8 and 3.<sup>28</sup> Gene ontology data indicated that innate immune response pathway was enriched in siControl but not siTNF-OMe-P. Therefore, we aimed to ascertain TLR activation capacity for all siRNAs, using a TLR7/8 response assay in PBMC. It is important to note that murine anti-TNF $\alpha$  siRNAs do not inhibit the expression of h-TNF $\alpha$ , a marker of TLR activation in this assay. As shown in **Figure 5a**, all chemically modified siRNAs exhibited reduced capacity to induce h-TNF $\alpha$  release by PBMC compared to unmodified siRNA (siTNF). We then examined TLR gene activation *in vivo* by comparing the differential expression of several *TLR* genes present in the gene array shown previously. TLR genes 2, 3, 4, 7, and 9 were significantly downregulated (two-way ANOVA) after siTNF-OMe-P treatment (**Figure 5b**) when compared to siTNF-treated mice, whereas siTNF-OMe was ineffective (data not shown).

### DISCUSSION

TNF $\alpha$  plays a major role in inflammatory responses and has therefore been identified as a major therapeutic target in different chronic inflammatory diseases, including IBD and arthritis. The importance of TNF $\alpha$  has also been demonstrated in a variety of animal models<sup>11,29</sup> using wild-type and TNF $\alpha$  knockout mice, as well as in DSS-induced colitis.<sup>15</sup> Consequently, neutralization of TNF $\alpha$  by antibodies, soluble receptors or antisense oligonucleotides has shown significant success in attenuating experimental colitis.<sup>3,15,18–21</sup> Zhang *et al.*<sup>30</sup> reported efficient silencing of TNF $\alpha$  mRNA after rectal delivery of unmodified siRNA in 5% DSS-treated mice, although no clinical improvement was observed.

In the present study we used the same TNF $\alpha$ -specific siRNA sequence as that described by Zhang *et al.*,<sup>30</sup> but added several chemical modifications to the molecule. Specifically, chemical modifications (LNA, 2'-O-methyl (OMe), and PS) known to



**Figure 5** Modulation of Toll-like receptor (TLR)-response mediated by small interfering RNAs (siRNAs). Differential absolute gene expression for several TLR genes was identified using an Affymetrix Mouse Gene ST 1.0 microarray. **(a)** Peripheral blood mononuclear cells (PBMC) were treated with various siRNAs /DOTAP (10 nmol/l) for 18 hours. Stimulation of innate immune response by siRNAs was monitored by measuring the levels of h-tumor necrosis factor (TNF $\alpha$ ) produced by PBMC. The data represent the mean  $\pm$  SEM,  $n = 3$ ; \* $P < 0.05$  and \*\* $P < 0.01$  versus siTNF. Statistical analysis was performed by ANOVA with Bonferroni *post-hoc* test. **(b)** Changes in gene expression induced by treatment with siTNF and siTNF-OMe-P. Statistical analysis was performed by two-way ANOVA. The data represent the mean  $\pm$  SEM,  $n = 8$ .

improve the stability and potency of siRNA were introduced into the same nucleotide sequence and were compared with propanediol modification at the 3'-end of the siRNA, which, according to previous results, exhibited an improved chemical stability in the context of antisense delivery.<sup>31</sup> Several studies have shown improved serum stability of single-stranded and double-stranded nucleic acids by blocking 3'-exonuclease activity, the most important nuclease activity in serum, with modifications at the 3'-end.<sup>32</sup> In this study, the silencing capacity of unmodified or chemically modified siTNFs was first tested in mouse peritoneal macrophages. This study showed that among the chemical modifications introduced into the siTNFs studied, propanediol modification at the 3'-end showed the greatest silencing capacity (Figure 1a). Similarly, siTNF-OMe-P was strikingly effective in the context

**Table 2** Gene ontology pathways enriched upon treatment with siControl or siTNF-OMe-PGO pathways enriched

siControl	siTNF-OMe-P
Apoptosis	3',5'-Cyclic-nucleotide phosphodiesterase activity
Basal lamina	Amino acid transmembrane transporter activity
Cell adhesion	Cell junction
Cell migration	Chemokine activity
Complement activation	Cytokine activity
Cytokine production	Intermediate filament
DNA repair	Keratin filament
Innate immune response	Ligand-dependent nuclear receptor activity
Leukocyte adhesion	Morphogenesis of an epithelium
Lymph node development	Negative regulation of angiogenesis
MAPKKK cascade	Regulation of cell differentiation
Positive regulation of interleukin-6 production	Sodium ion binding
Positive regulation of tumor necrosis factor production	Sodium ion transport
Regulation of apoptosis	Tight junction
Vascular endothelial growth factor	Water channel activity
Vasculogenesis	Amino acid transmembrane transporter activity

*Abbreviations:* OMe, 2'-O-methyl; siTNF, unmodified siRNA against tumor necrosis factor. Only pathways that are enriched in siControl but not in siTNF-OMe-P, or vice-versa, are shown.

of difficult to transfect cells such as 4T1 mammary carcinoma cells, or HeLa cells cotransfected with an expression plasmid for mTNF $\alpha$  (Figure 2a,b). The difference in efficacy could be partially accounted for by increased serum stability of siTNF-OMe-P (Figure 1b). This assay preferentially shows double-stranded RNA since staining is performed with ethidium bromide. All siRNAs, except for siTNF-OMe-P, showed the late appearance of a second, faster mobility band that could represent degradation products. Propanediol could stabilize siTNF-OMe-P molecule by presumably blocking 3'-exonuclease activity. However, it is not clear whether other biological variables including off-target effects, stimulation of cellular protective mechanisms, or RNA-induced silencing complex activation may be also justify successful siRNA-mediated silencing when utilizing OMe-P modification.<sup>28</sup>

We consistently found that when 5% DSS colitic mice were treated with the siRNAs described in this study, only siTNF-OMe-P was able to reduce TNF $\alpha$  mRNA and improve upon clinical endpoints such as the DAI, colon weight/length ratio, MPO activity, and histopathology, as compared to unmodified siTNF, siTNF-OMe, or siControl. Interestingly, unmodified siTNF worsened animal survival and caecal inflammation under visual inspection (Supplementary Figure S3b) as compared to siControl. These observations are not consistent with results published by Zhang *et al.*<sup>30</sup> using the same protocol with unmodified siRNA against TNF $\alpha$ . Authors showed an important reduction of murine TNF $\alpha$  mRNA in the distal colon of treated mice, whereas in our

study TNF $\alpha$  mRNA was not reduced as much. In several studies involving acute DSS colitis,<sup>33,34</sup> complete neutralization by mAb against TNF $\alpha$  or its absence<sup>35,36</sup> failed to block or even exacerbated disease parameters. This has prompted some authors<sup>15</sup> to suggest that disease protection in the acute form of DSS colitis may be dependent on the degree of TNF $\alpha$  inhibition attained. A small amount of TNF $\alpha$  may actually be protective in the early stages of disease development. Antisense siRNA (or low dose mAb treatment) may not totally block all TNF $\alpha$  production and, hence, might improve treatment efficacy in the acute model.<sup>15</sup>

The proinflammatory action of TNF $\alpha$  is critical for disease initiation, whereas its anti-inflammatory activity helps to resolve the disease. This dual role for TNF $\alpha$  has also been demonstrated in the pathogenesis of experimental autoimmune encephalomyelitis,<sup>37</sup> thereby indicating that it may be beneficial to maintain certain levels of TNF $\alpha$ .

Given the lack of information regarding the mechanism of action by which siRNAs against TNF $\alpha$  might contribute to improving colitis, we decided to perform a whole-genome differential expression analysis with colitic mice treated with the siRNAs featured in our study. Our major interest was to gain insight into the genes involved in the therapeutic effects observed upon local siTNF-OMe-P delivery. Only 60 genes were exclusively modulated by siTNF-OMe-P treatment. Most of them were unchanged as compared to healthy controls, and hence were found in normal colon epithelium. More interesting information was obtained by analyzing the enrichment of gene ontology pathways in either the siTNF-OMe-P or siControl group versus healthy controls. Upregulated pathways present in siControl are also described in other studies of differential gene expression induced by DSS,<sup>1,38</sup> and include cytokine production, innate immune response, lymph node development, and angiogenesis. None of those pathways were enriched by siTNF-OMe-P treatment. Instead, several pathways related to tissue repair, normal colon function and chemokine modulation were observed, suggesting that colon inflammation is inhibited and tissue repair is activated. Similar results have been found using antisense oligonucleotides targeting CD40 in a 2,4,6-trinitrobenzenesulphonic acid inflammatory model.<sup>39</sup> Interestingly, antisense complexes were also introduced using a lipoplex enema, suggesting that directly access to the colon epithelia is an interesting strategy for the treatment of colitis.

The importance of TLR activation in assessing the performance of siRNA drugs has only been recognized in the last couple of years.<sup>28</sup> TLR3 has been shown to mediate the protective effects of systemic poly (I:C) in DSS-induced acute colitis.<sup>40</sup> Interestingly, unmodified siTNF direct stimulation of TLR3 in the colon was not protective in our system, probably because of the concomitant stimulation of several proinflammatory cytokines and TLRs by the unmodified siRNA. siTNF also stimulated the expression of TLR7/8, 2, 4, and 13, whereas siRNA chemically modified with OMe-P were effective in partially suppressing these genes. These data are in agreement with results obtained by transfecting PBMC, a good model to test TLR7/8 activation,<sup>41</sup> with all siRNAs. Moreover, gene array data indicated that the double modification 2'OMe and propanediol conferred improved TLR activation with respect to 2'OMe alone. It has been shown that

incorporation of 2'OMe modifications can dramatically increase nuclease resistance in serum-rich environments as compared to unmodified double-stranded RNA.<sup>42</sup> Additionally, and depending on the position, as few as two 2'OMe modifications can prevent immune stimulation mediated through TLR-7 pathways.<sup>43</sup> The mechanism by which 2'OMe modification reduces immune stimulation is not well established. It has been suggested that human TLRs might be preferentially activated by pathogen-associated RNA that contains fewer modified nucleosides than does host RNA.<sup>44</sup> Addition of 2'OMe modifications to positions 1 and 2 of the passenger strand, as in the present study, inhibited the incorporation of this strand into the Ago2:RNA-induced silencing complex, thereby reducing off-target effects by blocking the interaction with Ago2.<sup>45</sup> The synergy between 2'OMe and propanediol modifications with respect to TLR inhibition has not been previously reported. Regardless of the mechanism of action for propanediol, our results *in vivo* were consistent with data obtained *in vitro*, clearly showing that siTNF-OMe-P was the only treatment resulting in improved objective clinical endpoints in 5% DSS colitic mice. In addition, the propanediol and 2'-O-methyl modifications are easy and unexpensive to be incorporated in RNA, yet a large benefit can be obtained with the use of these modifications. For these reasons, we believe the findings described in this study are of potential interest in the development of therapeutic applications of siRNAs.

In conclusion, this study has demonstrated that siTNF-OMe-P treatment in murine DSS colitis resulted in the reduction of the DAI, the colon weight/length ratio, the extent of neutrophil infiltration and the microscopic evidence of inflammation, findings which are in agreement with its silencing ability demonstrated both *in vitro* and *in vivo*. Furthermore, we describe the successful application of chemically modified siRNA against TNF $\alpha$  in DSS-induced colitis and the differential gene expression associated with this treatment. These results will help to develop siRNA therapeutics for the treatment of colon inflammation.

## MATERIALS AND METHODS

**Oligoribonucleotide synthesis.** Oligoribonucleotides were prepared using solid phase methodology. The syntheses were carried out with an Applied Biosystems (Foster City, CA) (Model 3400) DNA synthesizer using a 1- $\mu$ mol scale. The sense anti-TNF $\alpha$  sequences shown in **Table 1** siTNF-P (sense): 5'-GUG CCU AUG UCU CAG CCU C-dT-dT-(CH<sub>2</sub>)<sub>3</sub>-OH-3'; siTNF-OMe-P (sense): 5'-guG CCU AUG UCU CAG CCU C-dT-dT-(CH<sub>2</sub>)<sub>3</sub>-OH-3'; siTNF-PS-P (sense): 5'-GUG CCU AUG UCU CAG CCU C-dT\*dT\*(CH<sub>2</sub>)<sub>3</sub>-OH-3'; siTNF-LNA-P (sense): 5'-GUG CCU AUG UCU CAG CCU C-T-T-(CH<sub>2</sub>)<sub>3</sub>-OH-3' were assembled on a controlled pore glass support functionalized with dimethoxytrityl-propanediol<sup>46</sup> in order to obtain oligoribonucleotides carrying propanediol at the 3'-end. dT stands for thymidine. Lower case bold letters (**g**, **u**) refer to 2'-O-methyl-RNA units. An asterisk (\*) indicates the presence of PS linkages in the polymer backbone, while the **T** represents a thymidine where the sugar is modified with a LNA nucleoside. Detailed synthesis and quality control of oligonucleotides are provided as **Supplementary Materials and Methods** and **Supplementary Figure S1**. The sequences obtained from commercial sources were: sense nontargeting negative control (sense sequence 5'-caG UCG CGU UUG CGA CUG G-dT-dT-3'), antisense nontargeting negative control (antisense sequence 5'-CCA GUC GCA AAC GCG ACU G-dT-dT-3'), antisense or guide anti-TNF $\alpha$ : 5'-GAG GCU GAG ACA UAG GCA C-dT-dT-3'.

**Preparation of siRNA molecules.** Modified and unmodified sense strands were dissolved in Tris buffer (50 mmol/l NaCl, 10 mmol/l Tris, pH 8.0) and annealed with equimolar amounts of the corresponding unmodified anti-sense strand dissolved in the same buffer. The resulting solution (0.1 ml) was heated at 90°C and allowed to cool slowly within 2 hours. Then 10 µl of 3 mol/l sodium acetate were added and the resulting siRNAs were precipitated by addition of 0.275 ml of ethanol. The sample is kept in the freezer overnight and centrifuged (15 minutes, 13,400g, 4°C). The pellet is dissolved in the appropriate buffer and used in the silencing experiments.

The anti-TNFα siRNA has previously been shown to efficiently downregulate murine TNFα mRNA.<sup>26</sup> To save resources, we only present data from O-methyl modified scrambled siRNA as siRNA control (siControl) because it is representative of results obtained with unmodified, O-methyl, and O-methyl-propanediol scrambled siRNA *in vitro* and *in vivo* (Supplementary Figure S2).

**siRNA degradation assay and RNA gel electrophoresis.** One microgram duplex of each siRNA was incubated with 50% fetal bovine serum at 37°C. Samples were incubated with 1% SDS prior to be analyzed after 0 minute, 1 hour, 4 hours, and 24 hours of serum incubation, by nondenaturing polyacrylamide gel electrophoresis. Double-stranded RNA was visualized using an Ethidium Bromide bath for 30 minutes, in order to determine the percentage of intact siRNA.

**Cell culture, transfection, and cellular assays.** HeLa cells were cultured under standard conditions. HeLa cells were transfected with 250 ng of the plasmid expressing murine TNFα plasmid using lipofectin (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. One hour after transfection, TNFα expressing HeLa cells were transfected with 50 nmol/l of each siRNA, using oligofectamine (Invitrogen). The TNFα concentration was determined from cell culture supernatant by enzyme-linked immunosorbent assay kit (BenderMedSystems, Vienna, Austria) following the manufacturer's instructions.

Murine 4T1 cells were cultured under standard conditions. One hundred nmol/l of each siRNA duplex was incubated with lipofectamine 2000 before being added to the 4T1 cells. After 24 hours, the amount of TNFα produced by the cells was analyzed by enzyme-linked immunosorbent assay.

For peritoneal macrophage collection, healthy mice were sacrificed and 10-ml harvest medium was injected into their peritoneum. Fluid was collected after 10 minutes. The amount of harvested cells was adjusted (10<sup>4</sup> cells/well) and transfected with 100 nmol/l of siRNA using DOTAP (Roche, Mannheim, Germany), following the manufacturer's instructions. After 20 hours of transfection, cells were stimulated with lipopolysaccharide (10 ng/ml) for 10 hours and the TNFα released in the supernatant was measured by enzyme-linked immunosorbent assay.

PBMC were obtained from human blood using Ficoll density gradient separation. After 3 hours incubation at 37°C, adherent cells were transfected with 10 nmol/l of siRNAs for 18 hours using DOTAP (Roche), following the manufacturer's instructions. Culture supernatants were then collected and human TNFα production upon immunostimulation was assessed by enzyme-linked immunosorbent assay (Bender MedSystems).

**DSS-induced colitis model in C57BL/6 mice.** Experiments were performed on female C57BL/6 mice (Harlan Iberica, Barcelona, Spain). Animals were housed under standard conditions at 22°C and 70–80% relative humidity, in a 12 hours light/dark cycle. All animal procedures conformed to EU regulations and were approved by the local ethical committee.

Colitis was induced by addition of 5% DSS (MP Biomedicals, Santa Ana, CA; PM 36–50kDa) to drinking water. Nine healthy animals (no DSS in drinking water) were used as noncolitic controls.

Food and drink were provided *ad libitum* and intake was monitored daily throughout the study.

**In vivo administration of siRNAs.** To create lipoplex siRNA preparations, 2 µl lipofectamine 2000 (Invitrogen) was mixed with 48 µl

OptiMEM and incubated for 5 minutes, according to the manufacturer's instructions. Twenty nanomolar siRNA was suspended in 50 µl OptiMEM were then added to this mixture and incubated at room temperature for 20 minutes. This solution was immediately administered to rectums of anesthetized mice (4 nmol/mouse). All siRNA preparations were administered twice, on days 2 and 4. Each rectal administration consisted of a 20 µl solution of liposomal siRNA and was delivering using a P20 pipettor.

**Evaluation of the DAI.** Throughout the experiment, animals were examined daily for the following variables: general state and appearance, weight loss, stool consistency, and food and drink intake. DAI scores were defined as follows: for weight: 0, no loss; 1, up to 5%; 2, 5%–10%; 3, 10%–15%; and 4, >15% weight loss; for stool: 0, normal; 1, soft stool; 2, semiliquid; 3, diarrhea but dry tail; and 4, diarrhea with wet tail; and for bleeding: 0, no blood; 1, presence in stool; 2, presence in anus; and 3, gross blood. The DAI parameter correlates well with the histopathological evaluation of inflammation and lesions in intestinal crypts.<sup>47</sup>

**Colonic MPO activity.** Neutrophil infiltration into tissue colon was quantified by measuring MPO activity as previously described<sup>48</sup> for use in a 96-well plate. The change in optical density at 450 nm was measured at 3-minutes intervals.

One unit of MPO activity was defined as the amount that degraded 1.0 µmol of peroxide per minute at 25°C. The results were expressed as U/mg wet tissue.

**Histology and histological score.** After flushing with cold phosphate-buffered saline, a whole-length longitudinal strip was fixed with paraformaldehyde 4%. Haematoxylin/eosin-stained sections were examined blindly and scored according to widely used criteria.<sup>49</sup>

**Statistical analysis.** All the data obtained were plotted and statistically analyzed using the software package GraphPad Prism version 5.0 for Windows. All treatment groups were compared using a one-way ANOVA and Bonferroni *post-hoc* test (\**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001). Only significant differences among the groups are indicated in the charts.

**Gene expression profiling.** Sixteen hours after the second administration of siRNAs, the colon of treated and untreated animals was obtained for RNA isolation. Twenty-five nanogram total RNA was used for whole transcriptome amplification (Sigma, St Louis, MO) according to manufacturer's recommendations. Thirty milligram distal colon (0.8 cm from the rectum) from each of eight mice per group was used for RNA extraction using RNeasy minikit (Qiagen, Dusseldorf, Germany). Distal colon was quickly flash with phosphate-buffered saline before collection onto RNAlater before RNA extraction. Ten microgram complementary DNA were obtained after retrotranscription of mRNA, and subsequently fragmented, biotinylated, and hybridized (unpooled) to Mouse Gene ST 1.0 microarrays (Affymetrix), as described previously.<sup>50</sup>

**Microarray data analysis.** Significance analysis of microarrays and gene ontology functional classification were carried out as described in Supplementary Materials and Methods. All datasets from the array have been submitted to GEO (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31906>).

## SUPPLEMENTARY MATERIAL

**Figure S1.** Quality control of representative oligonucleotides and siRNAs.

**Figure S2.** Silencing efficiency for various chemically modified siControl.

**Figure S3.** Phenotypic changes in a DSS murine colitis model after specific siRNAs administration.

**Table S1.** Differentially expressed genes.

**Materials and Methods.**



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