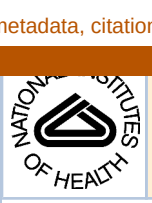

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An Endogenous TNF-α Antagonist Induced by Splice-switching Oligonucleotides Reduces Inflammation in Hepatitis and Arthritis Mouse Models

Maria A Graziewicz[1], **Teresa K Tarrant**[2], **Brian Buckley**[1], **Jennifer Roberts**[3,4], **LeShara Fulton**[3,4], **Henrik Hansen**[5], **Henrik Ørum**[5], **Ryszard Kole**[1,3,4], and **Peter Sazani**[1]

1*Ercole Biotech, Inc., Research Triangle Park, North Carolina, USA*

2*Division of Rheumatology, Allergy and Immunology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

3*Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

4*Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

5*Santaris Pharma, Hørsholm, Denmark*

Abstract

Tumor necrosis factor-α (TNF-α) is a key mediator of inflammatory diseases, including rheumatoid arthritis (RA), and anti–TNF-α drugs such as etanercept are effective treatments. Splice-switching oligonucleotides (SSOs) are a new class of drugs designed to induce therapeutically favorable splice variants of targeted genes. In this work, we used locked nucleic acid (LNA)–based SSOs to modulate splicing of TNF receptor 2 (TNFR2) pre-mRNA. The SSO induced skipping of TNFR2 exon 7, which codes the transmembrane domain (TM), switching endogenous expression from the membrane-bound, functional form to a soluble, secreted form (Δ7TNFR2). This decoy receptor protein accumulated in the circulation of treated mice, antagonized TNF-α, and altered disease in two mouse models: TNF-α-induced hepatitis and collagen-induced arthritis (CIA). This is the first report of upregulation of the endogenous, circulating TNF-α antagonist by oligonucleotide-induced splicing modulation.

Introduction

Tumor necrosis factor-α (TNF-α) is a key cytokine in rheumatoid arthritis (RA) and other inflammatory diseases.[1] Currently available anti–TNF-α biological drugs such as etanercept, a dimerized TNF receptor 2 (TNFR2) receptor:Fc fusion protein, and anti–TNF-α monoclonal antibodies, infliximab and adalimumab, bind TNF-α and block its inflammatory effects.[2] TNF-α activity is mediated through two membrane-bound receptors, TNFR1 and TNFR2.[1] In response to TNF-α exposure, both are downregulated by metalloprotease-induced shedding of the extracellular domains (ECDs), which bind TNF-α very poorly. Downregulation of TNFR2 is also achieved by alternative splicing to the soluble Δ7/8TNFR2 form, which is upregulated in the inflammatory state,[3] and lacks exons 7 and 8; exon 7 encodes the transmembrane domain

Correspondence: Peter Sazani, Ercole Biotech, Inc., PO Box 12295, Research Triangle Park, Durham, North Carolina 27709, USA. E-mail: E-mail: sazani@ercolebiotech.com.

(TM). The modest upregulation of Δ7/8TNFR2 in patients is evidently not sufficient to abrogate the inflammatory effects of TNF-α in RA.

The induction of exon skipping or other alternative splicing pathways by splice-switching oligonucleotides (SSOs) is a technology in which splice variants may be produced as potential therapeutics.[4–7] Unlike antisense downregulation of gene expression via RNase H or RNA interference degradation pathways, SSOs modulate alternative splicing of targeted pre-mRNA, upregulating expression of desirable protein isoforms, while simultaneously downregulating undesirable isoforms. Modified oligonucleotide backbones in SSOs prevent pre-mRNA degradation by RNase H, impart high specificity toward their target sequences, and increase resistance to nucleases in intracellular and serum environments.

The effectiveness of SSOs has been demonstrated in cell culture against several target genes.[8–16] Systemic SSO delivery *in vivo*, initially in a transgenic mouse model of alternative/aberrant splicing,[17] or against endogenous MyD88 and Duchenne muscular dystrophy target pre-mRNAs, demonstrated the functional and therapeutic consequences of splicing modulation.[18–20] Most recently, in a clinical trial, intramuscularly delivered SSOs restored dystrophin expression in Duchenne muscular dystrophy patients.[21] This rapidly expanding field was recently reviewed in detail.[4]

In vivo, depending on their chemical composition, there are significant differences in the functional tissue distribution and potency of SSOs.[17] Recent studies showed that locked nucleic acids (LNAs)/phosphorothioate backbone SSOs had extremely high potency in the liver.[22] The high activity of LNA SSOs in the liver, the fact that TNFR2 is expressed in the liver, and that exon 7 alone codes for the TM, led us to hypothesize that LNA SSOs targeted to exon 7 of TNFR2 pre-mRNA would shift splicing in the nucleus of hepatocytes, upregulating the Δ7TNFR2 protein, which would be secreted from the liver and accumulate in the circulation of treated animals (Figure 1), while simultaneously downregulating functional, membrane-bound liver TNFR2. Thus, the SSOs would instruct the liver to become a “factory” for the production of the soluble TNF-α antagonist, Δ7TNFR2 protein. Here we show that the “liver as a factory” approach yields, in a dose- and sequence-specific manner, biologically significant levels of Δ7TNFR2 and anti–TNF-α activity in the blood of SSO-treated mice, which reduced TNF-α-induced hepatitis[23] and collagen-induced arthritis (CIA).[24]

Results

Induction and anti–TNF-α activity of SSO-induced soluble Δ7TNFR2 protein

To induce murine Δ7TNFR2 protein, a series of LNA SSOs targeted to exon 7 sequences was designed and screened for splicing modulation in mouse L929 cells (Figure 2a). The observed positional effects presumably reflect the relative ability of the SSO to block the interaction of the splicing machinery with sequence elements (*e.g.*, splice sites or exonic splicing enhancer).[4] From this screen of just 12 SSO, LNA SSO3274, which specifically and potently shifted TNFR2 splicing to the Δ7TNFR2 isoform, was identified and used for further investigation *in vivo*. SSO3305, which also yielded significant splice switching, was not chosen because it interfered with reverse transcription–PCR of the full-length TNFR2 messenger RNA (mRNA) and because it induced lower Δ7TNFR2 protein than SSO3274 (Supplementary Figures S1 and S2).

The Δ7TNFR2 protein is structurally very similar to the soluble TNFR2 protein lacking the TM protein constructed by Moosmayer *et al*. (P80TM-), which has high affinity to TNF-α, due to dimerization via the intracellular domain.[25] Unlike the monomeric, naturally shed TNFR2 ECD present in the sera of untreated animals, soluble secreted Δ7TNFR2 contains both the ECD and intracellular domain, with the most of the TM deleted by exon 7 skipping (see Figure

1). We exploited this difference to develop an enzyme-linked immunosorbent assay (ELISA) that specifically detects Δ7TNFR2 protein in serum, while eliminating the signal from the shed ECD, by using a capture antibody that recognizes the intracellular domain, and a detection antibody that recognizes the ECD (see Materials and Methods and Supplementary Figure S3). Thus, Δ7TNFR2 was detected from a zero background, indicating that Δ7TNFR2 is not naturally expressed at the mRNA or protein level (see Figure 2) and is only induced by SSO treatment. This highly specific assay was used in the experiments described below to accurately measure Δ7TNFR2 protein induction by SSO3274 *in vivo*.

Mice were injected once daily with LNA SSO3274 at 25 mg/kg/day intraperitoneally (IP) for 5 days and bled 5–35 days after the last injection. SSO3274 induced 8,000–10,000 pg/ml of Δ7TNFR2, which, remarkably, remained detectable in the circulation for 35 days (Figure 2b). The protein production corresponded to a strong shift in TNFR2 splicing detected in liver RNA isolated at the indicated days at the time of killing (Figure 2b, bottom); SSO3274 produced no liver toxicity (Supplementary Figure S4). Small intestine and colon also showed Δ7TNFR2 induction after SSO3274 treatment; however the liver, due to its size, TNFR2 expression level, and the degree of splice shifting, is likely the main contributor of circulating Δ7TNFR2 (Supplementary Figure S5). These data agree with previous biodistribution studies for LNA SSOs, which show liver as one of the tissues that accumulate the most compound.[22]

To quantify the anti–TNF-α activity in the sera of LNA SSO3274–treated mice, a classical L929 cell-based cytotoxicity assay was employed. This assay showed that cell treatment with TNF-α plus the serum from mice receiving no or control SSOs led to ~80% cell kill (20% cell survival), reflecting no anti–TNF-α activity (Figure 3a). Importantly, cells treated with TNF-α plus the serum collected 5 or 27 days after mouse injections with SSO3274 (Figure 3a, TNF + 3274) showed ~60% cell survival, indicating that the soluble Δ7TNFR2 protein in the serum acted as a TNF-α antagonist, binding to and inactivating the TNF-α, and preventing its cytotoxic effect on the L929 cells. Overall, these data indicate that the induced Δ7TNFR2 TNF-α antagonist remained active in the circulation of treated mice for at least 27 days after SSO treatment.

To confirm that the observed effects of Δ7TNFR2 are caused by direct binding to TNF-α, and not a nonspecific phenomenon, we added increasing concentrations of an inactivating, anti-TNFR2 antibody, that competes with TNF-α for binding to Δ7TNFR2. The anti-TNFR2 antibody itself was not toxic, and as expected, prevented Δ7TNFR2 protein from binding TNF-α, neutralizing the protective effect of Δ7TNFR2 in the L929 cytotoxicity assay, in a dose-dependent manner (Figure 3b).

The potency of the Δ7TNFR2 protein was compared with that of etanercept, an approved anti–TNF-α drug. Etanercept was injected into mice at 50 μg, IP once daily for 5 days. Serum from SSO- or etanercept-treated mice was diluted with serum from untreated mice, and the anti–TNF-α activity was plotted versus mouse Δ7TNFR2 or etanercept protein concentration, respectively. Figure 3c shows that *in vivo*, the half maximal effective concentration (EC_{50}) of Δ7TNFR2 was tenfold lower than that of etanercept recovered from mouse serum. *In vitro*, etanercept not exposed to the *in vivo* environment, or recombinant human or mouse Δ7TNFR2 protein, had equal EC_{50} values against mouse TNF-α (Supplementary Table S1). These results likely reflect that, *in vivo*, etanercept was subjected to degradation that reduced its activity, while the SSO continuously induced fresh Δ7TNFR2 into the serum, replacing degraded or inactivated protein. Overall, the data suggest that, compared to injections of exogenous etanercept, the SSO-induced Δ7TNFR2 protein, continuously expressed from the endogenous gene, is tenfold more potent in the serum of treated mice. Note, however, that etanercept is a humanized protein, and has greater pharmacokinetic properties in humans than in mice.

SSO3274 treatment in hepatitis and arthritis mouse models

The SSO-induced Δ7TNFR2 protein was next tested for its anti–TNF-α activity in a mouse model of inflammatory liver disease. Injection of galactosamine (GalN) followed by TNF-α leads to rapid onset of liver inflammation.[23] Galactosamine depletes intracellular UTP,[26] leading to transcription inhibition and metabolic inactivation in hepatocytes, thus sensitizing the liver to the toxic effects of TNF-α. Serum levels of released liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), provide a quantitative measure of hepatocyte damage.[27]

Mice were injected IP once daily for 10 days with saline, control LNA SSO3083, or LNA SSO3274 at 25 mg/kg/day. GalN and TNF-α were then administered (Figure 4a), which induced peak serum concentration of AST and ALT 24–28 hours later (ref. 28 and Supplementary Figure S6). Saline injections had no effect; 28 hours after GalN/TNF treatment, there was up to a 20-fold serum increase in levels of both AST and ALT over background (Figure 4b and c, white bars). In contrast, in LNA SSO3274–injected mice there was no increase in the ALT/AST levels (Figure 4b, black bars), indicating that the active SSO protected the liver from the TNF-α insult and prevented liver damage. Treatment with SSO3274 also prevented leukocyte infiltration into the liver, indicating prevention of the inflammation (see Figure 4d). The effects were sequence specific, as there was no protection and no statistically or biologically significant difference ($P > 0.2$, saline versus SSO3083, ALT or AST) between the effects of saline and the control SSO3083 (Figure 4c). SSO3083 is targeted to human β-globin, and like SSO3272, which is targeted to TNFR2 but yields no splice switching (Figure 2), and induced no Δ7TNFR2 protein or anti–TNF-α activity *in vivo*.

Etanercept was also examined in the TNF-α-induced hepatitis model. A single dose, either 20 μg or 20 ng, intravenously (IV), predicted to produce ~10 μg/ml and 10 ng/ml of etanercept in the blood, respectively, was administered 5 hours before GalN/TNF injection. The latter is equal to the concentration of Δ7TNFR2 achieved after SSO3274 injection (Figure 2b). The results (Supplementary Figure S7) show that while injection of 20 μg etanercept prevented ALT/AST induction, 20 ng had no effect, suggesting that at comparable concentrations, SSO3274-induced Δ7TNFR2 protein was more potent in mice than etanercept.

LNA SSO3274 was also examined in the CIA mouse model of RA, in which two injections of bovine collagen (CII) are given at days 0 and 21 (ref. 24). The first CII injection elicits an initial immune reaction and establishes the memory response, while the booster (day 21) triggers the memory response, resulting in acute inflammation of the joints. The CIA model is TNF-α dependent, and disease onset begins at day 25 with a rapid increase in paw swelling, which plateaus by days 50–65.

For treating CIA, mice were injected once daily with LNA SSO3274 at 25 mg/kg/day IP for 5 or 10 days (Figure 5a). The resulting total SSO doses, 3 and 6 mg respectively, induce Δ7TNFR2 protein in DBA/1 mice, required for CIA experiments, in a dose-dependent manner (Supplementary Figure S8). The SSO treatment was initiated at day 11, after the first CII injection has activated memory effector lymphocytes and induced IgM and IgG antibodies. Proportional to the total dose, SSO3274 induced 2,000 and 4,000 pg/ml of Δ7TNFR2 protein at 5- and 10-day treatments, respectively (Figure 5b). These levels are lower in the DBA/1 mice than those in previously used FVB/N mice (~10,000 pg/ml, Figure 2b). Nevertheless, in both groups, the Δ7TNFR2 protein and its anti–TNF-α activity (Figure 5c) persisted in the serum until at least day 50 of the CIA, 30 days after the last injection of SSO.

The 10-day SSO treatment ending on day 20 delayed significant paw swelling and clinical scores caused by CIA for 14 days (days 27–40) (Figure 5d and e), which remained below the level of saline-injected control mice until day 45 of the experiment. The course of the disease

was inversely related with the level of Δ7TNFR2 protein detected by ELISA in the sera of 10-day treated mice (Figure 5d versus b). Importantly, all groups of mice had nearly identical incidence of disease (Supplementary Figure S9), confirming that SSO treatment did not prevent disease, but suppressed its severity until day 45. The 5-day SSO treatment was ineffective, because of lower, evidently insufficient, production of Δ7TNFR2 protein.

SSO3274 dose-maintenance

On the basis of Figure 2b, which shows persistence of Δ7TNFR2 for 27 days after SSO injections, we hypothesized that once significant levels of Δ7TNFR2 were achieved by several daily doses, they could be maintained by less frequent dosing. Thus, groups of FVB/N mice were injected with a 5-day loading dose, followed by a single SSO injection every 2nd, 3rd, 4th, or 5th day (maintenance dose), for up to 37 days thereafter. All groups (Figure 6a) show a robust induction of Δ7TNFR2 protein at day 1 post-loading dose. Mice that received a maintenance dose every 5th day (Figure 6a, white bars) maintained high levels of Δ7TNFR2 for at least 37 days, compared with decreases in protein seen by day 27 without maintenance dosing (Figure 2b). Impressively, mice receiving maintenance doses every 4th, 3rd, or 2nd day showed increased Δ7TNFR2 serum levels. The major implications of these data are that (i) high levels of Δ7TNFR2 protein can be maintained or enhanced by relatively infrequent SSO dosing, and therefore it is a feasible treatment for chronic inflammation and (ii) that periodic dosing does not lead to decreased activity of the SSO with time. This confirms our findings that SSO-mediated induction of Δ7TNFR2 does not elicit an immune response against the protein (Supplementary Figure S10).

Δ7TNFR2 induction in primary human hepatocytes

SSO3274 is mouse specific and cannot be used for clinical development. Therefore, a panel of LNA SSOs, targeted toward skipping of human TNFR2 exon 7, was synthesized and tested in primary human hepatocytes, the clinical target site of SSO action. These SSOs induced soluble human Δ7TNFR2 protein in the media of treated cells (Figure 6b), with SSO3378, 3379, and 3384 shifting splicing >75%. Importantly, the human target sequences of these SSOs are identical to those in *Rhesus macaques*, and thus their potency and toxicity can be evaluated in detail in nonhuman primates before clinical trials.

Discussion

Previous studies have described a natural soluble secreted splice variant of TNFR2, Δ7/8TNFR2, which is upregulated in the inflammatory state (~300 pg/ml).[3] However, no protective effect of this splice variant has been demonstrated in RA patients. These levels are evidently insufficient to alter the course of disease, and as the anti–TNF-α activity of Δ7/8TNFR2 has not been determined,[3] its physiological role is unclear. Here we show that SSOs induced the Δ7TNFR2 protein, which has potent anti–TNF-α activity, to 10 ng/ml (Figure 2b), 30-fold higher than the Δ7/8TNFR2 in RA patients. Furthermore, while TNFR2 function is downregulated in the inflammatory state by receptor shedding, expression at the transcription level is often enhanced,[29,30] increasing the potential Δ7TNFR2 yield of the SSO. Thus, SSOs targeting TNFR2 may be effective in RA or hepatitis patients.

Mouse treatment with LNA SSO3274 for 10 days induced a pronounced, prolonged, dose-, sequence-, and Δ7TNFR2 protein–dependent anti-inflammatory effect, which altered the course of disease in two TNF-α-mediated disease models. Notably, SSO-induced Δ7TNFR2 appeared more potent than etanercept in the TNF-α-induced hepatitis (Figure 4, Supplementary Figure S7). SSO3274 treatment before the onset of symptoms also delayed acute CIA, in which two collagen injections are administered (Figure 5). The delayed course of the disease was similar to that induced by etanercept in a milder mouse CIA model, in which just a single,

initial collagen injection is given.[31] In those experiments, etanercept was injected at 50 μg/mouse once daily for 14 days, which should result in ~25 μg/ml serum, a concentration 6,000-fold higher than that of Δ7TNFR2 in our experiments (~4 ng/ml).

In contrast to etanercept and other biologics, LNA SSO3274 itself has no anti–TNF-α activity, but instead induces the sustained production of the Δ7TNFR2, TNF-α antagonist. Upon systemic injection, LNA oligonucleotides are cleared within 1 hour from the circulation into tissues,[32] including liver, where they reside intact with a half-life of 2–3 weeks. Thus, they continuously induce production of Δ7TNFR2 mRNA and protein, maintaining anti–TNF-α activity in the circulation for ~35 days (Figures 2b, 3a and 5). Furthermore, once the therapeutic, steady-state level of Δ7TNFR2 is reached, Δ7TNFR2 production can be maintained and enhanced by infrequent dosing (Figure 6a); this loading/maintenance regimen was successfully used in clinical trials of a cholesterol-lowering antisense compound.[33]

SSOs may have several advantages over other drugs[34,35] that are currently being used to reduce the action of TNF-α. First, they induce a splice variant of the endogenous *TNFR2* gene, which may be better tolerated by patients and be less likely to have reduced efficacy over time due to immune resistance. Second, the constant, persistent level of Δ7TNFR2 protein achieved by SSO may allow less frequent drug dosing than required by other anti–TNF-α products. It also avoids drastic changes in drug concentration seen with biologics,[36] and therefore, the negative effects on immune surveillance caused by anti–TNF-α treatment, a primary concern with TNF-α Abs,[37] may be avoided. Third, SSOs have the dual action of upregulating the decoy-binding receptor in the peripheral circulation while downregulating the cell surface receptor, which is particularly relevant in liver inflammation. Fourth, LNA SSOs may be orally bioavailable;[22] traditional antisense oligonucleotides containing the MOE modification have been evaluated for oral bioavailability in clinical trials.[38] Finally, because the oligonucleotides are synthesized chemically, they would be less expensive to produce than current anti–TNF-α protein drugs. Although the extent of these advantages remains to be determined, development of these novel drugs will give physicians an additional therapeutic option for RA patients, possibly including those (30% of all patients) that never see significant improvement with the use of currently available drugs.[39,40] Although certain sequences of LNA oligonucleotides recently showed hepatotoxicity,[41] we have seen no LNA-induced toxicity in any of the mice we have treated,[22] and a recent report shows LNA to be nontoxic in monkeys.[42]

Materials and Methods

Reagents and cell culture

See Supplementary Materials and Methods

Mice and injections

FVB/N mice were used in all experiments except CIA, in which DBA/1 mice were used. IP injections were 200 μl; tail-vein blood samples were 60 μl; LNA SSO injections were IP at 25 mg/kg/day once daily. To assay Δ7TNFR2 protein and anti–TNF-α activity, mice were bled and sera collected after the SSO injections. At the time of killing, mice were asphyxiated with carbon dioxide, blood was taken by cardiac puncture, and livers were removed. For 50 μg etanercept injections, the drug was dissolved in sterile saline at 250 μg/ml. Lower dose dosing solutions were diluted accordingly. Fresh solution was made each day of injection, which was administered at 200 μl IP or 100 μl IV. All animal experiments were conducted in accordance with Institutional Animal Care and Use Commitee guidelines.

ELISA

Mouse blood (~60 μl) was clotted for 1 hour at 37 °C and centrifuged for 10 minutes at 14,000 rpm (Jouan BRA4i centrifuge; Jouan, Saint-Herblain, France). The concentration of mouse Δ7TNFR2 induced by SSO3274 was quantified in 96-well plates coated using 100 μl of 10 μg/ml of a capturing antibody against C terminus (intracellular domain) of mouse TNFR2 (Abcam, Cambridge, MA), and Quantikine kit (R&D Systems, Minneapolis, MN), with an N-terminal-specific TNFR2 primary antibody conjugated to horseradish peroxidase and substrate solution (hydrogen peroxide and stabilized chromogen), according to the manufacturer's instructions. As a standard, mouse Δ7TNFR2 obtained from the culture media of wild-type HeLa cells transfected with plasmid expressing His-tagged Δ7TNFR2 and purified on Ni-column was used (see Protein Preparation).

For etanercept (human) and human Δ7TNFR2 induced by SSOs in primary human hepatocyte media, the Quantikine kit (R&D Systems) for human TNFR2 was used per the manufacturer's instructions. Human Δ7TNFR2 protein was measured against the standard provided with the kit; etanercept was measured against a standard curve of etanercept, with concentrations based on the prescription details.

Reverse transcription–PCR

Approximately 5 mg of tissue, or cells in culture, was dissolved in 1 ml Tri Reagent (Molecular Research Center, Cincinnati, OH) and total RNA was isolated per the manufacturer's instructions. Approximately 200 ng of total RNA was subjected to reverse transcription–PCR using the rTth polymerase (Applied Biosystems, Foster City, CA) per the manufacturer's instructions. The mouse reverse primer was 5′-TCT CTC TGC AGG CAC AAG GG-3′; the mouse forward primer was 5′-AAC GTG GTG GCC ATC CCT GG-3′. The human reverse primer was 5′-CTT ATC GGC AGG CAA GTG AGG-3′; the human forward primer was 5′-CGT TCT CCA ACA CGA CTT CA-3′. The PCR proceeded as follows: 95 °C, 60 seconds; 56 °C, 30 seconds; 72 °C, 60 seconds for 22 cycles total. The reaction solution contained Cy5-labeled dCTP (GE Healthcare Bio-Sciences, Piscataway, NJ) for visualization (1 μl per 50 μl). The PCR products were separated on a 10% nondenaturing polyacrylamide gel, and Cy5-labeled bands were visualized using a Typhoon 9400 Scanner (GE Healthcare Bio-Sciences, Piscataway, NJ). Scans were quantified using ImageQuant (GE Healthcare Bio-Sciences, Piscataway, NJ) software.

L929 cytotoxicity assay

Serum collected from mice was diluted to 10% in 100 μl L929 media, plus 1 μg/ml actinomycin D and 0.1 ng/ml mouse TNF-α for 30 minutes. This mixture was then applied to the L929 cells plated in 96-well plates (10^4 cells per well). After 24 hours later, cell viability was assayed by adding 20 μl MTS reagent (CellTiter 96 AQueous One Solution Reagent; Promega, Madison, WI) and measuring optical density at 495 nm, normalized to cells treated with 10% serum from untreated mice and without added actinomycin D or TNF-α. For dose–response experiments, sera from SSO-treated mice were serially diluted with serum from untreated mice and the anti–TNF-α activity was assayed, and plotted against the concentration of Δ7TNFR2, as determined by Δ7TNFR2-specific ELISA. Etanercept recovered from treated mice was assayed in the same fashion.

For pure etanercept, mouse and human Δ7TNFR2 studies, fresh protein was diluted in 100 μl L929 media from 0.4 to 444 ng/ml, and incubated with 10% mouse serum from untreated mice containing actinomycin D and TNF-α for 30 minutes before being applied to cells. EC_{50} values were determined by standard nonlinear regression analysis using GraphPad Prizm (GraphPad Software, San Diego, CA).

L929 cell cytotoxicity assay with anti-TNFR2 antibody

Samples were prepared in 100 μl L929 media supplemented with 10% fetal bovine serum. Mouse TNF-α, actinomycin D, and purified mouse Δ7TNFR2 (see Protein Preparation) were added to a final concentration of 0.1 ng/ml, 1 μg/ml, and 2 ng/ml, respectively. Anti-mouse sTNFR2 polyclonal antibody (R&D Systems, Minneapolis, MN) was added to each sample at the indicated final concentration. The samples were then incubated for 30 minutes at room temperature and evaluated in the L929 cytotoxicity assay as described earlier.

GalN/TNF

Mice were injected IP daily with either LNA SSO3274 (10 mice at 25 mg/kg/day) or saline (15 mice) for 10 days, or once with etanercept IV. Five hours after etanercept or twenty-four hours after the final SSO injection, all mice were injected IP with 20 mg galactosamine [D-(+)-galactosamine hydrochloride] followed by 3 ng IV injection of mouse TNF-α 20 minutes later. Twenty-eight hours after TNF-α injection, blood was taken and the mice were killed. ALT/AST measurements were by the University of North Carolina Histology Core. Livers were removed for paraffin embedding and hematoxylin & eosin staining, and slide mounting was performed by the Pathology Core at University of North Carolina (http://cancer.med.unc.edu/research/cores/facility.asp?facilityID=12). Images were taken using an Axiovert microscope (Carl Zeiss, Thornwood, NY).

CIA

DBA/1 mice were immunized subcutaneously with bovine type II collagen subcutaneously (100 μg in complete Freund's adjuvant on day 0 and 100 μg in incomplete Freund's adjuvant on day 21), at the base of the tail using a 27G needle with a volume of 100 μl (1:1 adjuvant:collagen). Mice were assessed for general health daily for 3 days after immunization, then every 3–4 days for weight, paw swelling, and global assessment of disease. Swelling at the ankle of all four paws was measured with a caliper and the change in paw thickness from the pretreatment baseline was recorded. Clinical index is a clinical measurement of the degree of arthritis severity and takes into account arthritis that is present beyond the ankle swelling, such as the toes or tenosynovium. Each paw was assessed by a blinded observer; maximum score is 3 per paw for a total of 12 per mouse (Table 1).

Protein preparation

HeLa cells (0.5×10^6 cells per well in 6 wells) were transfected with 1.5 μg/well of plasmid DNA coding a c-terminal His-tagged human or mouse Δ7TNFR2. Media were collected after 48 hours, filtered through a 1-μm filter, and loaded onto 1-ml HisTrap (Ni) column using a Fast Protein Liquid Chromatography (GE Healthcare). The Δ7TNFR2 was eluted with 400 mmol/l imidazole buffer (20 mmol/l Tris pH = 7.5, 150 mmol/l NaCl, 400 mmol/l imidazole). The fractions were pooled, concentrated and exchanged to phosphate-buffered saline with a VivaSpin 20 (30,000 MWCO; Viva Science) centrifuge filter unit. The final protein concentration was measured by ELISA as described earlier.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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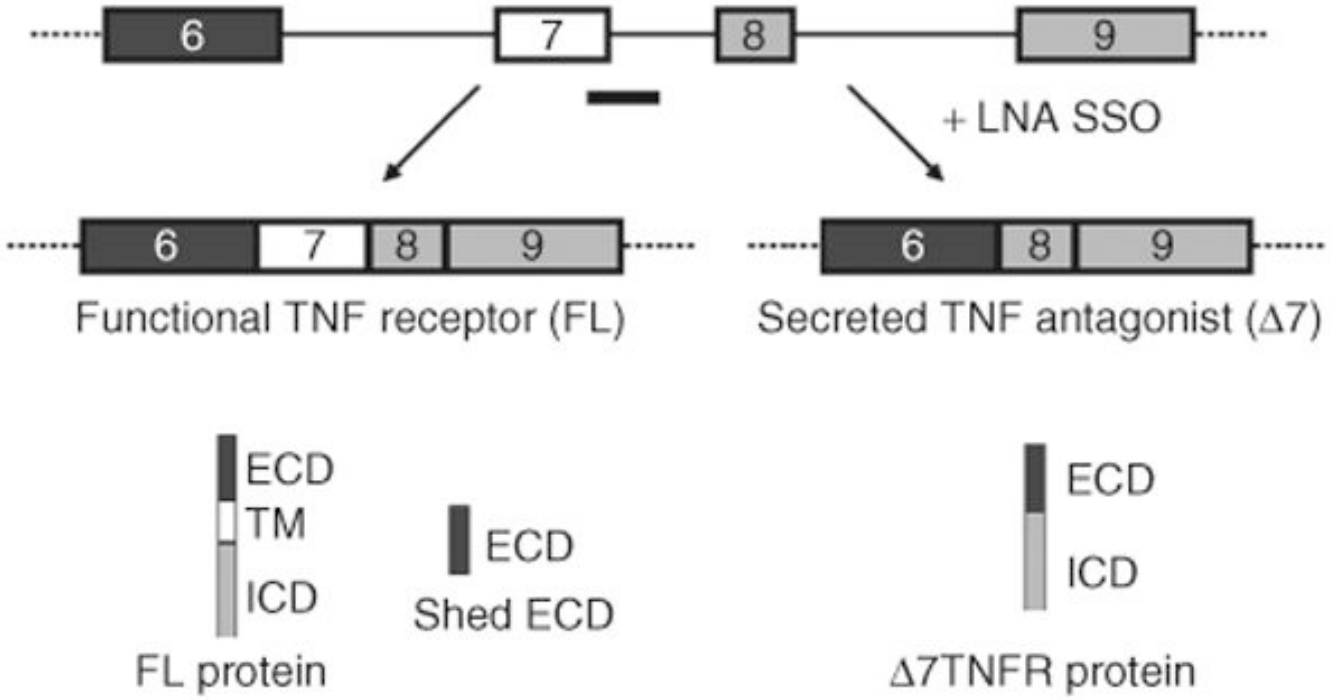


Figure 1. The Δ7TNFR2 splice variant

The full-length (FL), functional, membrane-bound tumor necrosis factor receptor 2 (TNFR2) is translated from messenger RNA (mRNA) containing all 10 coding exons. Targeting of exon 7 in pre-mRNA with LNA SSOs (black bars) induces a soluble isoform, Δ7TNFR2, that lacks a transmembrane domain (TM), retains the extracellular and intracellular domains (ECD and ICD, respectively) and is secreted by cells. Importantly, the shed form of soluble TNFR2 contains only the ECD. Black, extracellular domain; white, transmembrane domain; gray, intracellular domain. LNA, locked nucleic acid; SSOs, splice-switching oligonucleotides.

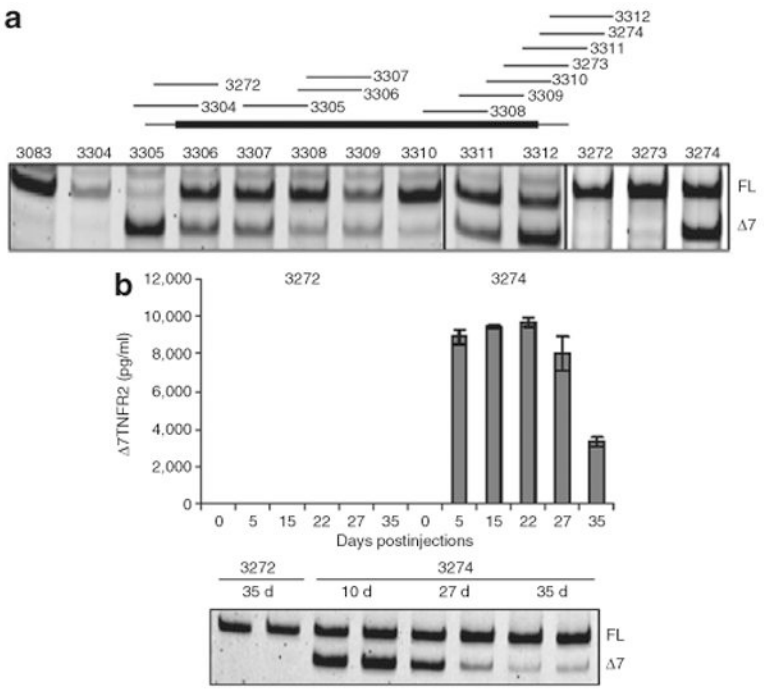
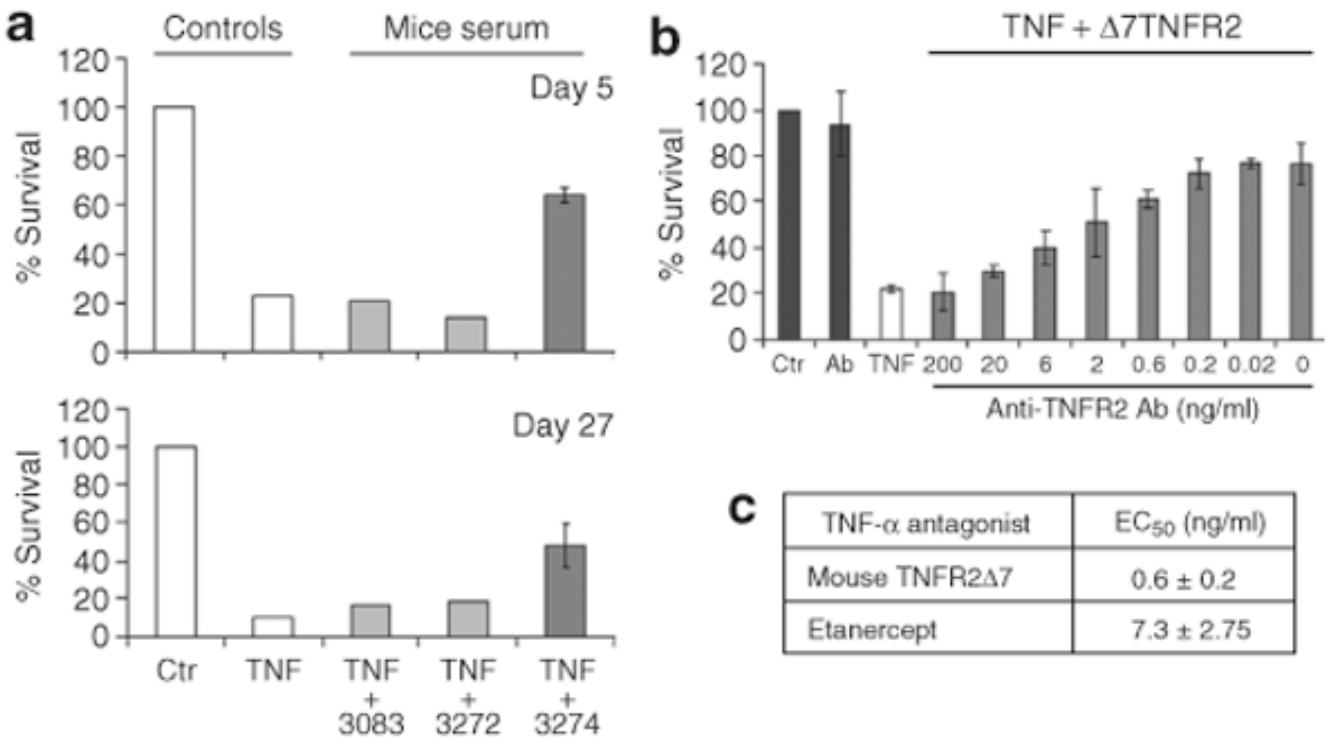


Figure 2. Induction of Δ7TNFR2 by locked nucleic acid (LNA) SSO3274

(**a**) LNA splice-switching oligonucleotides (SSOs) targeted to mouse tumor necrosis factor receptor 2 (TNFR2) pre-mRNA from 20 bp upstream to 20 bp downstream and including exon 7. Thick line, exon; thin line, intron; short lines, LNA SSOs. L929 cells were transfected with the indicated SSOs at a final concentration of 50 nmol/l. After 24 hours, cells were lysed, RNA was isolated and analyzed for splice switching by reverse transcription–PCR (RT-PCR). FL, full-length TNFR2 messenger RNA (mRNA); Δ7, Δ7TNFR2 mRNA splice variant. (**b**) Mice (n = 5 per group) were injected intraperitoneally with SSO3274 or control SSO3272 at 25 mg/kg/day once daily for 5 days. Serum was collected 4 days before injections began (0) and at the indicated number of days after the last SSO injection (gray). Top, samples were analyzed by enzyme-linked immunosorbent assay. Bottom, mice were killed at indicated days and total liver RNA was analyzed by RT-PCR for TNFR2 pre-mRNA splice switching. Each lane represents analysis of liver RNA from a single treated mouse. Data for days 10 and 27 are from separate experiments.



TNF-α antagonist	EC_{50} (ng/ml)
Mouse TNFR2Δ7	0.6 ± 0.2
Etanercept	7.3 ± 2.75

Figure 3. Anti–TNF-α activity of Δ7TNFR2 protein

(**a**) LNA SSOs were administered to mice as described at 25 mg/kg/day once daily for 10 days. The serum was collected 5 or 27 days after the last injection, diluted to 10% and applied to L929 in the presence of actinomycin D and TNF-α (ActD/TNF-α). After 24 hours, cell viability was determined using MTS assay and compared with controls. Ctr, cells treated with 10% serum from untreated mice and no ActD/TNF; TNF, cells treated with 10% serum from untreated mice and ActD/TNF; TNF +, cells treated with 10% serum from mice treated with the indicated SSO and ActD/TNF. TNF + 3274 ($n = 3$), all other ($n = 1$). Note that serum from SSO3272, 3083, and untreated mice yielded a reproducible lack of protection from TNF-α. (**b**) Anti-TNFR2 antibodies (Abs) neutralize the anti–TNF-α effect of Δ7TNFR2. The L929 cytotoxicity assay was performed as described in **a**, except that the indicated concentrations of an anti-TNFR2 Ab was added at the indicated concentrations. Ctr, L929 cell receiving no ActD/TNF-α, anti-TNFR2 Ab, or Δ7TNFR2; Ab, cells receiving 200 ng/ml anti-TNFR2 Ab only; TNF, cells receiving ActD/TNF-α only. (**c**) Half maximal effective concentration (EC_{50}) values calculated from **a** (see Materials and Methods). LNA, locked nucleic acid; SSO, splice-switching oligonucleotide; TNF-α, tumor necrosis factor-α; TNFR2, TNF receptor 2.

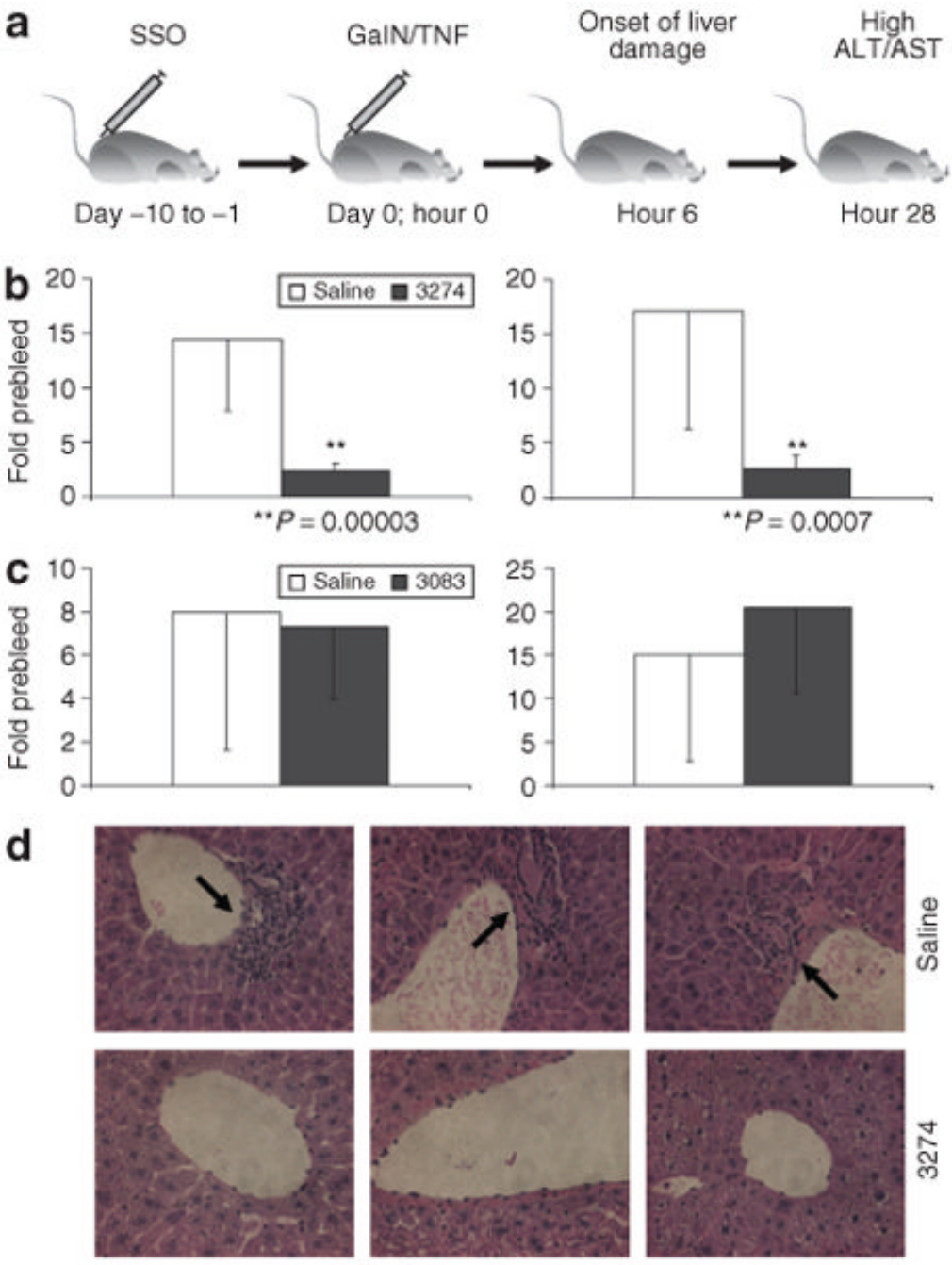


Figure 4. Tumor necrosis factor-α (TNF-α)-induced hepatitis model

(**a**) Mice were treated intraperitoneally with saline, control SSO3083 (see Figure 2a), or Δ7TNFR2 inducing SSO3274 at 25 mg/kg once daily for 10 days. After 24 hours (day 0, hour 0), mice were injected with 20 mg galactosamine (GalN) followed by 3 ng TNF-α 20 minutes later, which induces an acute inflammatory response in the liver. (**b**) The extent of hepatocyte damage caused by the inflammatory insult is reflected by increased levels of liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), released by hour 28 into the blood stream of control, saline-injected, mice (white bars). Treatment with locked nucleic acid SSO3274 (black bars) prevents liver damage and enzyme release. (**c**) Control SSO3083 (black bars) showed no preventative effect. Saline treatment (n = 15); splice-

switching oligonucleotide (SSO) treatment ($n = 10$). P values for saline versus control SSO3083 in ALT or AST were $P > 0.2$. (**d**) Livers from mice treated as in **b** were removed at the time of killing (28 hours), imbedded in paraffin and stained with hematoxylin & eosin. Arrows, sites of inflammatory infiltrate. TNFR2, TNF receptor 2.

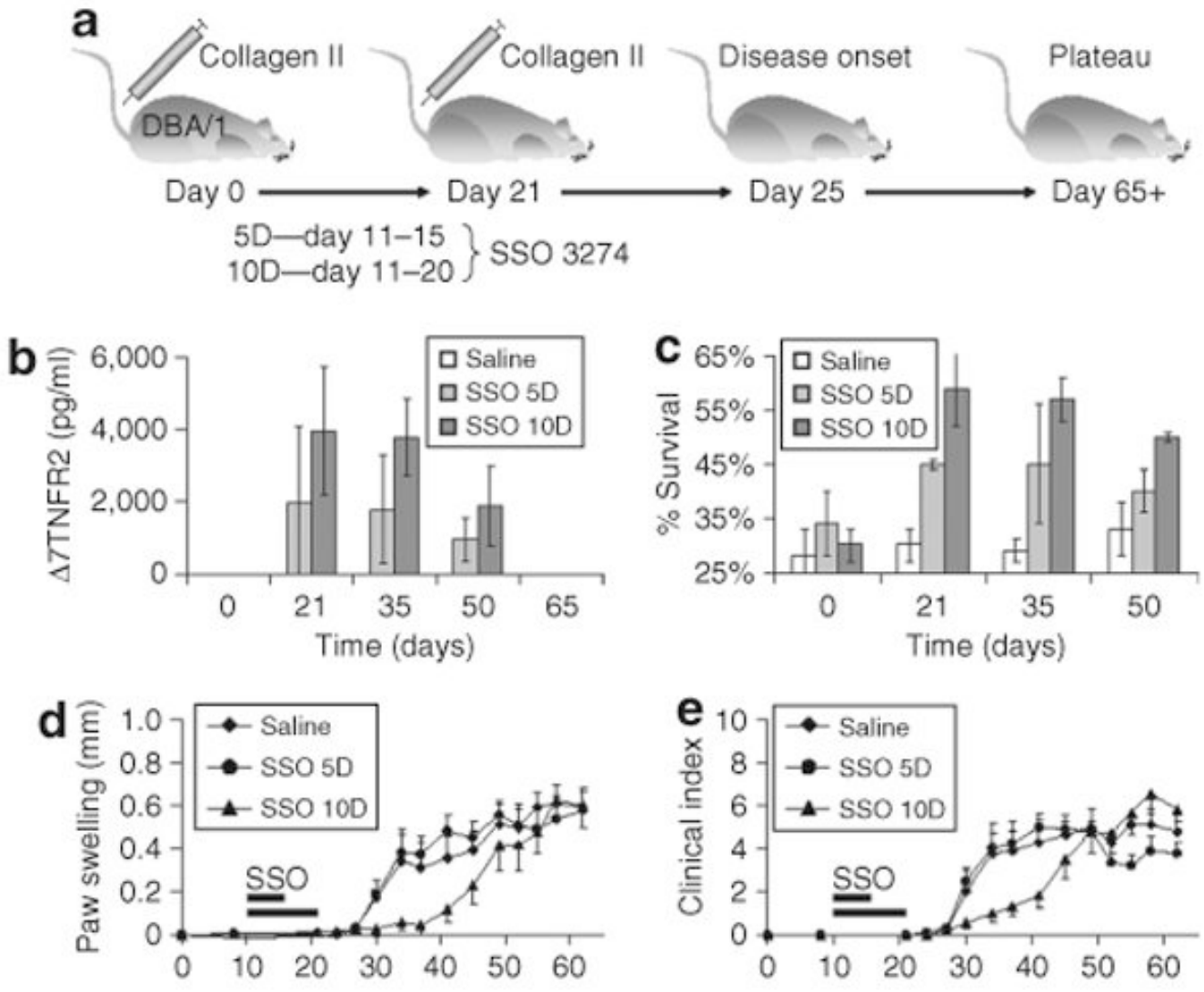


Figure 5. Locked nucleic acid (LNA) SSO3274 activity in collagen-induced arthritis (CIA) mice

(**a**) Time course of CIA model. Mice (n = 10) were treated with either saline from day 11–20 (saline) or 25 mg/kg/day of SSO3274 from day 11–15 or 11–20 (SSO 5D and 10D, respectively). (**b**) Serum was collected from mice on the indicated day of the experiment and analyzed by Δ7TNFR2 specific enzyme-linked immunosorbent assay, or (**c**) anti–TNF-α L929 cytotoxicity assay. (**d**) Administration of LNA SSO3274 for 10 days delayed and reduced the onset and extent of the course of the disease as measured by paw swelling or (**e**) clinical index. Measurements in **d** and **e** were performed as described in Materials and Methods. SSO, splice-switching oligonucleotide; TNF-α, tumor necrosis factor-α; TNFR2, TNF receptor 2.

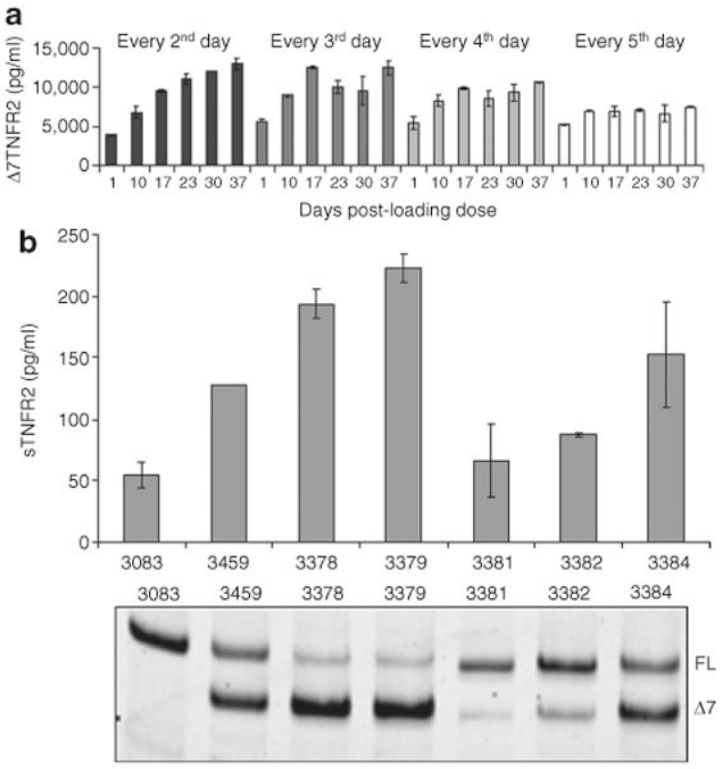


Figure 6. Therapeutic potential of Δ7TNFR2 inducing splice-switching oligonucleotide (SSO)

(**a**) Mice were injected with a loading dose of 5 days of once daily 25 mg/kg/day SSO3274, followed by a single 25 mg/kg maintenance dose every 2nd, 3rd, 4th, or 5th day for 37 days. Δ7TNFR2 was measured in serum samples as in previous figures. (**b**) The indicated SSO was delivered to primary human hepatocytes by cationic lipid transfection at a final concentration of ~30 nmol/l. Total RNA and extracellular media were collected 72 hours after transfection, and total RNA was analyzed by reverse transcription–PCR and medium was analyzed by enzyme-linked immunosorbent assay (ELISA) for Δ7TNFR2 messenger RNA and protein, respectively. Note that this ELISA detects both the shed form and the Δ7TNFR2 form, thus control (3083)-treated cells show a small background of shed tumor necrosis factor receptor 2 (TNFR2) extracellular domain. FL, full length.

Table 1

Clinical Scoring

Arthritis score	clinical presentation
0	Normal paw, no arthritis
1	Mild but definite swelling and/or redness of one major joint (wrist or ankle) or toe involvement (any number)
2	Moderate redness and swelling of wrist or ankle ± any number of toes
3	Maximal redness and swelling of entire paw including toes ± ankylosis