Citation: Molecular Therapy–Nucleic Acids (2012) 1, e52; doi:10.1038/mtna.2012.44

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A Selective Inhibitor of Human C-reactive Protein Translation Is Efficacious *In Vitro* and in C-reactive Protein Transgenic Mice and Humans

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Observational studies of patients with established rheumatoid arthritis (RA) document a positive correlation between C-reactive protein (CRP) blood concentration and worsening of RA symptoms, but whether this association is causal or not is not known. Using CRP transgenic mice (CRPTg) with collagen-induced arthritis (CIA; a rodent model of RA), we explored causality by testing if CRP lowering *via* treatment with antisense oligonucleotides (ASOs) targeting human *CRP* mRNA was efficacious and of clinical benefit. We found that in CRPtg with established CIA, ASO-mediated lowering of blood human CRP levels improved the clinical signs of arthritis. In addition, in healthy human volunteers the ASO was well tolerated and efficacious *i.e.,* treatment achieved significant CRP lowering. ASOs targeting CRP should provide a specific and effective way to lower human CRP levels, which might be an effective therapy in patients with established RA.
Molecular Therapy–Nucleic Acids (2012) **1**, e52; doi:10.1038/mtna.2012.44; published online 13 November 2012
Subject Category: Therapeutic proof-of-concept

Introduction

Systemic inflammation and erosive destruction of the joints are hallmarks of rheumatoid arthritis (RA);[1,2] the hands and feet being most often affected but other joints not spared.[3] Emerging data also indicate that compared with the general population, people with RA are at significantly increased risk of cardiovascular disease.[4,5] Several theories have been proposed to explain the underlying pathobiology of RA and its sequelae but none have been universally accepted nor conclusively demonstrated. For example, since the discovery of rheumatoid factor (antibodies against the Fc portion of immunoglobulin G) it has been widely accepted that RA has an autoimmune origin.[1] Accordingly, rheumatoid factor interacting with the Fc portion of IgG antibodies is thought to promote formation of immune complexes that, in turn, activate the complement system and bind to Fc receptors, thereby propagating the inflammation associated with established RA.[1,2,6] In alignment with the autoimmune hypothesis, a variety of inflammatory cells (macrophages, dendritic cells, etc.) are seen to infiltrate the synovium of patients with established RA.[1,2] Those cell types are thought to exert influence on both the onset of the disease and its subsequent clinical course. T cells are also postulated to be critical to RA onset and their interaction with macrophages, fibroblasts, and other cell types is thought to contribute to the production of deleterious cytokines (e.g., interleukin (IL)-2, IL-4, IL-10, and interferon-γ) once arthritis is established.[1]

It has long been recognized that for patients with established RA the concentration of C-reactive protein (CRP) in the blood correlates positively with disease severity and progression.[7] In the context of established RA, CRP can form complement-activating complexes[8,9] and bind to various Fc receptors,[10,11] so it should be as likely as rheumatoid factor to participate in the disease process. Indeed in patients with established RA, CRP is found within arthritic joints[12,13] and the synovial fluid[14] and its presence there has been used to differentiate inflammatory from noninflammatory RA.[14] Based on these findings and others, measurement of CRP blood level has been incorporated into clinical algorithms used to gauge RA disease activity.[15] Yet despite the recognized clinical utility of CRP measurement and all of the guilt by epidemiological association, still little is known about the biology of CRP in the context of established RA. No human study to date has directly investigated the role of CRP in active RA and the animal studies performed so far have had mixed results. Early studies of experimentally induced arthritis in rabbits established that the serum was the source of synovial CRP[16] and that intra-articular injection of rabbit CRP elevated knee joint temperature if arthritis was established but not if the joint was healthy.[17] This was the first study to support the expectation that CRP should exacerbate established RA. Later and unexpectedly, a study of experimentally induced arthritis using rabbit CRP transgenic mice (CRPTg)[18] showed that CRP might also be protective if it was present before disease onset and establishment. We recently used human CRPTg[19,20] in tandem with CRP deficient mice (*Crp*$^{-/-}$)[21] to examine more closely the strength and direction of CRP's contribution to inflammation, immunity, and emerging collagen-induced arthritis (CIA, an animal model of RA). The results of our study[21] reinforced those reported earlier,[18] *i.e.,* we showed

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Keywords: antisense therapy; CRP; rheumatoid arthritis
Received 25 July 2012; revised 27 August 2012; accepted 7 September 2012; advance online publication 13 November 2012. doi:10.1038/mtna.2012.44

that CRP (both human and mouse) was beneficial during the presymptomatic stages of early CIA since emergence of experimental CIA was hastened in $CRP^{-/-}$. While our results and those of Jiang *et al.*[18] would both suggest a possible benefit of CRP during the transition from health to emergence of RA or early RA in humans, neither study was designed to address the large body of clinical evidence showing that higher blood CRP level associates positively with worsening of symptoms in patients with established RA. In such patients, the level of blood CRP likely is raised in response to worsening of the RA-associated inflammation, and in that context CRP might activate complement or otherwise worsen the inflammation.

To more realistically model the clinical experience and thus ascertain the impact of CRP on ongoing disease, herein we studied the impact of pharmacological inhibition of human CRP in CRPTg with established CIA. We sought to determine whether such reduction of human CRP was of therapeutic benefit. To target human CRP, we developed an antisense oligonucleotide (ASO) that prevents translation of human CRP by promotion of selective degradation of human *CRP* mRNA. The results show that this strategy is efficacious, *i.e.,* treatment with ASOs lowered human CRP production by Hep3B cells and primary human hepatocytes *in vitro* and reduced blood CRP in CRPTg. Importantly, pharmacologic lowering of human CRP in transgenic mice with established CIA significantly reduced their clinical signs of arthritis. In addition, a clinical trial in healthy volunteers showed that the CRP lowering ASO is also effective and generally well tolerated in humans. If the results of our animal studies are translatable to humans with RA, then CRP should be disease-limiting during early RA and disease-promoting once RA is established. Accordingly, ASO-mediated reduction of blood CRP could be an effective new therapy for patients with established RA.

Results

Treatment with CRP-specific ASOs reduce hepatic CRP mRNA and serum CRP protein levels in CRPTg

In initial-screening experiments, three ASOs (ISIS 353512, ISIS 329993, ISIS 353491) were each found to significantly reduce human *CRP* mRNA production by dexamethasone plus cytokine (IL-6 plus IL-1β)-stimulated Hep3B cells, with the IC_{50} (half maximal inhibitory concentration) value for the lead compound (ISIS 329993) in the ~8 nmol/l range (**Supplementary Figure S1a**). Further assessment using human primary hepatocytes stimulated with dexamethasone plus cytokine (IL-6 and IL-1β) confirmed the potency of these ASOs, the IC_{50} value for ISIS 329993 being in the ~20 nmol/l range (**Supplementary Figure S1b**). Based on these results, ISIS 353512, ISIS 353491, and ISIS 329993 were chosen for further evaluation *in vivo*.

In the CRPTg used for this analysis, baseline serum human CRP (treatment day 0) was 12.7 ± 1.03 μg/ml (n = 35) with no statistically significant difference in serum CRP level among the various treatment groups (analysis of variance). Treatment with each of the three human CRP-specific ASOs significantly lowered serum human CRP level in CRPTg (**Figure 1**). By day 17, serum human CRP was lowered by 71, 86, and 94% by ISIS 353512, ISIS 329993, and ISIS 353491, respectively (**Figure 1**). For each human CRP-specific ASO, this effect was accompanied by parallel reduction of hepatic human *CRP* mRNA (**Figure 1**, inset). Thus by day 17 compared with animals administered 0.9% saline (vehicle), *CRP* mRNA in livers of mice that received the human CRP-specific inhibitors ISIS 353512, ISIS 329993, and ISIS 353491 was reduced by 34, 52, and 99%, respectively. The response to treatment with ISIS 141923, the control ASO, paralleled the response to vehicle in that it neither significantly lowered serum human CRP level nor reduced hepatic human CRP mRNA (**Figure 1** and the inset to **Figure 1**, respectively). None of the human CRP-specific ASOs reduced serum mouse CRP significantly below baseline level (39 ± 5.9 ng/ml) (**Figure 2a**) and none of them elevated liver enzymes (**Figure 2b**), attesting to their

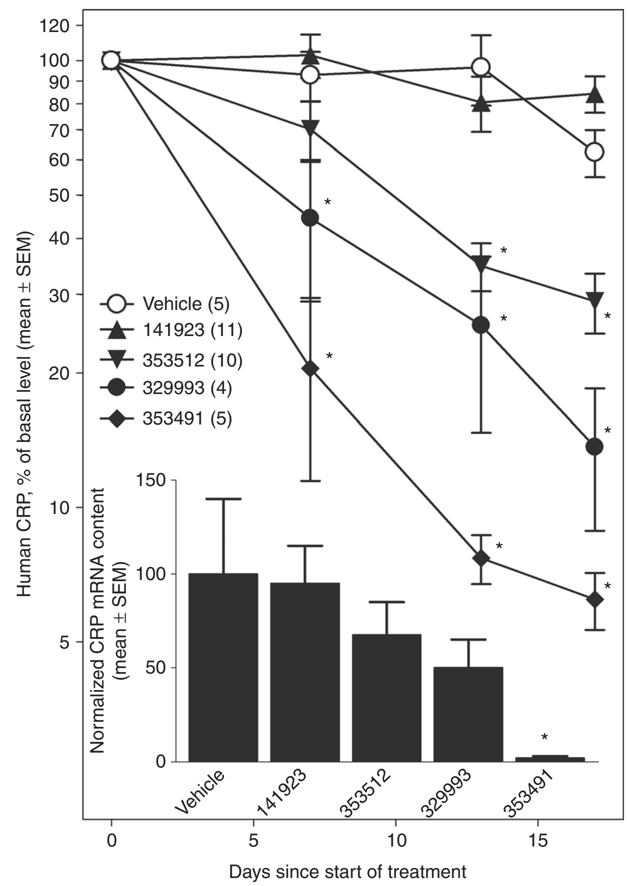

Figure 1 Treatment of CRPTg mice with CRP-specific ASOs lowers serum human CRP and hepatic human CRP mRNA. Administration of three different human CRP-specific ASOs (ISIS 353512, ISIS 329993, ISIS 353491; 25 mg/kg intraperitoneally every 4th day), but not administration of the control ASO (ISIS 141923) or vehicle, results in significant reduction of serum human CRP by 7 days after initiation of treatment. By the end of the treatment phase (day 17), serum human CRP was lowered by as much as 94% (by ISIS 353491). The number of mice is indicated and the asterisks (*) indicate $P < 0.005$ for one-sample t-tests. Inset: Hepatic human *CRP* mRNA levels measured on day 17 were lowered by treatment with CRP-specific ASOs. The asterisk (*) indicate $P < 0.005$ for one-sample t-tests. ASO, antisense oligonucleotide; CRP, C-reactive protein.

specificity for human CRP and good tolerability. Importantly, none of the treatment-related changes in serum IL-6 (**Supplementary Figure S6**) were significant and there was no correlation between change in serum IL-6 and change in CRP. Thus CRP lowering by ASOs was not attributable to IL-6, the main inducer of human CRP expression in both the CRPtg mouse and man.[19,20] ISIS 329993 was chosen for further testing due to the best balance of specificity, tolerability, and CRP reduction.

Lowering serum CRP in CRPTg mice improves the clinical signs of established CIA

We showed recently that in CRPTg mice human CRP blood level is robustly elevated (up to 30-fold above baseline) during the emergent and symptomatic phases of CIA,[21] with CRP levels peaking after each immunization with collagen and then slowly returning to baseline during the symptomatic period. In the present study in CRPTg mice with established CIA (*i.e.,* mice with an arthritis clinical score of ≥2.0 at the time of treatment, see **Supplementary Materials and Methods**), treatment with ISIS 329993 (but not control ASO ISIS 141923) led to significant reduction of human CRP serum levels within 10 days with the efficacious effect remaining significant for up to 20 days (**Figure 3a**). Thereafter, there was apparent development of tolerance in this particular preclinical model as evidenced by a return of serum CRP to approximately baseline levels. In the same animals, serum mouse CRP levels were never reduced (**Figure 3b**). Importantly, the efficacious effect of ASO therapy in CRPTg mice with active CIA was of clear therapeutic benefit, as evidenced by amelioration of arthritis clinical score or signs in CRPTg receiving the human CRP-targeting drug ISIS 329993 (AUC 133.2) but not in CRPtg receiving the control ASO (AUC 159.4) (**Figure 4**) nor in wild-type treated with ISIS 329993 (**Supplementary Figure S7**). In the subset of CRPtg mice whose arthritis was clinically milder when treatment began, the therapeutic effect of ISIS 329993 was more evident and prolonged. For example, when we excluded from our analysis any animals whose arthritis clinical score at the time of initiation of therapy was >5.0, the cumulative disease index (the sum of daily clinical scores for each mouse) was significantly lowered for mice treated with ISIS 329993 (98.6 ± 10.33, n = 20) compared with those receiving the control ASO (138.4 ± 18.37, n = 17) (P = 0.029, one-tailed Student's t-test) (**Figure 4**, inset). Notably, the period of maximum therapeutic effect coincided with the period of significant serum CRP lowering (compare **Figures 4** and **3a**). Importantly, among all arthritic mice (*i.e.,* regardless of their clinical score at

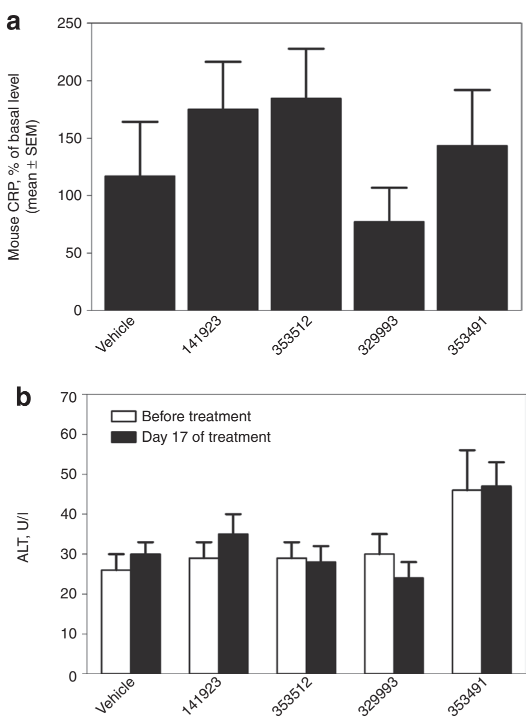

Figure 2 Treatment of CRPTg mice with CRP-specific ASOs does not reduce serum mouse CRP nor elevate serum transaminase. Serum was obtained from blood collected from the mice shown in **Figure 1**. Blood was obtained 24 hours before beginning treatment with the 0.9% saline vehicle or the indicated ASOs, and at the end of treatment 17 days later. Each ASO was administered intraperitoneally at a dose of 25 mg/kg every 4th day. (**a**) There was no significant lowering of serum mouse CRP levels. Values shown are day 17 levels relative to baseline levels. (**b**) There was no significant change in serum ALT levels. The number of mice in each treatment group is shown in **Figure 1**.

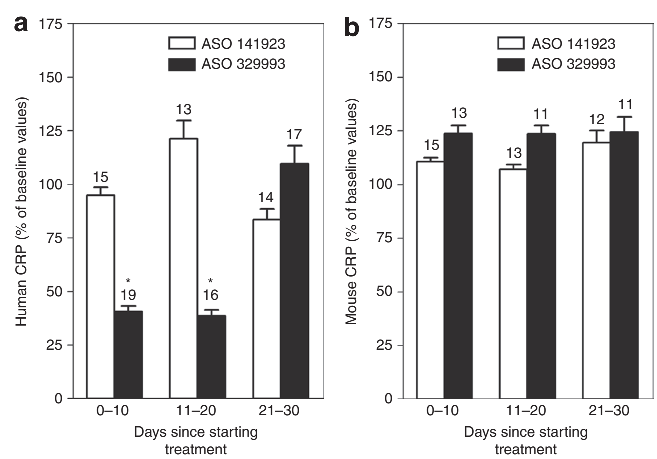

Figure 3 In CRPTg mice with established CIA, treatment with a human CRP-specific ASO lowers serum human CRP. CIA was induced in CRPTg mice and the disease allowed to emerge. After the arthritis clinical score reached ≥2.0, mice began receiving treatment with a human CRP-targeting drug (ISIS 329993, black bars) or a control ASO (ISIS 141923, white bars). Mice were randomly assigned to receive drug or placebo and each was delivered by intraperitoneal injection (25/mg/kg) every 4th day. Human and mouse CRP serum levels were measured during the ensuing symptomatic phase and each is plotted here as a percentage (mean ± SEM) of baseline values (*i.e.,* serum levels measured before induction of CIA). (**a**) For up to 20 days after clinical presentation of arthritis, human CRP serum levels were significantly lowered in CRPTg mice receiving ASO 329993 versus control ASO. The number of mice is indicated and asterisk (*) signifies $P < 0.05$ for Student's t-tests. (**b**) In the same animals, mouse CRP serum levels were not significantly affected by ASO treatment. ASO, antisense oligonucleotide; CIA, collagen-induced arthritis; CRP, C-reactive protein.

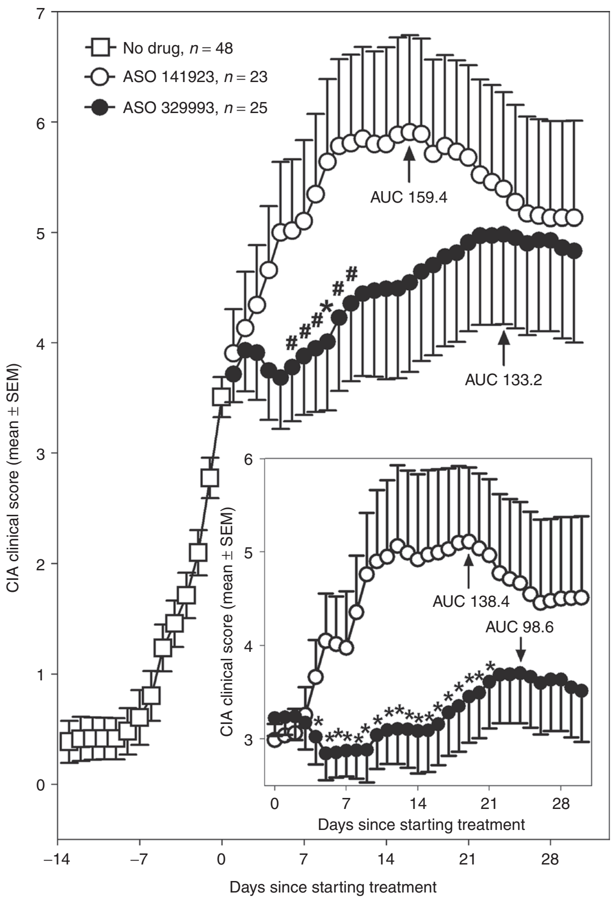

Figure 4 The clinical signs of arthritis are reduced in CRPTg mice with established CIA and treated with a human CRP-lowering ASO. CRPTg mice (*n* = 141) were immunized with type-II collagen as described in the **Supplementary Materials and Methods** and emergence of arthritis in each animal was monitored. Arthritis evolved in 48 animals (open squares) and was allowed to develop unhindered until the arthritis clinical score reached or surpassed 2.0. At that point each of these 48 mice was assigned (randomly) to one of the two treatment arms. ASOs were delivered by intraperitoneal injection (25 mg/kg) every 4th day and drug treatment continued for 28 days. Twenty-five mice received the human CRP-targeting drug ISIS 329993 (closed circles) and 23 mice received the control ASO ISIS 141923 (open circles). Arthritis clinical score was attenuated in CRPTg given the human CRP-targeting ASO. In the inset, animals whose arthritis clinical score at the time of initiation of therapy was >5.0 were excluded. The area under the curve (AUC) and the day of peak (average) arthritis clinical score (arrows) is shown. # and * signify $P < 0.1$ and $P < 0.05$, respectively, for Student's *t*-tests. ASO, antisense oligonucleotide; CIA, collagen-induced arthritis; CRP, C-reactive protein.

recruitment into the treatment phase) the proportion of treated animals whose arthritis clinical score was improved by 20, 50, and 70% compared with baseline (analogous to the American College of Rheumatology (ACR) criteria for improvement of RA symptoms in clinical trials) was always substantially greater for the cohort that received ISIS 329993 (**Figure 5**). The group receiving therapy with ISIS 329993 achieved 50 and 70% improvement more rapidly than did the control ASO-treated group (**Figure 5**).

Pharmacological effects of ISIS 329993 in humans

A phase I double-blind, placebo-controlled, dose escalation, first in human clinical study (ISIS 329993-CS1) designed to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of ISIS 329993 administered to healthy volunteers has been completed (see **Supplementary Materials and Methods** for details). As part of this study, a multiple-dose cohort evaluated the pharmacodynamic effect of ISIS 329993 (600 mg intravenously administered *via* 2-hour infusion) versus placebo (see **Supplementary Figures S3, S4,** and **S5**). To be enrolled, subjects were required to have serum CRP levels between 2 and 10 mg/l on two independent measurements within a 2-week period during screening. Ultimately 8 healthy subjects (2 women and 6 men, 18–53 years in age) whose median baseline CRP level (measured twice during screening, on days 1, 3, and 5 of a 7-day pretreatment run-in period and before dosing on day 1) ranged from 2 to 5 mg/l were enrolled. Subjects received loading doses on days 1, 3, and 5 followed by two weekly maintenance doses on days 8 and 15 for a total of five doses.

In each of the six individuals that received ISIS 329993 serum CRP levels were lowered below baseline value by day 22 after initiation of therapy (**Figure 6**). The median reduction from baseline in serum CRP measured on day 22 was 76% (range 54–83%). CRP levels remained lowered for at least 1 week after the last dose was administered. In the two subjects who received the placebo, serum CRP was either unchanged or elevated compared with baseline. The treatment with ISIS 329993 was well tolerated across the full dose range and with multiple doses; no serious adverse events occurred (see **Supplementary Materials and Methods**).

Discussion

Ours is the first report of safety and efficacy of a specific and direct CRP-lowering compound in humans. The small molecule inhibitor 1,6-bis(phosphocholine)-hexane that occludes the ligand-binding "B" face of CRP, was previously shown to abrogate the adverse effects of human CRP administered to rats undergoing experimentally induced myocardial infarction[22] but to our knowledge has yet to be tested in humans. Rather than blocking ligand binding by CRP or cross-linking CRP to promote its removal from the circulation, thus risking further complement activation and Fc receptor engagement,[10,11,23–25] we reduced CRP protein production *per se* using antisense inhibitors that specifically and selectively prevent the translation of *CRP* mRNA. CRP is very well suited for this approach because the protein's expression is regulated mainly at the level of transcription and it is synthesized primarily by hepatocytes–cells that readily accumulate antisense drugs and are sensitive to ASO pharmacology.[26–28] ASOs also accumulate in extrahepatic cells and tissues known to make CRP such as the kidney, alveolar macrophages, and adipocytes,[29] so the CRP-lowering effect of ASOs is likely global and efficient. Finally because of their much longer half-life compared with small molecule inhibitors,[27,30,31] ASOs can be administered comparatively infrequently to patients. Indeed, the ASO approach has been successfully used to target proteins not readily amenable to small molecule or antibody-based therapeutic interventions.[30–34]

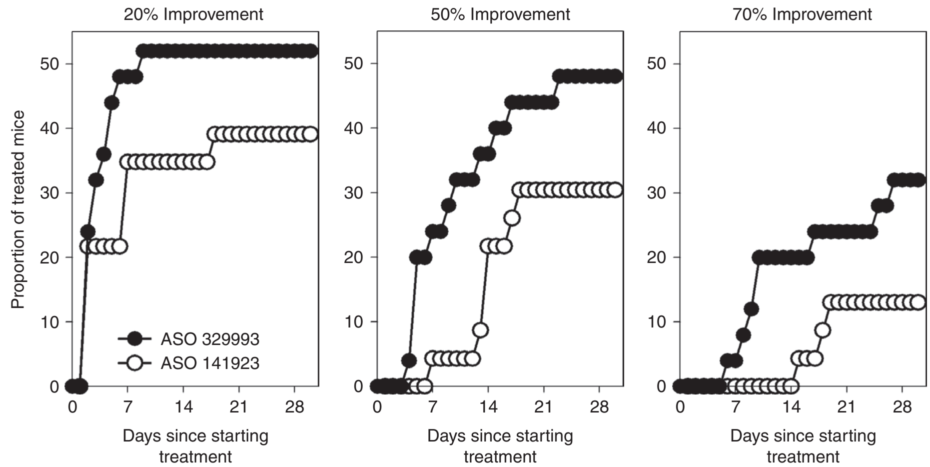

Figure 5 The proportion of mice with established CIA (%) that achieved 20, 50, and 70% improvement in arthritis clinical symptoms was increased by treatment with the human CRP-lowering ASO. Among the 48 mice with established arthritis (*i.e.,* regardless of their clinical score at recruitment into the treatment phase, see **Figure 4**), the proportion of mice whose arthritis clinical score was improved by 20, 50, and 70% (analogous to the ACR20, ACR50, and ACR70 clinical outcome measures; see ref. [44]) was increased for the cohort that received ISIS 329993 (closed circles) compared with the cohort that received the control ASO ISIS 141923 (open circles). Mice that received ISIS 329993 achieved 50 and 70% improvement more rapidly than did the control-treated group. ASO, antisense oligonucleotide; CIA, collagen-induced arthritis; CRP, C-reactive protein.

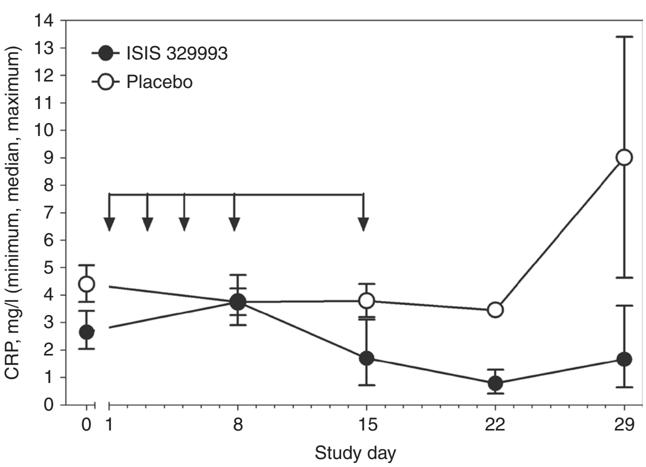

Figure 6 Efficacy of ISIS 329993 in healthy subjects with elevated CRP levels at screening. Eight subjects were treated for 3 weeks with ISIS 329993 (closed circles) or placebo (open circles) *via* 2-hour intravenous infusions on the days indicated (arrows). Serum CRP was lowered in subjects receiving ISIS 329993. See the **Supplementary Materials and Methods** for details. CRP, C-reactive protein.

The CRP-lowering ASOs we tested here were designed to target the human *CRP* mRNA transcript. All three of the human CRP-targeting inhibitors we eventually identified specifically reduced human *CRP* mRNA in human Hep3B cells and primary hepatocytes (**Supplementary Figure S1**). Moreover, hepatic human *CRP* mRNA and serum human CRP levels in CRPTg mice were lowered within 1 week after initiation of a modest dosing regimen (25 mg/kg every 4th day). ASOs are generally proinflammatory in rodents[35,36] but the ASOs we tested here were well tolerated in mice as evidenced by a lack of significant elevation of liver transaminase levels and IL-6. Also, since ISIS 329993 was designed to hybridize to the human *CRP* mRNA at nucleotides 1397-1416 (Genbank NM_000567.2), a region within the 3′ untranslated region that is entirely conserved in the cynomolgus monkey *CRP* transcript, we obtained similar levels of CRP reduction and tolerability in cynomolgus monkeys (data not shown). In healthy human volunteers, ISIS 329993 was well tolerated, had no unexpected safety issues across all doses tested (see **Supplementary Materials and Methods** and **Supplementary Table S4**), and had a predictable pharmacokinetic profile based on prior preclinical experience and disposition of other drugs in its class (see **Supplementary Materials and Methods** and **Supplementary Tables S2** and **S3**). Most importantly in healthy volunteers with elevated baseline CRP levels, serum CRP was reduced by as much as 83% following 3 weeks of dosing with ISIS 329993.

The proven ability of antisense inhibitors to reduce baseline expression of human CRP in CRPTg mice after treatment for a short duration with a low dose of drug suggests that these agents should be useful for intervention in both chronic and subacute disease processes in humans. We tested this prediction in an animal model arthritis and our study provides the first direct evidence that targeted lowering of human CRP could lessen the severity of established RA. Thus in CRPTg mice with established CIA, treatment with ISIS 329993 lowered both human CRP and arthritis clinical severity. This outcome is in alignment with the positive association of elevated plasma CRP level with symptoms of ongoing RA in humans. By extension we predict that in humans with established RA, in which plasma CRP is known to be elevated and known to associate with worsening of symptoms, pharmacological lowering of CRP should be of therapeutic benefit. We underscore that this therapeutic benefit is limited to cases of established RA, as earlier results from our laboratory[21] and another[18] both suggest that CRP may be of benefit during the early stages of newly emerging RA. Indeed in studies wherein CRPtg mice were treated prophylactically with ASO 329993 (starting 2 weeks before induction of CIA and continuing for 35 days thereafter), CRP lowering hastened and worsened development of RA (**Supplementary Figure S8**).

In sum, our animal studies suggest that the contribution of CRP is much like that of interferon-γ,[37] *i.e.,* disease-limiting during emergence of CIA and disease-promoting once CIA is established. Despite this it still remains unknown whether CRP plays a pathophysiologic role in RA. Ultimately, a randomized, placebo-controlled clinical trial will be needed to validate our predictions. Without a human specific-CRP drug, it has not been possible to conduct clinical trials to test the CRP → disease hypothesis. The CRP-specific ASO inhibitor that we described here could fill this gap and provide the impetus for future *in vivo* pharmacological, toxicological, and ultimately clinical studies that will help clearly delineate the role of CRP in human health and disease. A randomized, placebo-controlled, phase 2 study of ISIS 329993 in established RA is currently underway.

Materials and methods

ASOs. We designed 640 different second generation ASOs to specifically hybridize to human *CRP* mRNA, the nucleotide sequence of each being complementary to a region proximal to position 1690 or 1738 on the *CRP* mRNA transcript (GenBank accession no. M11725.1). For each ASO hybridization site on the *CRP* transcript, we used the SEQUENOM Mass ARRAY SNP discovery approach to verify absence of single-nucleotide polymorphisms. Each ASO was 20 nucleotides long and comprised a central unmodified core consisting of 10 or 14 nucleotides flanked by phosphorothioate linkages and three or five 2′-O-methoxyethyl (2′-MOE) modifications on the 3′ and 5′ flanking ends. The ASOs thus had a "3-14-3" or a "5-10-5" configuration. The nucleotide sequences of the lead compounds are (MOE modifications underlined): ISIS 353512: 5′-CCCATTTCAGGAGACCTGG-3′; ISIS 329993, 5′-AGCATAGTTAACGAGCTCCC-3′; ISIS 353491, 5′-GCACT CTGGACCCAAACCAG-3′. A fourth ASO (ISIS 141923, 5′-CC TTCCCTGAAGGTTCCTCC-3′), which is not complementary to any known mouse or human gene sequence, served as a control ASO. ASOs were purified as described.[32,38]

Animals. The human CRP transgene, its detection by PCR, and its human-like pattern of expression in CRPTg have been fully described elsewhere.[19,20] Human CRP is present in the blood of CRPTg at concentrations manifested in humans[39] *i.e.,* low levels under steady-state conditions (<1–10 μg/ml) and high levels during the acute phase response (~30–500 μg/ml). Mouse CRP continues to be expressed in CRPTg, but mouse CRP was neither targeted by the ASOs we tested nor is it a major acute phase protein.[40] Mice were housed at constant humidity (60 ± 5%) and temperature (24 ± 1 °C) with a 12-hour light cycle (6:00 AM to 6:00 PM) and maintained *ad libitum* on sterile bottled water and regular chow (Harlan Teklad, Madison, WI). Males 8–12 weeks old were used unless specifically noted otherwise. All animal use protocols were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals, 8th Edition (2010).

CIA. CIA was elicited using a previously described protocol[41] that evokes disease in approximately one-third of all immunized CRPtg animals.[21] Briefly, complete Freund's adjuvant containing 4 mg/ml *Mycobacterium tuberculosis* was emulsified 1:1 with a 4 mg/ml solution of chicken type-II collagen. Complete Freund's adjuvant and chicken type-II collagen were from Chondrex. (Redmond, WA). At the start of each experiment (day 0), 100 μl of a freshly prepared emulsion was injected intradermally using a 23-gauge needle at a site toward one side of the base of the tail. Three weeks later (day 21) a booster injection (100 μl of chicken type-II collagen emulsified in incomplete Freund's adjuvant) was administered at a site contralateral to the primary injection site. Thrice weekly thereafter until day 50 the clinical signs of arthritis were recorded for each paw. The arthritis clinical scoring system we used was described by Brand *et al.*[42] where 0 = no evidence of erythema and swelling, 1 = erythema and mild swelling confined to the tarsals or ankle joint, 2 = erythema and mild swelling extending from the ankle to the tarsals, 3 = erythema and moderate swelling extending from the ankle to the metatarsal joints, 4 = erythema and severe swelling encompassing the ankle, foot, and digits, or ankylosis of the limb. We used multiple individuals blinded to treatments to score mice. To verify the accuracy of our clinical scoring system, fore- and hind-limbs were removed from representative (humanely euthanized) mice, the soft tissue removed, and the articulated bones fixed in 10% formalin. These were decalcified and embedded in paraffin and sectioned (5 μm) and stained with hematoxylin and eosin to assess arthritic changes. To confirm changes in bone density and bone volume, we performed micro computerized tomography (micro-CT) scans as described[43] using a high resolution μCT imaging system (μCT40; SCANCO Medical, Wayne, PA). The region of interest was the tarsals and metatarsals of the paws. Both the histological analyses and micro-CT scans confirmed the presence of underlying arthritic changes in the paws of CRPTg with visual scores ≥2.0, thus verifying our visual scoring system and validating that mice whose paws had a clinical score ≥2.0 had established arthritis (**Supplementary Figure S2**).

Animal studies. To test if ASOs could achieve CRP lowering *in vivo* we used male CRPTg. At the start of each experiment (day 0) following appropriate anesthesia, blood (20 μl) was collected from the retro-orbital plexus of each mouse and then either vehicle (0.9% saline), control ASO (ISIS 141923), or one 3 human CRP-specific ASOs (ISIS 329993, ISIS 353491, and ISIS 353512) were administered (25 mg/kg) *via* intraperitoneal injection. Thereafter, drugs were injected every 4th day until day 15, with additional blood sample collections on days 7 and 13. On day 17 mice from each treatment group were killed, their tissues collected for analyses of human *CRP* mRNA, and their serum collected for measurement of human and mouse CRP and mouse transaminases.

To test in a preclinical setting if pharmacological lowering of human CRP blood level could reduce the clinical signs of established RA, we compared the fate of established CIA in CRPTg mice treated with ISIS 329993. ASO was dissolved in phosphate-buffered saline and ~200 μl doses were administered *via* intraperitoneal injections twice per week, the volume of each dose being adjusted slightly to achieve 25 mg/kg per injection. To ensure that animals had established arthritis before beginning CRP-lowering therapy, each mouse received its first dose of ASO on the day its clinical signs of arthritis first scored 2.0 or more. Mice that developed established CIA were assigned at random to receive either ISIS 329993 or control ASO. Treatments ended 30 days later.

Reverse transcription-PCR. Total RNA was extracted from cultured cells and freshly harvested tissues using Qiagen RNeasy isolation kits (Invitrogen, Carlsbad, CA), and 50 ng of RNA was subjected to reverse transcription-PCR using a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The primer probes used for human *CRP* mRNA quantification were: forward primer 5′-GGCCCTTCAGTCCTAATGTCC-3′, probe 5′-TCCTGAACTGGCGGGCACTGAAG-3′, and reverse primer 5′-GGTTTGGTGAACACTTCGCC-3′. The probe was labeled on the 5′ end with FAM (a 6-carboxyfluorescein reporter dye) and on the 3′ end with TAMRA (a 5(^{6})-carboxytetramethylrhodamine quencher dye). Following 40 amplification cycles, amplicons were quantitated using SDS analysis software (Applied Biosystems).

Measurement of CRP and alanine aminotransferase. We used commercially available kits to measure serum mouse CRP (Life Diagnostics, West Chester, PA) and *alanine aminotransferase* (Sigma, St Louis, MO), according to each manufacturer's instructions. Human CRP was measured using an ELISA developed in our laboratory.[20]

Human studies. Eight healthy volunteers (**Supplementary Table S1**) whose blood CRP levels ranged from 2 to 5 mg/l on two qualifying examinations were enrolled to evaluate the efficacy of ISIS 329993 in lowering plasma CRP. Individuals were randomized to receive 600 mg ISIS 329993 (n = 6 subjects) or placebo (n = 2 subjects). ASOs were administered *via* a 2-hour intravenous infusion on days 1, 3, 5, 8, and 15. This dose regimen was designed to rapidly achieve near steady-state levels of drug in tissues for evaluation of pharmacodynamics. Blood samples were collected before each infusion and on days 22 and 29 for CRP determination. See **Supplementary Materials and Methods** for additional details.

Statistical analysis. For statistical analysis, a mouse was considered to have CIA on the day its clinical score reached or surpassed 2.0, and a mouse was considered to have established CIA only if its symptoms were sustained or worsened for at least 2 days thereafter (before drug treatment). For each treatment group, daily average clinical scores and area under the curve was calculated and for each mouse cumulative disease index (the sum of daily clinical scores obtained during the symptomatic phase) was calculated. The proportion of mice whose arthritis clinical score was improved by 20, 50, and 70% compared with the day CIA was manifested (analogous to the ACR20, ACR50, and ACR70 criteria)[44] was determined. Pooled data are expressed as the mean ± SEM of seven separate experiments and the sample sizes are reported. Group comparisons were done using unpaired Student's t-tests or with one-way analysis of variance followed by post-hoc pairwise least-squared difference tests or Dunnett's analysis for multiple comparisons. Differences were considered significant when the P value was <0.05. Statistical analyses were performed using Graphpad Prism 3.02 (GraphPad Software, San Diego, CA) and Statview 5.0.1 (SAS Institute, Cary, NC).

Acknowledgments. The work reported in the manuscript was funded in part by National Institutes of Health grant 1R21DA026914 (to A.J.S.), a pilot and feasibility award (to A.J.S.) from the NIH-funded UAB Clinical Nutrition Research Unit (5P30DK056336), and a training grant (to N.R.J.) from the NIH-funded UAB training program in Rheumatic Diseases Research (5T32AR007450), and by ISIS Pharmaceuticals, Inc. Isis Pharmaceuticals holds a patent on the C-reactive protein lowering antisense drugs described in our manuscript. The authors declared no conflict of interest.

Supplementary material

Figure S1. Dose-dependent reduction in cytokine-induced *CRP* mRNA expression after treatment with ISIS 329993.
Figure S2. Agreement between arthritis clinical scores, bone density, and bone volume measured by micro-CT, and bone histology in mice with CIA.
Figure S3. Flow of study participants.
Figure S4. Treatment regimens.
Figure S5. Plasma concentration-time profiles for ISIS 329993 in subjects in the multiple-dose cohorts.
Figure S6. Treatment of CRPTg mice with CRP-specific ASOs does not significantly reduce serum mouse IL-6.
Figure S7. Treatment with a human CRP-lowering ASO reduces the clinical signs of established arthritis in CRPTg mice but not wild-type mice.
Figure S8. Treating CRPtg mice with the human CRP-specific drug ASO 329993 before CIA induction increases the severity of newly emerging CIA.
Table S1. Subject demographics and baseline characteristics.
Table S2. Summary of ISIS 329992 plasma pharmacokinetic parameters for single and multiple-dose cohorts.
Table S3. Plasma pre-dose and trough concentrations after multiple IV infusions of 600 mg ISIS 329993 in healthy volunteers (Cohort-III), mean ± SD.
Table S4. Summary of treatment emergent adverse events.
Materials and Methods.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)