THE ROLE OF C-REACTIVE PROTEIN IN ARTHRITIC DISEASE

by

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ABSTRACT

C-reactive protein (CRP) is an acute phase protein that is commonly used to evaluate systemic inflammation level. Blood CRP level also correlates very well with rheumatoid arthritis (RA) disease severity. However, CRP has many biological actions that lead one to suspect that it may be playing a role in the development or pathology of the disease. We have directly addressed whether this is the case by the use of novel mouse models (CRP deficiency or transgenic overexpression). We have shown that CRP positively affects collagen-induced arthritis development and progression. In order to thoroughly address the question, we have also reported a mechanism by which CRP may be exerting its influence on the system, through modulation of dendritic cells, which potently modulate T cell function. We have also shown that this is a split-paradigm; CRP’s multiple biological functions and abilities are differentially influential. In line with this, we have shown that lowering CRP level in mice with established arthritis is therapeutic. Overall, we have thoroughly shown that CRP impacts arthritic disease and in doing so have discovered important new facts that will change the way that RA and its management are considered. We have set the stage for further examination of the relationship between CRP and RA and have contributed to the development of a novel therapy.
DEDICATION

This dissertation is dedicated to Samuel Sum Yee Fan.

1954-2010

S.D.G.
ACKNOWLEDGMENTS

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INTRODUCTION

*Rheumatoid arthritis*

Rheumatoid arthritis (RA) is a systemic, inflammatory autoimmune disease that most visibly affects the major joints of the hands and feet causing debilitating joint destruction and bone erosion (1). While evidence of RA dates back thousands of years, we have only recently begun to understand the details of its complicated mechanism and progression. It has been proposed that a localized induction of innate immune responses and inflammation in the joint space causes both recruitment of T cells and priming of local dendritic cells (DCs) with newly liberated synovial autoantigens (2). These primed DCs then to travel to lymph nodes where they potentiate a T helper type 1 (Th1) differentiation of T cells, which will then continue to accumulate in the inflamed joints. Th1 cells produce cytokines that contribute to an increase in macrophages and fibroblasts in the affected joint which further promotes inflammation and local tissue destruction (3). At some point in the development of the disease, joint inflammation may reach a point where the local destruction becomes self-sustaining (2). Importantly, the joint destruction in RA also has systemic consequences as evidenced by an increased risk of cardiovascular disease in RA patients(4, 5).
**Collagen-induced arthritis**

Many of the advances in knowledge relating to RA pathogenesis have been achieved by the use of animal models of arthritis (6–9), such as collagen-induced arthritis (CIA) (10, 11). The method of induction of CIA is thought to mimic the sequence of events leading to RA in humans. Briefly, mice are injected at the base of the tail with an emulsion of type-II collagen (CII), which is found exclusively in the joints (12), and complete Freund’s adjuvant (CFA), which is a suspension of heat killed *Mycobacterium tuberculosis* in heavy mineral oil. The CII provides a novel, joint-specific antigen, and the CFA provides potent toll-like receptor 4 stimulation, a powerful inflammatory stimulus which helps drive the immune response. This initial injection is followed 21 days later by a secondary immunization that is identical to the first except that incomplete Freund’s adjuvant is used instead, which omits the heat killed bacteria (13). This induction protocol generates an immune response to a joint-specific antigen that recruits T cells and other disease-potentiating cells to the joint spaces and fosters the development of an arthritic disease in mice which mirrors many of the features of RA in humans, including the formation of invasive pannus (8, 14–20).

One specific way that mouse models can help elucidate the causal or pathogenic mechanisms of RA is through the use of gene knockout or transgenic systems. These systems involve the deletion or elimination of expression of a particular gene from the mouse or the inclusion of a new gene to be expressed in the mouse, either systemically or restricted to a specific cell type. In our studies we have utilized three main knockout or transgenic mice: the CRP knockout mouse (*Crp*−/−) (21), the human CRP transgenic mouse (*CRPTg*) (22, 23), and the FcγRIIB knockout mouse (*FcγRIIB*−/−) (24).
**C-reactive protein**

C-reactive protein (CRP) is a pentameric acute-phase protein produced principally in the liver and mainly by hepatocytes. It consists of five identical protomers assembled in a planar array, forming two opposing faces with different ligand binding properties. One side (B face) targets phosphocholine in a Ca\(^{++}\)-dependent fashion while the other side (A face) binds to complement C1q and various Fc receptors (FcRs) (25). While these interactions are the most well-known, CRP certainly can bind with a variety of other ligands as well (26). The main stimulator of CRP expression is IL-6, although other factors such as IL-1 can boost the IL-6 responsiveness (25). Blood levels of CRP in healthy caucasians fall between 1 and 3 mg/L, but levels up to 10 mg/L are seen in some individuals. Levels greater than 10 mg/L are usually indicative of an inflammatory condition. Acute phase levels can reach as high as 1000-fold above baseline (27).

CRP is known to aid in opsonization and clearance of microbes by binding phosphocholine and derives its name from its reactivity with the C-polysaccharide of *Streptococcus pneumoniae* (28). However, CRP is most widely recognized today because of its association with inflammatory and autoimmune diseases such as cardiovascular disease and rheumatoid arthritis. Indeed, clinical measurement of blood CRP level is indicated for the determination of disease severity and prognosis for many diseases including Chrohn’s disease, rheumatoid arthritis, and even some types of cancer (27). CRP’s utility is not restricted to its usefulness as a clinical marker, however. The recognized biological functions of CRP suggest that it likely also participates in these diseases. For example, CRP can bind complement and thus contribute to the formation of immune complexes which can enhance inflammation (29, 30). CRP can also bind several
FcγRs (31–33), which are present on many cells of the immune system such as neutrophils, macrophages, and dendritic cells (34). CRP’s interaction with the various activating and inhibitory FcγRs could allow it to influence phagocytosis, the release of cytokines, and the activation (or quiescence) of cells. Through these varied interactions CRP is able to link innate and adaptive immunity.

**Regulation of immune activation by FcγRs**

FcγRs are receptors that bind the fragment crystallizable portion of Immunoglobulin G. Expressed variably among cells of the immune system, there are 3 main activating receptors in mice (FcγRI, FcγRIII, and FcγRIV) and 5 in humans (FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA, and FcγRIIIB) and only 1 known inhibitory receptor in both (FcγRIIB) (35). These activating and inhibitory receptors act together to regulate cell activation and mediate a variety of important cell processes including antibody production, phagocytosis, and antigen presentation (36). Interaction with these receptors has been shown to play an important role in several autoimmune diseases (35). In systemic lupus erythamatosus (SLE) for example, dendritic cells (DCs) from human patients were shown to have an increased activating to inhibitory FcγR ratio compared to healthy volunteers (37). FcγRIIB seems to play a pivotal role in SLE, since it plays a large role in regulating the autoantibody production that is an important feature of this disease (35, 36, 38). In fact, FcγRIIB overexpressing DCs have been shown to enhance immunotolerance and lengthen survival time in a mouse model of SLE (39). Similarly, in a mouse model of multiple sclerosis (MS), it has been shown activating FcγRs are not involved in disease induction (40). Importantly, it was shown previously that FcγRIIB is
required for inhibition of the MS-like disease experimental autoimmune
encephalomyelitis (EAE) by CRP (41). FcγRs play an equally critical role in arthritis. For
example, mice deficient in the FcR common gamma chain required for cell signaling by
all activating FcγRs became resistant to development of arthritis (42), whereas FcγRIIB,
has been shown to be important for development and progression of arthritis (14). In fact,
deletion of FcγRIIB allowed mice known to be resistant to CIA to become fully
susceptible (43). In humans, a functional variant of FcγRIIB has been found to have a
positive association with RA severity (44), and upregulation of FcγRIIB on DCs has been
shown in patients with remission of RA (45).

Dendritic cell regulation of T cell activation and differentiation

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) of a
myeloid lineage. They share a common developmental precursor with macrophages, and
perform many similar functions (46). DCs constitutively sample their environment by
pinocytosis and readily phagocytose antigens in response to stimulation through surface
receptors such as Toll-like receptors and FcRs (33). While both macrophages and DCs
can act as APCs, DCs are much more potent regulators of T cell differentiation (47). T
cells are small lymphocytes known best for their cytotoxic (CD8+) or helper functions
(CD4+) (48). More recently, a subset of CD4+ T cells that express Forkhead box P3
(FOXP3) have been recognized for their regulatory function (49). Dendritic cells are able
to prime both CD8+ and CD4+ antigen-specific T cells through interaction of DC-bound
MHC-Class I and MHC-Class II, respectively, with the cognate T-cell-bound T cell
receptor. In addition to this binding, known as signal 1, several costimulatory molecules
are expressed on the surface of DCs and serve to modulate the message that T cells receive from DCs by binding receptors on the T cell. These costimulatory molecules constitute signal 2, and include such molecules as CD40, CD80, CD86, and OX40L. In addition to the binding that occurs between DCs and T cells, cytokines such as IL-12 and IFN-α may be released and serve to further guide the effective T cell response (47).

Since DCs are such potent regulators of T cell activity, and RA is a T cell-mediated disease (3), abnormalities in DCs are likely to be relevant for RA pathogenesis. Indeed, differences in development and function of DCs between RA patients and healthy volunteers have been noted (50). Additionally, localized inflammation at the joint during the initiation of RA may affect these patients differently. RA patients may have higher levels of certain DC populations or more reactive DCs than their healthy human counterparts (46). There is also evidence for increased levels of cytokines such as IL-13 that serve as potentiators of DC activation. DCs may even be recruited to the joint space of RA patients more aggressively than healthy volunteers due to an increase in chemokines like CCR5 and MIP-3α in the inflamed joint (46). In accordance with the increase in activated DCs in RA patients, therapies have been devised that seek to restore balance by boosting the level of tolerogenic DCs (51–53). Most of the current therapies focus on the isolation of DCs from the patient, followed by manipulation and reintroduction, though efforts to alter the balance and function of DCs in vivo could be effective as well. As further evidence of the validity of DC targeted therapy, RA patients who have responded to other, contemporary therapies have been shown to have increased tolerogenic activity by DCs (50).
We have seen that DCs and FcγRs, which are expressed on DCs, are implicated in the initiation and maintenance of RA and that CRP interacts with FcγRs, so it stands to reason that CRP could easily impact DCs and by extension RA. There is, however, currently some controversy as to what effect CRP has on DCs. A report looking at several proteins present ubiquitously in human plasma showed no affect of CRP on monocyte-derived DCs (MDDCs) at baseline levels (5µg/mL) and an inhibition of activation at acute phase levels (500µg/mL) (54). Another report shows that treatment with 10µg/mL CRP during development of MDDCs caused a reduction in the percentage of cells expressing the costimulatory molecules CD209, CD40, CD80, and CD86, a reduction in phagocytosis by treated DCs, and that these CRP-treated DCs stimulated less T cell proliferation from allogeneic cells when the DCs were irradiated, eliminating their ability to dynamically communicate with the T cells (55). The most recent report looked at the response of MDDCs to overnight CRP treatment at varying doses and found an increase in the costimulatory molecules CD40, CD80, CD83, and CCR7, with CD40 and CD80 showing a dose-dependent increase. This report also showed a significant increase in proliferation of T cells when autologous peripheral blood lymphocytes were incubated with overnight CRP-treated (60µg/mL) MDDCs for 8 days (56).

**CRP and arthritic disease**

With all the evidence showing that DCs and FcγRs are important factors in the pathogenesis of RA, and knowing that CRP interacts with DCs through FcγRs, we hypothesized that CRP is not simply a marker of inflammation and disease severity in
RA, but that steady-state levels of CRP modulate DCs, and in turn T cells, and that in this fashion CRP actually participates in the pathogenesis of RA.

We show herein that CRP deficiency exacerbates CIA, whereas transgenic overexpression of human CRP ameliorates it. Additionally, we provide new evidence for CRP’s influence on DCs and T cells, shedding light on a possible mechanism by which CRP exerts its protective effect. Deviation from normal CRP level (deficiency or transgenic overexpression) modestly increased total DC number and significantly altered the distribution of DCs throughout the peripheral lymphoid organs we tested. Treatment with CRP during development of BMDCs significantly increased surface expression of several activating costimulatory molecules including CD40, CD80, CD86, and OX40L, and CRP treatment during coculture of BMDCs and T cells significantly increased proliferation of T cells. Finally, we address the split paradigm of differential influence of CRP in early and late disease by showing that pharmacological lowering of CRP levels in mice with established CIA is, in fact, therapeutic. CRPTg mice with established disease that had their human CRP level significantly lowered with an antisense oligonucleotide specific for CRP saw significant improvement in the severity of their CIA disease. We also report the beginning of clinical trials of a safe, efficacious CRP-lowering drug.
COLLAGEN-INDUCED ARTHRITIS IS EXACERBATED IN C-REACTIVE PROTEIN-DEFICIENT MICE

by

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Format adapted and errata corrected for dissertation
Abstract

Objective: Blood C-Reactive Protein (CRP) is routinely measured to gauge inflammation. In rheumatoid arthritis (RA), heightened CRP is predictive of a poor outcome and lowered CRP indicative of a positive response to therapy. CRP interacts with the innate and adaptive immune systems in ways that suggest it may be causal in RA and, although this is not proven, it is widely assumed CRP makes a detrimental contribution to the disease process. Paradoxically, animal studies indicate CRP might be beneficial in RA.

Methods: We compared the impact of CRP deficiency versus transgenic over-expression on the inflammatory and immune responses using CRP deficient mice (Crp−/−) versus human CRP transgenic mice (CRPTg), respectively, and we compared the susceptibility of wild type, Crp−/−, and CRPTg to collagen-induced arthritis (CIA), a disease that resembles RA in humans.

Results: CRP deficiency significantly altered the inflammatory cytokine response evoked by challenge with endotoxin or anti-CD3 antibody, and heightened some immune responses. Compared to CIA in wild type mice, CIA in Crp−/− progressed more rapidly and was more severe whereas CIA in CRPTg was dramatically attenuated. Despite these disparate clinical outcomes, anti-collagen autoantibody responses during CIA did not differ among the genotypes.

Conclusion: CRP exerts an early and beneficial effect in mice with CIA. The mechanism of this effect remains unknown but does not involve improvement of the autoantibody profile. In humans the presumed detrimental role of heightened blood CRP during active RA might be balanced by a beneficial effect of baseline CRP manifest during the pre-clinical stages of disease.
Rheumatoid arthritis (RA) is a chronic, debilitating disease characterized by systemic inflammation and erosive destruction of the joints [1,2]. The hands and feet are the most commonly affected sites, but the disease can affect other joints such as the elbow, shoulder, knee, and hip [3]. Several theories have been proposed to explain the underlying mechanisms of RA, but none has been universally accepted nor conclusively demonstrated. Since the discovery of rheumatoid factor (RF; antibodies against the Fc portion of immunoglobulin G) it has been postulated that RA is an autoimmune disease [1]. It is thought that RF interacting with the Fc portion of IgG promotes formation of immune complexes that activate the complement system and bind to various Fc receptors (FcRs), thereby contributing to inflammation associated with RA [1,2, 4]. In concert with the autoimmune model, various kinds of inflammatory cells (macrophages, dendritic cells, etc) infiltrate the synovium of patients with RA [1,2] and are also thought to exert influence on the disease’s onset and clinical course. A critical role of T-cells is postulated; their interaction with macrophages, fibroblasts, and other cells thought to contribute to the production of deleterious cytokines (eg. IL-2, IL-4, IL-10, and IFN-γ) [1].

C-reactive protein (CRP) is a widely used blood marker of inflammation [5], and growing evidence indicates it plays an active role in host defense [6] and certain cardiovascular diseases [7]. It has long been recognized that in RA patients the concentration of CRP in the blood correlates positively with disease severity and progression [8]. Like RF, CRP can form immune complexes that activate complement [9, 10] and bind to FcRs [11, 12], so it is not unreasonable to predict CRP also participates in the RA disease process. Indeed, although many of CRP’s functions arguably are effected
in the fluid phase [13], CRP is found within the arthritic joint [13, 14] and synovial fluid [15], and its presence there can be used to differentiate inflammatory from non-inflammatory arthritis [15]. CRP blood level has also been incorporated into clinical algorithms used to measure RA disease activity [16]. Despite all of this “guilt by association” still little is known about the biology of CRP in the context of arthritis. In fact no human study to date has directly investigated the contribution of CRP to RA, and the animal studies performed so far have had mixed results. For instance early studies of experimentally induced arthritis in rabbits established that the serum was the source of synovial CRP [17], and that intra-articular injection of (rabbit) CRP elevated knee joint temperature if arthritis was present but not if the joint was healthy [18]. These findings, pointing to CRP as a potentiator of already existing inflammation in RA, are in alignment with the clinical observations. In contrast, a more recent study of experimentally induced arthritis using rabbit CRP transgenic mice [19] showed that (rabbit) CRP was protective with the protective effect being exerted during a short time at the very beginning of disease initiation. The potential relevancy of this observation to the pre-clinical stages of RA has still not been investigated.

To gain new evidence for a contribution of CRP to RA, for the first time we used human CRP transgenic mice (CRPTg) [20, 21] in tandem with a newly engineered CRP deficient strain (Crp−/−) [this report] to examine the strength and direction of CRP’s contribution to inflammation, immunity, and collagen-induced arthritis (CIA). We found that compared to wild type mice; (a) Crp−/− expressed less blood TNF-α and IL-10 and more IL-6 following i.p. endotoxin challenge and less blood IFNγ, IL-2, and IL-4 after i.v. injection of anti-CD3 antibody, (b) Crp−/− had an enhanced antibody response to
vaccination with the thymus independent antigen TNP-ficoll, and \( C r p^{-/-} \) exhibited more rapid clinical progression and more severe clinical symptoms of CIA. Conversely, CRPTg, which we previously showed make less IL-10 [22], had a weaker immune response to TNP-ficoll, mounted a robust human CRP acute phase response during the inductive phase of CIA, and had only mild symptoms of disease. Surprisingly, the aggressiveness of CIA in \( C r p^{-/-} \) and its mildness in CRPTg was not because of heightened or depressed autoantibody responses, respectively, as the anti-collagen II responses of \( C r p^{-/-} \) and CRPTg were not different from that of wild type. We do not dispute that CRP might have a detrimental effect during active RA, but we caution that this can only be achieved in the context of already established disease. Our new observations, which align best with earlier ones made by others [19], indicate that CRP is probably beneficial during the early stages of experimentally induced arthritis in mice and during the pre-clinical stage of RA in humans.
Materials and Methods

**Animals:** Transgenic mice carrying lox \( P \) targeted \( Crp \) alleles (floxed \( Crp \)), wherein exon 2 of \( Crp \) was flanked by lox \( P \) sites (Fig. 1A), were generated using a conditional targeting vector derived using the Lambda KOS system (Lexicon Pharmaceuticals, Inc.). Floxed \( Crp \) mice were mated with protamine-Cre recombinase transgenic mice to generate hybrid offspring carrying one Cre-deleted \( Crp \) allele (Fig. 1A). These were intercrossed to generate CRP deficient mice (\( Crp^{-/-} \)) and backcrossed to the C57BL/6 strain (The Jackson Laboratories). Quantitative real-time PCR (qRT-PCR) and Western blot assays performed using standard methods confirmed absence of CRP mRNA (Fig 1B) and protein (Fig 1C) in \( Crp^{-/-} \). ELISA done on acute-phase sera (collected 24hr after i.p. injection of endotoxin) confirmed absence of blood CRP in \( Crp^{-/-} \) and approximately 50% reduction of CRP in \( Crp^{+/+} \) (Fig 1D). The human CRP transgene, its detection by PCR, and its human-like expression in CRPTg (also strain C57BL/6) have been fully described elsewhere [20,21]. Human CRP is present in the blood of CRPTg at concentrations relevant to humans [5] i.e., low levels under steady-state conditions (<1 to 30 \( \mu \)g/ml) and high levels during an endotoxemia or infection-induced acute phase response (100 to 500 \( \mu \)g/ml). Mouse CRP is still expressed in CRPTg, but mouse CRP is not a major acute phase protein [23]. For certain experiments \( Crp^{-/-} \) were reconstituted (by breeding) with CRPTg to generate mice that express only the human form of CRP.

Mice were housed at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12 hour light cycle (6 AM to 6 PM), and maintained ad libitum on sterile bottled water and regular chow (Harlan Teklad). Mice were 8-12 weeks old when used and both sexes were combined unless specifically noted. All animal use protocols were approved by the
Institutional Animal Care and Use Committees at the University of Alabama at Birmingham and Boehringer Ingelheim Pharmaceuticals and were consistent with the *Guide for the Care and Use of Laboratory Animals* (NIH publication 96-01, revised 1996).

**Measurement of inflammatory and immune responses:** To cause sterile peritonitis, 0.5 ml of zymosan A (Sigma-Aldrich; 2 mg/ml in sterile 0.9% NaCl) was injected i.p. into each animal. Mice were sacrificed 4 h later and their peritoneal cavities lavaged with ice-cold phosphate buffered saline (PBS). The total leukocyte count was determined for the peritoneal exudates and differential cell counts performed using Giemsa-stained cytospin preparations. Aseptic endotoxemia was induced by i.v administration of 200 μl pyrogen-free 0.9% NaCl containing 200 ng Escherichia coli lipopolysaccharide (LPS) serotype 055:B5 (Sigma) plus 1 mg D-galactosamine (D-GalN; Sigma). One hour later mice were anesthetized and blood collected. To activate T-cells, mice received 10 μg of hamster anti-mouse CD3 antibody (eBioscience) i.p., and blood was collected 3 hours later. To measure the delayed-type hypersensitivity (DTH) response, mice were immunized s.c. with 100 μg of ovalbumin (OVA fraction V) emulsified with 50 μl complete Freund's adjuvant (both from Sigma) in 50 μl. Six days later, each mouse was challenged by injection (into one ear pinnae) of 200 μg OVA in 10 μl of saline. The DTH response was assessed by measuring the thickness of the ear prior to and 24 h after challenge using an engineer’s micrometer (Mitutoyo 2804F-10); the difference between the two measurements giving an index of ear swelling. Carrageenan induced paw edema was induced by giving an intraplantar injection of 0.3% carrageenan (Sigma) in a 20 μl
volume into the left hind paw using a 26 gauge needle. Paw volume was measured by determining fluid displacement upon immersion in water, and the difference in volume measured prior to and 3 h following carrageenan administration is reported. To measure the antibody response mice were immunized via i.p. injection of 10 \( \mu \)g TNP-Ficoll (Biosearch Technologies), and pre- and post-immunization plasma was obtained to determine anti-TNP antibody titers. The methodology used to measure in vitro proliferative responses and IFN\( \gamma \)-producing ability of isolated mixed splenocytes, induction of dinitrofluorobenzene (DNFB) and fluorescein isothiocyanate (FITC) induced contact hypersensitivity (CH), and induction of cutaneous anaphylaxis are detailed in the Supplementary Information.

**Collagen-induced arthritis:** Collagen-induced arthritis was elicited using a previously described protocol [24]. Briefly, complete Freund’s adjuvant (CFA) containing 4mg/ml *Mycobacterium tuberculosis* (*M.tb*) was emulsified 1:1 with a 4 mg/ml solution of chicken type-II collagen (CII). CFA and CII were from Chondrex, Inc. (Redmond, WA). At the start of each experiment (day 0), 100 \( \mu \)l 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. On day 21, a booster injection (100 \( \mu \)l of CII emulsified in incomplete Freund’s adjuvant, IFA) was administered at a site contralateral to the primary injection site. Thrice weekly thereafter, and until day 50, the clinical signs of arthritis were recorded for each paw. The clinical scoring system we used was described by Brand et al. [25] 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 to score mice (NRJ, MAP, AJS) and we verified the accuracy of our scoring by micro computerized tomography (micro-CT) and histological analysis of representative arthritic limbs [26] (see Supplemental Information, Fig S1). For statistical analysis a mouse was considered to have presented with CIA on the day its clinical score reached 2, and a mouse was considered to have full blown CIA only if its symptoms were sustained thereafter. The rate of progression of disease was estimated by calculating the slope of the linear regression of days since clinical presentation (abscissa) versus clinical score (ordinate). This regression was done on data collected for the first week following disease onset.

**Measurement of CRP, cytokines, and antibodies:** Anti-TNP ELISA used TNP-BSA coated plates (Biosearch Technologies), biotinylated goat anti-mouse IgM (Caltag), and streptavidin-HRP (Caltag). Plasma cytokines were measured using ELISA kits from R&D systems. Serum mouse CRP was measured using the mouse C-Reactive Protein kit from Life Diagnostics, Inc. according to the manufacturer’s instructions, and human CRP was measured using an ELISA developed in our laboratory [21]. The latter does not detect mouse CRP and has a lower limit of detection of approximately 20 ng of human CRP per ml of mouse serum. Anti-CII IgG was measured using ELISA grade CII and mouse anti-CII IgG standards from Chondrex, Inc (Redmond, WA).
**Statistical Analysis:** All pooled data are expressed as the mean ± SEM, without transformation, and the sample size is given. Group comparisons were done using unpaired Student’s t-tests or with one-way ANOVA followed by post-hoc pairwise least-squared difference (PLSD) 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 or Statview 5.0.1.
Results

**CRP deficiency alters inflammatory and immune responsiveness.**

*Crp* deletion altered the inflammatory response to endotoxin challenge; *Crp*⁻/⁻ having a significantly weakened serum IL-10 and TNFα response compared to wild type (71 ± 11 versus 179 ± 17 pg/mL IL-10 and 1498 ± 201 versus 2786 ± 395 pg/mL TNF-α, respectively) and a significantly strengthened IL-6 response (4007 ± 117 versus 3193 ± 109 pg/mL IL-6, respectively) (Fig. 2A). Carrageenan induced paw inflammation was also affected, being significantly worsened in *Crp*⁻/⁻ compared to wild type (0.54 ± 0.05 ml of swelling versus 0.40 ± 0.03 ml, respectively) (Fig 2B). However, *Crp* deletion had no effect on the total number of inflammatory cells recruited to the body cavity during zymosan-induced peritonitis (data not shown) nor the proportion of each type of inflammatory cell (neutrophils, monocytes, mast cells, eosinophils) recruited to the body cavity (Fig 2 C). Likewise, deletion of *Crp* had no effect on FITC or DNFB induced contact hypersensitivity and OVA induced active cutaneous anaphylaxis (Supplementary Information, Fig S2). These results suggest that under certain circumstances, mouse CRP dampens the inflammatory response.

For mice treated with a T cell targeting anti-CD3 antibody, the plasma IL-2 and IFN-γ responses of *Crp*⁻/⁻ were significantly impaired compared to wild type (666 ± 63 versus 1267 ± 134 pg/mL IL-2 and 293 ± 28 versus 538 ± 16 pg/mL IFN-γ, respectively) (Fig. 3A), whereas the IL-4 response was not. The impaired production of these cytokines in *Crp*⁻/⁻ given anti-CD3 was likely due to a trans-effect of FcγR-bearing cells, and not because of an intrinsic defect in T cells, as stimulation of *Crp*⁻/⁻ mixed splenocytes with
anti-CD3 in vitro induced less proliferation and less IFN-γ production than wild type splenocytes only if the anti-CD3 was presented in soluble form (see Supplementary Information, Fig S3). Further, T cell proliferation and cytokine production by Crp<sup>−/−</sup> versus wild type splenocytes did not differ if splenocytes were directly stimulated with the superantigen SEB or the mitogen ConA (see Supplementary Information, Fig. S3). In accordance with this interpretation, Crp deletion significantly strengthened the DTH response to OVA (Fig. 3B), a response thought to be T cell initiated but macrophage driven [27]. Interestingly, the antibody response of Crp<sup>−/−</sup> immunized with the thymus independent antigen TNP-Ficoll was significantly stronger than that of wild type (Fig. 3C) whereas that of CRPTg was weaker (Fig. S4). These results suggest that under certain circumstances mouse CRP can influence both T cell and B cell responses, albeit indirectly.

**CRP deficiency exacerbates CIA.**

Given the observed pleiotropic effects of mouse CRP deficiency and human CRP expression on inflammatory and immune responses in mice, the clinical data linking rise and fall of blood CRP to worsened and improved RA severity, respectively, and the paradoxical reports of CRP mediated benefits in mouse models of RA, we investigated the impact mouse CRP deficiency and human CRP excess each have on the incidence, onset, and progression of CIA. Micro-CT and histopathological analysis (Fig. S1) revealed gross pathology that was typical of mouse CIA, with no readily observable differences among the genotypes. Likewise incidence of CIA was similar among the 3 genotypes and its clinical onset was uniformly manifest at approximately 4 weeks after
induction (Table 1). Importantly however, following its clinical presentation the progression of CIA was fastest (Table 1, Fig 4A) and its severity was greatest (Fig. 4, B and C) for Crp−/−. Conversely for CRPTg, in which elevation of human CRP well above baseline values was observed (Fig. 5A), the tempo of CIA was slowest (Table 1, Fig 4A) and its severity lowest among the three genotypes (Table 1, Fig. 4,A-C). Importantly, an experiment done using Crp−/− mice reconstituted (by breeding) with the human Crp transgene showed that replenishment with human CRP restores resistance to CIA (Fig S5). Despite the disparate effects, the anti-CII IgG autoantibody responses of wild type, Crp−/−, and CRPTg were not different (Fig. 5B). These findings show that CRP exerts a significant and beneficial effect on the development of arthritis in mice – an effect seemingly unrelated to the autoantibody response.
Discussion

Our comprehensive comparison of wild type, Crp\(^{-/-}\), and CRPTg mice reveals that CRP exerts a significant influence on both the inflammatory and immune responses. At least in mice, CRP actively participates in these processes and is not simply associated with them. CRP has been shown to have numerous effects in vitro that indicate it should be able to interact with the inflammatory and immune systems on multiple levels in vivo. For example, the protein’s interaction with phosphocholine [9, 28] on biologically relevant ligands [6, 7, 29] could allow CRP to target the sources of inflammation, whereas its interaction with the complement proteins C1q and Factor H [30] could allow it to influence immune complex formation and its interaction with various FcRs [11, 12, 21] could allow it to influence antigen presentation and thereby cellular and humoral immunity. Based on these known abilities of CRP it is perhaps not so surprising that Crp deletion influenced the cytokine response evoked by challenge with both endotoxin and anti-CD3 antibody, altered the delayed type hypersensitivity response to OVA, and strengthened the antibody response to immunization with the thymus independent antigen TNP-Ficoll. In fact we showed earlier that transgenic expression of human CRP had the opposite effects on some cytokine and immune responses, for example leading to increased production of IL-10 [22] and decreased production of IgM autoantibodies [31], and we showed here that the immune response to TNP-Ficoll was weaker in CRPTg than wild type (Fig. S4).

As expected based on its effect on inflammatory and immune responsiveness, CRP deletion did influence the course of CIA. In alignment with an earlier report showing that elevation of rabbit CRP during induction of antigen-induced arthritis suppressed disease
in rabbit CRP transgenic mice [19], our data from $Crp^{−/−}$ and CRPTg together suggest an early and beneficial effect of CRP in CIA. Notably compared to wild type mice, $Crp^{−/−}$ showed more rapid progression of disease with more severe symptoms, whereas CRPTg showed slower progression of disease with milder symptoms. As expected replenishment of $Crp^{−/−}$ with the human $Crp$ transgene restored their resistance to CIA. In their sum these findings strongly suggest that CRP confers benefit during the early inductive phase of CIA. We think this beneficial effect requires accessory cells. Indeed, in preliminary experiments wherein we induced arthritis by direct injection of arthritogenic monoclonal antibodies, thereby bypassing the need for accessory cells and T cells, we have observed no difference in onset, progression, or severity of arthritis in wild type versus $Crp^{−/−}$ mice (data not shown).

At first glance our findings might seem to be in conflict with the large body of evidence showing that higher blood CRP level associates positively with worsening of symptoms in RA patients. With one important caveat, i.e. that our observations in mice with CIA might not extrapolate to humans with RA, we believe this apparent paradox can be resolved. We propose that during health, baseline blood CRP is sufficient to enable tonic suppression of inflammation that would otherwise predispose to autoimmunity. Thus in mice, deletion of $Crp$ renders animals prone to a more rapidly evolving and more severe form of CIA because the inflammatory response is de-suppressed, whereas transgenic overexpression of $Crp$ has the opposite effect. It is important to understand that in the mouse model we employed, the influence of CRP (or lack of CRP) is exerted (or not) already prior to disease induction. In humans this beneficial, tonic-suppressive effect of CRP would go unrecognized as it would be manifest during the pre-clinical
stage of RA. In the context of clinically diagnosed, active arthritis on the other hand, levels of blood CRP could be raised either in response to worsening of the associated inflammation, as most assume, or in an effort to dampen it, as we propose. This model fully accounts for our findings and the positive association of elevated blood CRP level with symptoms of ongoing RA.

In this study we provide the first direct evidence that CRP deficiency alters the course of experimentally induced arthritis in mice. Others recently described a separately generated CRP deficient C57Bl/6 mouse strain [32], but whether that strain – like ours – is susceptible to collagen-induced arthritis or not remains to be tested. Regardless, our observation of worsened CIA in the CRP deficient mouse suggests that baseline CRP maintains health rather than promotes disease. Further study is needed to identify the means by which baseline CRP provides tonic suppression of inflammation and autoimmunity in CIA, but based on observations we made in another model of experimentally induced autoimmunity [22,33] we think the beneficial effect of CRP is likely mediated by FcγRIIB expressing cells. The disruption of a protective CRP → FcγRIIB pathway may be one reason why FcγRIIB deficient mice also show increased susceptibility to CIA [34]. Our findings suggest we may have to rethink CRP epidemiology as it relates to CRP biology in the context of arthritis. Since patients seldom visit rheumatologists before symptoms of RA manifest, much of the data pointing to a positive correlation of higher blood CRP level with worsening disease is from patients with active RA rather than pre-clinical RA. Our model predicts that in healthy people baseline blood CRP level should be inversely correlated with future RA risk. In people whose baseline level of CRP is insufficient to maintain the tonic-suppressive
effect there is progression of disease. Ultimately an observational study will be needed to validate these predictions. In the future we hope to illuminate CRP’s mechanisms of action in arthritis and point the way toward novel therapies.
ACKNOWLEDGEMENTS

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Supplementary Information

Supplementary Methods

Contact hypersensitivity (CH) and passive cutaneous anaphylaxis (PCA): To elicit a CH reaction mice were painted on the shaved abdomen with 200 µL of 1% fluorescein isothiocyanate (FITC) or the same volume of 0.5% 2,4-dinitrofluorobenzene (DNFB) (both from Sigma) dissolved in acetone : dibutylphtalate, 1:1. Six days later mice were challenged by painting the dorsal side of one ear pinna with 10 µL of FITC or DNFB. The CH response was determined 24 hours after challenge by measuring ear thickness as described in the main text for DTH. For PCA, one ear was injected with 10 µl monoclonal anti-dinitrophenyl (DNP) clone SPE-1 (a mouse IgE produced on location at Boehringer Ingelheim). In this case ear thickness was measured before and 24hr after injection of anti-DNP, to ascertain the background swelling reaction. To stimulate the PCA reaction, mice were then injected intravenously with 100 µl of 0.3 mg/ml DNP-human serum albumin (SIGMA A-6661) in PBS, and 10 minutes later ear swelling was measured a final time. The resulting change in ear thickness is proportional to the mast cell-mediated inflammatory response.

Splenocyte isolation and stimulation: Spleens were isolated from wild type and Crp−/− mice and single-cell suspensions prepared by crushing through a 40-µm cell strainer (BD Pharmingen). Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 100 mM EDTA, pH 7.4), and the remaining cells washed twice with RPMI 1640 (BioWhittaker Europe). Splenocytes were suspended in fresh medium
(RPMI 1640 with l-glutamine, 5% autologous serum, 5% antibiotic-antimycotic (Invitrogen Life Technologies), seeded into 96-well flat-bottom culture plates at a cell density of $1 \times 10^6$ cells/well in triplicate, incubated at 37°C in 5% CO$_2$, and stimulated. Stimulation was with 6.25 ng/ml phorbol 12-myristate 13-acetate (PMA) in combination with 625 ng/ml ionomycin (calcium salt from Strepotmyces conglobatus (SIGMA), 1.25 μg/ml T-cell mitogen concanavalin A (ConA; Sigma. St. Louis, MO), 6.25 μg/ml Staphylococcal enterotoxin B (SEB; Sigma. St. Louis), or 1.25μg/mL anti-TCR α-mouse CD3ε (Pharmingen), all in an end-volume of 200 μl. Anti-CD3 was presented either in solution (anti-CD3sol) or as a plate-bound version (anti-CD3pb). Supernatants were harvested after 48h and cytokine levels assessed by ELISA (R&D Systems). Separate plates were used to measure cell proliferation, and in this case 16 hours prior to harvesting the cells were pulsed with 0.5 μCi $^3$H-thymidine. $^3$H-thymidine incorporation was determined using a liquid scintillation counter.

**Joint Histology and Imaging:** Fore- and hind-limbs were removed from humanely euthanized mice, the soft tissue removed, and the articulated bones fixed in 10% formalin. Decalcified bones were embedded in paraffin and sectioned (5 μm) and serial sections were stained with haematoxylin and eosin (H&E). To assess bone density and volume we performed micro computerized tomography scans as described [1] using a high resolution μCT imaging system (μCT40; SCANCO Medical, Wayne, PA). The region of interest was the tarsals and metatarsals of the paw. Histological analyses and micro-CT scans confirmed the development of typical CIA in each genotype and verified the validity of our clinical scoring system (Supplementary Figure S1).
Supplementary References:

Table I. Clinical traits of collagen-induced arthritis in CRP deficient (Crp<sup>−/−</sup>), wild type, and human CRP transgenic (CRPTg) mice.

<table>
<thead>
<tr>
<th></th>
<th>onset (mean ± sem days)</th>
<th>incidence (%)</th>
<th>progression* (slope, r²)</th>
</tr>
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<tbody>
<tr>
<td>Crp&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>28.18 ± 2.53</td>
<td>40.7</td>
<td>0.757, 0.945</td>
</tr>
<tr>
<td>wild type</td>
<td>28.18 ± 1.87</td>
<td>30.8</td>
<td>0.365, 0.924</td>
</tr>
<tr>
<td>CRPTg</td>
<td>27.50 ± 2.35</td>
<td>32.0</td>
<td>0.083, 0.570</td>
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* The slope and r² value for the linear regression of days since presentation with arthritis versus clinical score, calculated for days 1 through 7 (see Figure 4A).
Figure 1: Targeted deletion of the mouse Crp gene. CRP deficient mice were generated by Cre-Lox recombination wherein exon 2 (E2) of the Crp gene was flanked by loxP sites to direct Cre-recombinase mediated deletion (A). The arrows indicate positions and directions of elongation of sense and anti-sense primers (BL25-3, BL25-33, BL25-27) used to discriminate Crp from floxed Crp and Cre-deleted Crp. The resultant CRP deficient mice (Crp^{-/-}) do not express Crp mRNA (B; quantitative real-time PCR of liver total RNA from 3 wild type mice and 3 Crp^{-/-} mice) nor do they express the CRP protein (C; Western blot of plasma proteins from mice shown in panel B and D; ELISA done on sera from n = 5 mice of the indicated genotype). The arrow in panel C points to the position of migration of the mouse CRP monomer.
Figure 2: Targeted deletion of mouse Crp alters the inflammatory response.
Compared to wild type mice (dark bars), Crp−/− mice (open bars) had significantly less serum IL-10 and TNF-α and significantly more serum IL-6 after endotoxin challenge (A; n = 23 mice of each genotype. Note break in y-axis.) and a significantly greater inflammatory response to carrageenan injection (B; n = 13 Crp−/− and n = 20 wild type). In contrast, recruitment of peritoneal exudates cells (PECs) into the peritoneal cavity during zymosan-induced peritonitis was unaffected. (C; n = 12 mice per genotype). The results shown in each panel are from 2 separate experiments. In panel A, * and ** indicates p < 0.006 and p < 0.0001, respectively, for PLSD tests. In panel B, * indicates p = 0.01 for Students t-test.

Figure 3: Targeted deletion of Crp alters the immune response.
The serum cytokine response to i.p. administered anti-CD3 antibody was reduced for Crp−/− (open bars) compared to wild type (dark bars) (A; n = 21 mice per genotype), whereas the ovalbumin induced delayed-type hypersensitivity response (B; n = 10 mice per genotype) and the TNP-Ficoll elicited anti-TNP antibody response (C; IgM antibodies measured on day 7 after immunization, n = 4 females and 5 males of each genotype) were significantly enhanced. The results shown in each panel are from 2 separate experiments. **, p < 0.002 for PLSD tests (A, C) or t-test (B).
Figure 4: CIA is exacerbated by targeted deletion of the mouse Crp gene and dampened by transgene-expressed human CRP.

CIA was induced in wild type (•, n = 36), Crp/− (○, n = 27), and CRPTg (●, n = 25) mice and arthritis symptoms were monitored for 50 days, as described in the Materials and Methods. Following initial clinical presentation, Crp/− had the most rapid progression of disease, whereas disease was nearly static for CRPTg (A; see also Table 1). Furthermore, compared to wild type mice, disease symptoms were significantly worse in Crp/− (open bars) and significantly better for CRPTg (black bars) than for wild type (gray bars) (B and C). The results shown are from 3 separate experiments. *; p < 0.0001 for ANOVA and **; p < 0.0001 for PLSD tests.
Figure 5: Deletion of mouse C-reactive protein (CRP) do not alter the anti-type II collagen (anti-CII) IgG response during collagen-induced arthritis. (A) Robust elevation of serum human CRP level (filled circle) was observed in CRPTg one day after initial immunization with CII and CFA (30-fold increase above baseline) and again one day following the booster injection (3.6-fold increase), and levels of human CRP slowly returned to normal during the symptomatic phase. In comparison, in the same animals mouse CRP (open circle) was only modestly elevated. (B) Serum anti-CII IgG levels did not differ between Crp−/− (open circle), wild type (filled diamond), and CRPTg (filled circle).
Supplementary Figure 1: Good agreement between visual scores of CIA, bone density and bone volume measured by micro-CT and bone histology. Representative forelimbs and hindlimbs with visual scores of grade 0, 2, and 4 were analyzed by micro-CT scanning (top) and H&E staining (bottom). Note that bone erosions evident by micro-CT (top) become more apparent as visual score increases, and that both the calculated bone density and bone volume correlated with the visual score (middle graphs). The lower micrographs show H&E stained sections of a healthy joint (visual score of 0) and a diseased one (visual score of 4).
Supplementary Figure 2: No difference in FITC and DNFB contact hypersensitivity (CH) and OVA induced passive cutaneous anaphylaxis (PCA) between wild type and Crp<sup>−/−</sup> mice. Each experiment was done using <i>n</i> = 10 - 12 mice of each genotype.
Supplementary Figure 3: Mixed splenocytes from Crp<sup>−/−</sup> mice respond less robustly to anti-CD3 if anti-CD3 is presented in solution. (A) The proliferative response of mixed splenocytes from Crp<sup>−/−</sup> was significantly less than those from wild type when the stimulant used was soluble anti-CD3 (anti-CD3sol), but not when the stimulant used was plate-bound anti-CD3 (anti-CD3pb). Similarly, IFN<sub>γ</sub> production (B) by Crp<sup>−/−</sup> mixed splenocytes was significantly less than wild type only when the stimulant used was anti-CD3sol. The response to phorbol myristate acetate (PMA), concanavalin A (ConA), and Staphylococcal enterotoxin B (SEB) are shown for comparison. The aggregate data shown are from two experiments that used mixed splenocytes from 4 male and 4 female mice of each genotype, with each in vitro stimulant tested in triplicate. *; p<0.05 for t-tests between stimulant-matched groups.
Supplementary Figure 4: Weakened antibody response in CRPTg mice immunized with TNP-Ficoll. Wild type and CRPTg mice were immunized with TNP-Ficoll, and serum anti-TNP antibody levels were measured for 28 days. CRPTg showed a reduced antibody response compared to wild type.
Supplementary Figure 5: Reconstitution with the human Crp transgene restores resistance to CIA in Crp\(^{-}\). Crp\(^{-}\) were bred with CRPTg to obtain mice expressing only human CRP. CIA was induced in 6 Crp\(^{-}\), 6 Crp\(^{-}\)/CRPTg, and 5 wild type mice and clinical scores were measured for 50 days. Human CRP was sufficient to provide resistance to CIA. The abscissa indicates days since the booster injection with IFA (2\(^{\circ}\)).
C-REACTIVE PROTEIN ALTERS DISTRIBUTION, PHENOTYPE, AND FUNCTION
OF MURINE DENDRITIC CELLS

by

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ALEXANDER J. SZALAI

In preparation for (Arthritis & Rheumatism)

Format adapted for dissertation
Abstract

Objective: We have previously reported that C-reactive protein (CRP) deficiency in mice exacerbates collagen-induced arthritis (CIA), a mouse model of rheumatoid arthritis, which is a T cell mediated disease. In order to decipher the mechanism by which CRP impacts CIA disease severity, we examined the affect that CRP has on dendritic cells, which have a significant impact on T cell polarization and activity.

Methods: The distribution of dendritic cells (DCs) in wild type, Crp deficient (Crp<sup>−/−</sup>), and human CRP transgenic (CRPTg) mice was analyzed by flow cytometry. We also examined the effect that CRP has on the phenotype and function of bone marrow derived dendritic cells (BMDCs) by examining surface expression of costimulatory molecules and T cell proliferation in response to antigen by flow cytometry.

Results: Crp<sup>−/−</sup> mice showed fewer DCs in the bone marrow, with an increase in the spleen, CRP treatment increased the surface expression of costimulatory molecules including CD80, CD86, CD40 and OX40L in an FcγR IIB independent manner, and CRP had an overall potentiating effect on T cell proliferation.

Conclusion: The data herein provide a reasonable mechanism by which CRP may impact T cell mediated autoimmune diseases. CRP alters the distribution of DCs in the mouse body and increases most costimulatory molecules on DCs, driving proliferation of T cells.
C-reactive protein (CRP) is a pentameric acute-phase reactant that participates in the pathology of inflammatory and autoimmune diseases as diverse as atherosclerosis (1–4) and experimental autoimmune encephalomyelitis (EAE) (5, 6). We have recently shown that CRP plays a role in the biology of yet another autoimmune disease: collagen-induced arthritis (CIA), an animal model of rheumatoid arthritis (7). While most literature examining rheumatoid arthritis regards CRP as a molecule that is simply an effective measure of systemic inflammation and disease severity (4, 8), we have shown that the presence and amount of CRP makes a significant difference in CIA (7). While our previous study provided good evidence that CRP is involved in CIA, it was not designed to determine by what mechanism CRP exerts its affect. In the current study we have endeavored to discern this mechanism of CRP action.

CRP participates in many different biological interactions that may be of relevance to CIA. CRP can form immune complexes that activate complement (9, 10) and bind to FcRs (11, 12), CRP is found within the arthritic joint (13, 14) and synovial fluid (15), and its presence there can be used to differentiate inflammatory from non-inflammatory arthritis (15). CRP blood level has also been incorporated into clinical algorithms used to measure RA disease activity (8).

FcγRs are membrane bound receptors that bind the Fc portion of IgG, and are expressed on a variety of immune cells, including antigen presenting cells such as DCs. There are three activating (FcγRI, FcγRIII, and FcγRIV) and one inhibitory (FcγRIIB) FcγR known in mice (16). CRP’s interactions with FcγRs are of particular interest to us for several reasons. Based on our previous observations in different experimentally induced autoimmune models (5, 17) we think that CRP’s beneficial effect in CIA may be
mediated by FcγRIIB expressing cells. Also, like Crp−/−, FcγRIIB deficient mice also show an increased susceptibility to CIA (18). Activating FcγRs appear to be involved as well, since mice deficient in the common gamma chain for FcγRI and FcγRIII have been shown to be resistant to experimental arthritis (19). In humans, disease severity has been shown to have a positive association with a functional variant of FcγRIIB (20), and RA patients in remission have displayed upregulation of FcγRIIB on their DCs (21).

CRP’s effects on DCs may have a direct effect on CIA, because CIA is a T cell mediated disease (22), and DCs are the most potent regulators of T cell activation and differentiation (23). The exact effect that CRP has on DCs is currently contested, however. Only three studies have examined this question, all using human monocyte derived dendritic cells (MDDCs), and all showing differing results. The first study looked mainly at activation of MDDCs and showed no affect at baseline levels (5µg/mL) with inhibition at acute phase levels (500µg/mL) (24). The second study found that 10µg/mL of CRP during MDDC development resulted in fewer cells expressing activating costimulatory molecules and that these treated cells effected less T cell proliferation (25). The most recent study found that overnight treatment of MDDCs increased costimulatory molecules and that treated MDDCs potentiated T cell proliferation (26). Our study aims to clarify the topic by examining CRP’s effect on mouse BMDCs and what role FcγRIIB plays in this effect.

Our examination of CRP’s influence on DCs, and by extension T cells, has provided evidence for one mechanism by which CRP exerts this influence. We show herein that absence of CRP increases the number and alters the distribution of DCs in the mouse body with an increase of DCs in the spleen at the expense of the bone marrow. CRP not
only altered the DC composition of major peripheral lymphoid organs, but also imposed phenotypic changes on the DCs themselves. CRP treatment of bone marrow during development into DCs (BMDCs) caused an increase in costimulatory molecules, including CD40, CD80, CD86, and OX40L. Furthermore, CRP treatment was shown to potentiate T cell proliferation. Taken together, our data shed new light on a mechanism by which CRP may affect CIA and potentially other T cell mediated autoimmune diseases.
Materials and Methods

**Animals:** The creation and characterization of $Crp^{-/-}$ has been reported previously (7).

The human CRP transgene, its detection by PCR, and its human-like expression in CRPTg (also strain C57BL/6) have also been fully described elsewhere (27, 28). Human CRP is present in the blood of CRPTg at concentrations relevant to humans (4, 29) i.e., low levels under steady-state conditions (<1 to 30 µg/ml) and high levels during an endotoxemia or infection-induced acute phase response (100 to 500 µg/ml). Mouse CRP is still expressed in CRPTg, but mouse CRP is not a major acute phase protein (30). Wild type C57BL/6 mice were bred in our facility. FcγRIIB deficient mice were purchased from Taconic Farms, Inc. OT II mice (B6.Cg-Tg(TcraTcrb)425Cbn/J) are Tc stage transgenic specific for residues 323-339 of ovalbumin and were purchased from Jackson Laboratories, stock number 004194.

Mice were housed at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12 hour light cycle (6 AM to 6 PM), and maintained *ad libitum* on sterile bottled water and regular chow (Harlan Teklad). Mice were 8-12 weeks old when used and both sexes were combined unless specifically noted. 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* (NIH publication 96-01, revised 1996).

**Cell Culture:** To generate BMDCs, bone marrow from WT or FcγRIIB deficient mice was cultured in RMPI 1640 (Gibco) containing 10% FBS, 1% penicillin/streptomycin (Gibco), 20ng/mL GM-CSF, and 100ng/mL IL-4 for 7 days with media changes on days
3 and 5. Mouse recombinant GM-CSF and IL-4 were purchased from Shenandoah Biotechnology, Inc, (Warwick, PA). BMDCs were cocultured with T cells at a ratio of 1:5 in IMDM with GlutaMAX™-I (Gibco) containing 10% FBS, 1% penicillin/streptomycin (Gibco), 0.1% beta mercaptoethanol (Gibco), 1% non-essential amino acids (Gibco), and 1% sodium pyruvate (Gibco) for 3 days. Human CRP was purchased from US Biological. Anti-mouse CD3 and anti-mouse CD28 antibodies were purchased from Biolegend. All cultures were kept in a 37°C water jacketed incubator at 5% CO₂.

**Flow Cytometry:** Anti-mouse CD86 (V450), OX40L (biotin-PE), CD4 (V450) and CD11b (V500) were purchased from BD, anti-mouse CD11c (PE-Cy7) and CD80 (PerCP-Cy5.5) were purchased from Biolegend, and anti-mouse MHC-II (IA/IE) (AlexaFluor 700), CD40 (FITC), and 7-AAD were purchased from eBioscience. CFSE was purchased from Invitrogen, All samples were run on a BD LSR-II cytometer and analysis was done with BD FACSDiva version 6.1.3.

**Statistical Analysis:** All pooled data are expressed as the mean ± SEM. Group comparisons were done using one-way ANOVA followed by post-hoc Bonferroni’s multiple comparison test. Differences were considered significant when the p value was <0.05. Statistical analyses were performed using Graphpad Prism 3.02.
Results

*Dendritic cell number and distribution is altered by CRP deficiency.*

Having seen that the absence of CRP had a significant impact on CIA (7), and knowing that CIA is a T cell mediated disease (22), we examined *Crp*<sup>−/−</sup> and wild type (WT) mice to determine whether changes in CRP level had an effect on the number and distribution of DCs which are potent regulators of T cell activation and differentiation (23). Spleen, lymph nodes, and bone marrow were isolated from healthy male mice, prepared for flow cytometry, and stained for CD11c, CD11b, and Siglec H to distinguish conventional and plasmacytoid DCs. The total number of DCs was increased by approximately 15% in *Crp*<sup>−/−</sup> (Fig 1A). Furthermore, in *Crp*<sup>−/−</sup>, this increase was accompanied by a shift in the distribution of DCs to the spleen, at the expense of other tissues tested, especially the bone marrow (Fig 1B). Transgenic overexpression of CRP caused a very slight increase in cDCs in the spleen and a greater reduction in the lymph nodes (data not shown).

*CRP alters dendritic cell phenotype in vitro.*

Once we had established that differences in CRP level cause a change in DC number and organ distribution, we wanted to examine whether CRP changes the phenotype of DCs as well. We isolated bone marrow cells from mice and cultured them in RPMI-1640 with GM-CSF, IL-4, and increasing doses of CRP. After 7 days cells were harvested and processed for flow cytometry. CRP did not significantly affect cell
viability (Fig 2A), and the percent of CD11c+ cells generated during culture was only significantly affected at the 100 µg/ml constitutive dose (Fig 2B). In general, CRP caused an increase in surface expression of costimulatory molecules, including CD80, CD86, CD40, and OX40L. With the exception of CD 80, for which there was a dose dependent increase, all significant increases were seen at the 100 µg/ml dose, either constitutively (DCs exposed to CRP since day 0) or with overnight treatment only (DCs given CRP overnight on day 6). In comparison, expression of MHC Class II was increased to a lesser extent, and the β-2 integrin CD11b was even somewhat decreased (Fig 2C). DCs generated from FcγRIIB−/− bone marrow cells showed a similar overall response to CRP, with the exception of expression of MHC Class II where FcγRIIB−/− cells showed a significant increase at both 100 µg/ml treatments (data not shown).

**CRP alters dendritic cell function in vitro.**

Next, we wanted to know if the phenotypic changes we observed with the DCs affected their function. We tested these functional changes by examining how CRP affected the DCs’ ability to serve as antigen presenting cells to T cells. To determine the effect on T cell proliferation, BMDCs were grown for 7 days as described earlier and paired with CFSE-labeled splenic T cells from OT-II mice for an additional 3 days in the presence of increasing doses of CRP or anti-mouse CD3 and anti-mouse CD28 or media alone. After the 3 day co-incubation, cells were harvested and processed for flow cytometry. Representative histograms for CFSE dilution are shown (Fig 3A, B). In the absence of antigen, CRP treatment had no effect on T cell proliferation (data not shown). No effect of CRP on either median CFSE fluorescence or the percentage of T cells that
proliferated was observed (Fig 3C). However, when a more detailed approach of examining specific generations of T cell proliferation was undertaken some significant effects were revealed (Fig 3D). With wild type DCs, 1µg/ml CRP treatment significantly increased the percentage of cells that proliferated 3 or more generations. 100µg/ml CRP treatment significantly lowered the percentage of cells that proliferated 2 generations. Interestingly, when DCs from FcγRIIB −/− mice were used as APCs the effect of CRP was more often significant when compared to wild type. 1 and 10µg/ml CRP treatments significantly decreased the percentage of cells that did not proliferate while increasing the percentage that proliferated 3 or more generations (Fig 3D). Nearly all significant differences were decreases in the percentage of cells that proliferated 0 or 1 generations and increases in the percentage of cells that proliferated 2 or 3 or more generations. Overall, CRP treatment increased proliferation.
Discussion

We have shown previously that CRP is protective against progression of CIA (7), but it does not seem to be due to an FcγRIIB dependent tolerization of DCs as was expected. However, our study provides new details of how CRP modulates the development of dendritic cells and the proliferation of T cells. Large perturbations in baseline CRP level (none or transgenic overexpression) significantly altered the distribution of DCs in the lymphoid tissues we examined. An elevation of DCs in the spleen at the expense of the bone marrow in Crp−/− logically fits with our previous findings, suggesting that CRP deficiency removes a critical brake regulating DC egress from the bone marrow. The increased egress of cells from the bone marrow and augmentation of the spleen population is likely to influence the overall character of the immune system.

CRP treatment significantly affected the surface expression of costimulatory molecules on DCs, which is also widely regarded to be a highly influential factor in the determination of T cell activation and differentiation (31). Our results did not show evidence of a tolerogenic phenotype as expected, but instead showed an increase in the level of surface expression of CD40, CD80, CD86, and OX40L. Recent reports examining the effects of CRP on human monocyte-derived dendritic cells (MDDCs) have shown conflicting results, but one study did show an increase in the level of surface expression of CD40 and CD80, concurring with our dose dependent findings for CD80 (26). Additionally, the reduction in surface expression of CD11b that we see concurs with the increase in T cell proliferation and suggests a possible increase in Th-17 cells (35, 36).
Overall, we found that CRP treatment potentiated T cell proliferation. The overall median CFSE fluorescence and percentage of cells proliferating with CRP treatment were not significantly different from media alone, but examination of the degree to which cells proliferated did show some significant differences and nearly all were increases in the extent of proliferation. The previously mentioned study also found that CRP treated DCs increased T cell proliferation (26). The increases in proliferation with CRP treatment administered during the coculture of DCs and T cells is either a result of fast-acting responses from DCs during coculture or a direct effect on T cells themselves. Studies examining the interaction between CRP and T cells have not been undertaken.

We now know that CRP has an effect on DCs, altering their distribution, increasing costimulatory molecule expression, and potentiating T cell proliferation, but there are other ways that CRP could be affecting DCs as well. CRP is known to bind to FcγRs and mediate phagocytic uptake of bacteria and apoptotic cells (11, 32). This could be of importance since alterations in phagocytosis have been implicated in autoimmune disease (33). Also, it is not known how CRP’s affect on T cell proliferation affects the balance of Th subtypes. A change in the ratios of these subtypes could have major implications in arthritic disease (34).

While our previous study examined the effect of CRP on development of CIA, our current use of Tcr transgenic mice specific for OVA peptide in concert with the activated phenotype we see from our DCs more closely mirrors an established arthritic disease state. As a result of being Tcr transgenic, the vast majority of these T cells are ready and able to quickly mount a strong response upon encountering OVA. Seeing that CRP potentiates DC activation and proliferation of T cells in this setting, it lends
credence to the idea of CRP as a detrimental factor during established arthritic disease. In light of this fact, lowering CRP during established arthritis may prove to be therapeutic.
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Grabbe. 2007. Active MAC-1 (CD11b/CD18) on DCs inhibits full T-cell activation.

Figure 1 CRP affects the number and localization of DCs.
Bone marrow, spleen, and inguinal lymph nodes were harvested from male WT or Crp<sup>−/−</sup> mice and examined by flow cytometry for markers of plasmacytoid (Siglec-H<sup>+</sup>) or conventional (CD11b<sup>+</sup>) dendritic cells (CD1<sup>+</sup>). A: Change in the amount of CRP from wild type levels increased the total number of cells (plasmacytoid plus conventional) by ~15%. B: Crp<sup>−/−</sup> had a significantly altered distribution of both plasmacytoid and conventional dendritic cells with an increased localization to the spleen at the expense of the bone marrow. *, **, and *** are \( p < 0.05, p < 0.01, \) and \( p < 0.001, \) respectively.
Figure 2: CRP increases expression of costimulatory molecules and MHC-II
Bone marrow from wild type mice was cultured with or without CRP to determine CRP’s affect on development of bone marrow derived dendritic cells. A: CRP had no significant effect on cell viability during the course of development. B: Only constitutive treatment with 100μg/mL CRP significantly reduced the percentage of cells that were CD11c+ at day 7. C: CRP significantly increased the surface expression of several costimulatory molecules at day 7. *, **, and *** are $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.
Figure 3: CRP potentiates T cell proliferation.
Bone marrow derived dendritic cells cultured for 6 days, were loaded with OVA overnight, and were then incubated with CFSE labeled, naïve splenic T cells from OT II mice for 3 additional days in the presence of CRP, or anti-CD3 and anti-CD28. Proliferation was assayed by CFSE dilution via flow cytometry. A: Representative histograms for CFSE dilution in controls; B: Representative histograms for CFSE dilution in T cells cocultured with CRP and wild type DCs; Numbers represent CRP treatment amount in µg/mL.
Figure 3: CRP potentiates T cell proliferation.
C: Median CFSE fluorescence and percentage of cells proliferating (dimeric than the parent population) in T cells cocultured with CRP and WT or FcgRIIB deficient DCs. While no significant differences were found, dose dependent trends are evident; D: Percentage of T cells by proliferative generation. * and ** are $p < 0.05$ and $p < 0.01$, respectively.
SELECTIVE INHIBITION OF HUMAN C-REACTIVE PROTEIN IS EFFICACIOUS IN C-REACTIVE PROTEIN TRANSGENIC MICE WITH COLLAGEN-INDUCED ARTHRITIS

by

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Abstract

Objective: Epidemiological and clinical studies of patients with established rheumatoid arthritis (RA) indicate a positive correlation between C-reactive protein (CRP) blood concentration and worsening of symptoms, and CRP can be found deposited in arthritic joints of people with RA. Whether this association arises because blood CRP elevation is a cause for RA or because it is a response to the underlying inflammation is unknown. To address this question in CRP transgenic mice with established collagen-induced arthritis (CIA), an animal model of RA, we tested if specific lowering of human C-reactive protein was of any therapeutic benefit.

Methods: We designed antisense oligonucleotide (ASOs) to specifically target human CRP mRNA and thus inhibit translation of the human CRP protein. We screened 640 ASOs for their ability to impair CRP mRNA production by human Hep3B and primary hepatocytes in vitro and selected three of these (ISIS 329993, ISIS 353491, and ISIS 353512) for validation. The safety and CRP lowering capacity of these 3 drugs was ascertained in vivo using human CRP transgenic mice (CRPTg). The best drug candidate (ISIS 329993) was then used to ascertain if targeted reduction of blood CRP was of therapeutic benefit in CRPTg with established CIA and to confirm that it could effectively reduce blood CRP in healthy human volunteers.

Results: The three ASOs identified were found to significantly reduce CRP mRNA levels in Hep3B cells and primary human hepatocytes stimulated with dexamethasone plus IL-6 and IL-1β, with IC₅₀ values for the most effective compound (ISIS 329993) in the ~8nM
to ~20nM range. In CRPTg after two weeks of treatment with the CRP-specific compounds hepatic human CRP mRNA level was reduced by 51-99% compared to vehicle-treated CRPTg, and plasma human CRP level was lowered by 71-94%. Mouse CRP blood level was unaffected, and all three ASOs were well tolerated in CRPTg mice as evidenced by absence of elevation of serum transaminases. In healthy human volunteers lowering of blood CRP was achieved by treatment with 600mg/kg ISIS 329993, and in CRPTg mice with established CIA lowering of blood human CRP levels by treatment with ISIS 329993 was associated with improvement in the clinical signs of arthritis.

Conclusions: Antisense oligonucleotides provide a safe, specific, and effective way to lower human CRP levels in CRPTg mice and humans. The significant amelioration of CIA disease symptoms in CRPTg mice receiving CRP lowering therapy suggests that human CRP participates in the arthritic process in this animal model. By extension, these results suggest that ASO mediated lowering of human blood CRP might be an effective therapeutic treatment for patients with established RA.
Systemic inflammation and erosive destruction of the joints are hallmarks of rheumatoid arthritis (RA) [1, 2]. The hands and feet are most often affected but other joints are not spared in RA [3], and emerging data indicate that people with RA are at significantly increased risk of cardiovascular disease compared to the general population [33, 34]. Despite considerable research, the causal mechanisms of RA and its sequelae have yet to be conclusively explained. However, since the discovery of rheumatoid factor (RF; antibodies against the Fc portion of immunoglobulin G) it has been widely accepted that RA has an autoimmune origin [1]. Accordingly, RFs interacting with the Fc portion of IgG are thought to promote formation of immune complexes that then activate the complement system and bind to Fc receptors (FcRs), thereby propelling the inflammation associated with established RA [1,2,4]. In alignment with the autoimmune model various types of inflammatory cells (dendritic cells, macrophages, etc) have been shown to infiltrate the synovium of patients with established RA [1, 2], and those cells are thought to exert influence on both the disease’s initial onset and its subsequent clinical course. For example, T-cells are postulated to be critical to RA onset whereas their interaction with fibroblasts, macrophages, and other cells is thought to contribute to production of harmful cytokines (e.g. IL-2, IL-4, IL-10, and IFN-γ) [1] once arthritis is established.

It has long been recognized that in patients with established RA (i.e., clinically documented RA) the concentration of C-reactive protein (CRP) in the blood correlates positively with disease severity and progression [8]. Like RF, CRP can form complement activating complexes [9, 10] and it can bind to various FcRs [11, 12], so it is likely that CRP participates in the RA disease process. Indeed, CRP is found within arthritic joints [13, 14] and in the synovial fluid [15] of patients with established RA, and its presence
there can be used to differentiate inflammatory from non-inflammatory arthritis [15]. Based on these and other findings CRP blood level has been incorporated into clinical algorithms used to measure RA disease activity [16]. Yet despite this “guilt by association” still little is known about the biology of CRP in the context of established arthritis. To date no human study 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 [17] and that intra-articular injection of (rabbit) CRP elevated knee joint temperature if arthritis was established but not if the joint was healthy [18]. This was the first study to point to the possibility that CRP might exacerbate established RA.

Seemingly paradoxically, a study of experimentally induced arthritis in rabbit CRP transgenic mice [19] showed that (rabbit) CRP could also be protective, with that protective effect exerted prior to disease onset. More recently we used human CRP transgenic mice (CRPTg) [20, 21] in tandem with CRP deficient mice (Crp−/−) to examine more closely the strength and direction of CRP’s contribution to the emergence of incident collagen-induced arthritis (CIA, an animal model of RA). The results of our study [22] reinforced those reported by others earlier [19], i.e. we showed that CRP (human and mouse CRP) was beneficial during the pre-symptomatic stages of experimentally induced arthritis. Neither our study [22] nor the one by Jiang et. al. (19), was designed to address the large body of evidence showing that higher blood CRP level associates positively with worsening of RA symptoms in patients with established disease, i.e. we experimentally induced CIA in healthy mice and monitored subsequent onset and progression of incident disease. This is in contrast to the clinical observations
which are limited to the association of blood CRP with waxing and waning of already established RA. In the latter context, blood CRP level likely is raised in response to worsening of underlying inflammation, and thereby could activate complement or otherwise worsen the inflammation.

To ascertain the impact of CRP on ongoing disease and to more realistically model the RA clinical situation we studied herein CRPTg with established CIA. We sought to determine if, as predicted, pharmacological reduction of human CRP blood level might be of therapeutic benefit. To target human CRP we developed an antisense oligonucleotide (ASO) that prevents translation of human CRP mRNA by promotion of its selective degradation. The results show for the first time that this pharmacological strategy works, i.e., exposure to ASOs lowered human CRP mRNA production by Hep3B cells and primary human hepatocytes in vitro and reduced blood CRP levels in CRPTg. The ASO strategy was also safe and effective at lowering blood CRP level in healthy human volunteers. Importantly, pharmacologic lowering of human CRP in transgenic mice with established CIA significantly reduced the clinical signs of arthritis. If the results of these animal studies are translatable to humans, reduction of blood CRP levels could be an effective new therapy for patients with established RA.

**Materials and Methods**

**Antisense oligonucleotides:** We designed 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 # M11725.1). 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. Approximately 640 ASOs were purified as described (35, 36) and evaluated for their ability to reduce dexamethasone plus cytokine (IL-6 plus IL-1β) stimulated CRP mRNA expression in Hep3B cells (37). In those initial screening experiments mRNA was isolated and quantitated by reverse transcriptase polymerase chain reaction (RT-PCR; see below), and the ASOs ISIS 353512, ISIS 329993, and ISIS 353491 were found to significantly reduce CRP mRNA levels. The IC₅₀ value for the lead compound (ISIS 329993) was in the ~8 nM range (Fig 1A). Further assessment using human primary hepatocytes stimulated with dexamethasone plus cytokine (IL-6 and IL-1β) confirmed the potency of these 3 ASOs, with the IC₅₀ value for ISIS 329993 in the ~20 nM range (Fig 1B). Based on these preliminary results ISIS 353512, ISIS 353491 and ISIS 329993 were chosen for further evaluation in vivo. The nucleotide sequences of these lead compounds are as follows (MOE modifications underlined): ISIS 353512: 5’-CCCATTTACGGAGACCTGG-3’; ISIS 329993, 5’- AGCATAGTTAAGAGACCTCCC-3’; ISIS 353491, 5’-GCACTCTGGACCCAAACAG-3’. A fourth ASO (ISIS 141923, 5’-CCTCCCTGAAAGTTCCCTCC-3’), which is not complementary to any known mouse or human gene sequence, served as a placebo.

**Animals:** The human CRP transgene, its detection by PCR, and its human-like pattern of expression in CRPTg have been fully described elsewhere [20, 21]. Human CRP is present in the blood of CRPTg at concentrations experienced by humans [5] i.e., low
levels under steady-state conditions (<1 to 10 µg/ml) and high levels during the acute phase response (30 to 500 µg/ml). Note that mouse CRP continues to be expressed in CRPTg, but mouse CRP was neither targeted by the ASOs tested nor is it a major acute phase protein [23]. Mice were housed at constant humidity (60 ± 5%) and temperature (24 ± 1°C) with a 12 hour light cycle (6 AM to 6 PM) and maintained *ad libitum* on sterile bottled water and regular chow (Harlan Teklad). 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).

**Collagen-induced arthritis:** Collagen-induced arthritis (CIA) was elicited in CRPTg mice using a previously described protocol [24] that in our hands evokes disease in approximately one third of all immunized animals [22]. Briefly, complete Freund’s adjuvant (CFA) containing 4mg/ml *Mycobacterium tuberculosis* (*M.tb*) was emulsified 1:1 with a 4 mg/ml solution of chicken type-II collagen (CII). CFA and CII were from Chondrex, Inc. (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 CII emulsified in incomplete Freund’s adjuvant, IFA) was administered at a site contralateral to the primary injection site. The clinical signs of arthritis were gauged and recorded for each paw by multiple individuals at least thrice weekly thereafter until day 50. The clinical scoring system we used was described by Brand et al. [25] 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. 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 haematoxylin and eosin (H&E) to assess arthritic changes. To confirm changes in bone density and bone volume we performed micro computerized tomography (micro-CT) scans as described [26] using a high resolution µCT imaging system (µCT40; SCANCO Medical, Wayne, PA) available at our institute’s NIH funded ( P30N5057098) Small Animal Phenotyping Core facility. 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 bony changes in the paws of CRPTg with clinical scores ≥ 2.0 (Fig. 2). This verified our visual scoring system and validated that paws with a score ≥ 2.0 had established arthritis.

**Animal Studies:** To confirm that ASOs could achieve CRP lowering in vivo we used male CRPTg. At the start of the experiment (d 0) following appropriate anesthesia, heparinized blood (20 µl) was collected from the retro-orbital plexus of each mouse and then either vehicle (0.9% saline), placebo (ISIS 141923), or one of the 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 were sacrificed, their tissues collected for analyses of human CRP mRNA, and their plasma collected for measurement of human and mouse CRP and mouse IL-6 and transaminases. To test if pharmacological lowering of human CRP blood level might reduce the clinical signs of established RA, we compared the fate of established CIA in CRPTg mice treated with ISIS 329993 versus placebo. Each 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 arthritis was established 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 (Fig. 2). Mice were assigned at random to the two drug treatment groups. Drug treatments ended 30 days later.

**RT-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 RT-PCR using a Prism 7700™ Sequence Detector (Applied Biosystems, Foster City, CA). The primer probes used for human CRP mRNA quantification were as follows:

forward primer 5’-GGCCCTTCAGTCCTAATGTCC-3’,

probe 5’-TCCTGAACTGCGGGCACTGAAG-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).

For normalization we amplified GAPDH mRNA. Following 40 amplification cycles, amplicons were quantitated using SDS analysis software (Applied Biosystems, Foster City, CA).

**Measurement of CRP, IL-6, and alanine aminotransferase (ALT):** We used commercially available kits to measure serum mouse CRP (Life Diagnostics, Inc.), mouse IL-6 (Life Diagnostics, Inc.), and ALT (Sigma, St Louis, MO) according to each manufacturer’s instructions. Human CRP was measured using an ELISA that was developed [21].

**Statistical Analysis:** For statistical analysis a mouse was considered to have presented with 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 thereafter. For each treatment group daily average clinical scores and area under the curve (AUC) was calculated and for each mouse cumulative disease index (CDI; the sum of daily clinical scores obtained during the symptomatic phase) was calculated. The proportion of mice whose clinical score was improved after therapy by 20%, 50%, and 70% (analogous to the American College of Rheumatology ACR20, ACR50, and ACR70 criteria [27]) was determined. Pooled mouse data are expressed as the mean ± SEM of 7 separate experiments and the sample sizes are reported. Group comparisons were done
using unpaired Student’s \( t \)-tests or with one-way ANOVA followed by post-hoc pairwise least-squared difference (PLSD) 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 and Statview 5.0.1.

**Results**

*Pharmacological lowering of human CRP plasma protein and hepatic mRNA levels in CRP\(^T\)g mice*

Human CRP, mouse CRP, and mouse IL-6 plasma levels were 12.7±1.03 µg/ml, 39±5.9 ng/ml, and 30.6±4 pg/ml, respectively, at baseline (day 0) and there were no statistically significant differences in baseline plasma protein levels among the various treatment groups (ANOVAs, \( p \geq 0.815 \)). Administration of each of the three different human CRP specific ASOs resulted in significant reduction of plasma human CRP levels by 7 days after initiation of treatment (\( p \text{<} 0.005 \), one-sample \( t \)-tests). These reductions ranged from 30% lower than baseline (ISIS 353512) to 80% lower than baseline (ISIS 353491) (Fig. 3). By the end of the treatment phase (day 17) CRP was lowered by no less than 71% (ISIS 353512) and by as much as 94% (ISIS 353491) (Fig. 3). Reduction of human CRP levels was not accompanied by lowering of mouse CRP (Fig. 3 inset), attesting to the specificity of each compound. Interestingly, IL-6 was significantly reduced in mice treated with ISIS 329993 (Fig. 3 inset), indicating a secondary anti-inflammatory influence of this ASO. For each human CRP-specific ASO, the CRP-lowering effect was accompanied, as expected, by reduction of hepatic human \( CRP \) mRNA (Fig 4A). Compared to animals administered saline only, \( CRP \) mRNA was
greatly reduced in mice that received the human CRP specific inhibitors ISIS 353512 (34% reduction), ISIS 329993 (52% reduction), and ISIS 353491 (99% reduction).

Importantly, there was no difference in human CRP blood levels measured for mice treated with vehicle versus the placebo control, ISIS 141923 (Fig. 3), and the latter had no affect had no effect on CRP mRNA levels (Fig. 4A). All ASOs were well tolerated as judged by absence of elevation of plasma ALT (Fig. 4B)

*Pharmacological lowering of human CRP blood level is associated with clinical improvement of established collagen-induced arthritis in CRPTg mice*

We recently showed that in CRPTg mice human CRP blood level is robustly elevated (up to 30-fold above baseline) during the inductive phase of CIA [22], likely as the result of the direct stimulating effects of CFA used during immunization. By comparison, during the CIA symptomatic phase human CRP blood levels are less robustly elevated and more variable [22]. Nevertheless, in the present study of CRPTg mice with established CIA (i.e., those with a clinical score ≥ 2.0) treatment with ISIS 329993 (but not placebo) led to significant reduction of human CRP blood levels for up to 20 days after disease establishment (Fig 5A). Beyond this the human CRP lowering effect of ISIS 329993 was not evident. In the same mice mouse CRP levels were not lowered by ISIS 329993 at any time (Fig. 5B), attesting to the specificity of the ASO for human CRP.

This significant, early lowering of human CRP in CRPTg mice with active CIA was of clear therapeutic benefit as evidenced by amelioration of arthritis clinical symptoms in CRPTg receiving the human CRP targeting drug ISIS 329993 (AUC 133.2), but not in those receiving the placebo (AUC 159.4) (Fig. 6). Notably the beneficial effect was most
evident during the timeframe when CRP lowering was most pronounced i.e., up to about day 21. Further, the beneficial effect of ASO 329993 treatment was more pronounced and more prolonged in mice whose disease was milder at the onset of therapy. Thus when we excluded animals whose clinical score at the time of initiation of therapy was > 5.0 (Fig 6 inset), the cumulative disease index (CDI; the sum of daily clinical scores for each mouse) was significantly lower for mice receiving ASO 329993 (98.6±10.33, n=20) than placebo (138.4±18.37, n=17) ($p=0.029$, 1-tailed Student’s $t$-test). Lastly, when all arthritic mice were considered (i.e., regardless of their clinical score at recruitment into the treatment phase) (Fig 6), 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) was always substantially greater for the cohort that received ASO 329993 (Fig.7). Also, the specific drug treated group achieved 50% and 70% improvement more rapidly than did the placebo treated one (Fig.7).

**Pharmacological lowering of blood CRP in humans**

In 8 human subjects enrolled by ISIS pharmaceuticals baseline CRP level ranged from 2 to 5 mg/L. In all subjects that received ISIS 329993 (600mg/wk) blood CRP level was lowered below baseline within 3 weeks of drug dosing (Supplementary Fig 1). Blood CRP lowering was significant 1 and 2 weeks after the last dose of drug (Day 22 and Day 29, respectively) compared with baseline levels ($p=0.0313$). The median reduction in blood CRP measured one week following the last dose (Day 22) was still 76% (range 54 to 83%). CRP levels remained elevated in the 2 subjects who received placebo.
**Discussion**

Notwithstanding the many informative studies of CRP biology performed *in vitro* and in animals, a clinically practical and specific inhibitor of CRP is needed before a true understanding of the physiologic role of this acute phase protein in humans can be ascertained. Ours is not the first attempt at providing one. A small molecule inhibitor of CRP [1,6-bis(phosphocholine)-hexane] has been developed that occludes the ligand-binding ‘B’ face of CRP and thus reportedly blocks its ability to activate complement (32). Since (i) complement activation is known to occur in concert with arthritic disease (26,27), (ii) complement activation products are found in the arthritic joint (25) and (iii), CRP is known to activate complement and co-localize with complement fragments deposited in the arthritic joint (25), then administration of 1,6-bis(phosphocholine)-hexane could presumably be of some benefit in RA patients if CRP is causal in the disease process. Indeed the compound has already been shown to block the exacerbating influence of exogenously administered human CRP in a rat model of experimentally induced myocardial infarction (32). However, since ligand-bound CRP also might interact with FcγRs which are known to contribute to the RA process [35], treatment with 1,6-bis(phosphocholine)-hexane might not block all of the potentially detrimental pathways engaged by CRP in an RA patient. Also, it is not clear what consequences circulating CRP decamers (two pentamers crosslinked by 1,6-bis(phosphocholine)-hexane) might have in RA, since presumably these complexes could be deposited and potentially exacerbate local inflammation. Therefore, despite the fact that 1,6-bis(phosphocholine)-hexane appears to be of use in animal models of heart disease [32], its shortcomings may ultimately limit its therapeutic potential in
autoimmune diseases like RA

Rather than depleting circulating CRP by promoting its clearance from the blood, like 1,6-bis(phosphocholine)-hexane does, the approach to blood CRP lowering that we adopted was to limit CRP protein production altogether using antisense inhibitors to specifically and selectively prevent the translation of CRP mRNA. CRP is very well suited for inhibition using ASO technology because the protein’s expression is regulated mainly at the level of transcription and because it is synthesized primarily by hepatocytes, cells known to readily accumulate antisense drugs and to be sensitive to ASO pharmacology (40, 41, 49). ASOs also accumulate in extrahepatic cells and tissues known to make CRP, such as the kidney, alveolar macrophages, and adipocytes, (25, 41, 44), so the CRP lowering effect is efficient. Also, because of their much longer half-lives compared to small molecule inhibitors (41, 46, 48), ASOs can be administered comparatively infrequently to patients, and the approach has been successfully used to target other proteins not readily amenable to small molecule or antibody based therapeutic interventions [36, 45, 46, 47, 48].

The ASOs we tested here in vitro and in vivo were designed to target human CRP mRNA transcripts. All three of the human CRP targeting inhibitors we identified specifically reduced human CRP mRNA in human Hep3B cells and primary hepatocytes. Moreover, hepatic human CRP mRNA and plasma human CRP levels in CRPTg mice were lowered after only short-term administration (within one week) with a modest dosing regimen (25 mg/kg every 4th day). 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 human and cynomolgus
monkey CRP transcripts, ISIS pharmaceuticals obtained similar results in cynomolgus monkeys using ISIS 329993 (data not shown). Though ASOs are generally proinflammatory in rodents (42, 50), the ASOs we tested here were well tolerated, as evidenced by a lack of elevation of liver transaminase levels. Indeed, IL-6 levels were unchanged or even reduced in these animals.

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 antisense drug suggests that these agents should be useful for intervention in CRP-driven chronic and acute disease processes. We tested this prediction, albeit in an animal model, for the first time and our study provides direct evidence that targeted lowering of human CRP should lessen the severity of established RA. Thus in CRPTg mice with established CIA, treatment with ISIS 329993 lowered both human CRP and arthritis severity. This outcome is in alignment with the positive association of elevated blood CRP level with symptoms of ongoing RA in humans. By extension it is predicted that in humans with established RA, wherein blood CRP is known to be elevated, pharmacological lowering of CRP should be of therapeutic benefit. We note, however, that in CRPTg the CRP-specific ASOs we tested did exhibit tachyphylaxis that might limit its clinical use. Furthermore, as a caution, we propose that blood CRP levels should not be lowered so much that any potentially beneficial effects [22] of the protein are also eliminated.

Despite our compelling results, it still remains unknown whether CRP plays a pathophysiologic role in RA in humans. Ultimately an observational study 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→RA hypothesis. The CRP-specific ASO
inhibitors that we describe here could fill this gap and could provide the impetus for future in vivo pharmacological, toxicological, and ultimately clinical studies that will help clearly delineate the role of CRP in RA in humans. As a step towards this goal ISIS pharmaceuticals has obtained early evidence that ISIS 329993 is well tolerated in humans and very effective at lowering CRP.
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Supplementary Methods

**Human Studies:** Eight healthy volunteers whose blood CRP levels ranged from 2 to 5 mg/L on 2 qualifying examinations (determined 2 weeks apart) were enrolled to evaluate the efficacy of ISIS 329993. Individuals were randomized to receive ISIS 329993 ($n = 6$ subjects) or placebo (ISIS 141923; $n = 2$ subjects). ASOs were administered via 2-hr intravenous infusions on days 1, 3, 5, 8, and 15. This regimen was expected to result in maintenance of a target dose of 600mg/wk, with ASO levels in organs reaching 65-75% of steady-state levels by day 8 and 80-85% by day 17. Blood samples were collected prior to each infusion and on days 22 and 29 for CRP determination. All human studies were performed by ISIS pharmaceuticals and are simply being reported here for technical completeness.
Figure 1: Dose-dependent reduction in cytokine-induced CRP mRNA expression after ASO treatment.
A. After treatment with increasing doses of ISIS 329993, CRP mRNA expression was stimulated in Hep3B cells by addition to media of 1μM dexamethasone, 400 U/mL IL-1β and 200 U/mL IL-6. Hep3B cells were exposed to ASO for 48 hr. CRP mRNA was quantified by RT-PCR and expression normalized to stimulated cells not exposed to ASO. B. After ASO treatment, CRP mRNA expression was stimulated in human primary hepatocytes as described above. The primary hepatocytes were treated with ASO for 24 hr and CRP mRNA was quantified by RT-PCR and expression normalized to stimulated cells not exposed to ASO. In both cases basal expression is relative to the induced cells. Data are means ± SD (n=4-5 wells/treatment group).
Figure 2: Agreement between clinical scores determined visually, bone density, and bone volume measured by micro-CT, and bone histology for paws from mice with CIA. Representative hindpaws of mice with CIA visual scores of 0, 2, and 4 were analyzed by micro-CT scanning and H&E staining. Note both the calculated bone density and bone volume correlated with the visual score (top graphs), and that bone erosions detected by micro-CT (middle) become more apparent as visual score increases, and that). The lower micrographs show H&E stained sections of a healthy joint (visual score of 0) and a diseased one (visual score of 4).
Figure 3: Treatment of CRPT g mice with CRP specific ASOs inhibits the production of human CRP protein. Administration of each of three different human CRP specific ASOs (25 mg/kg), but not a control ASO (ISIS 141923) or vehicle (PBS), resulted in significant reduction of plasma human CRP levels by 7 days after initiation of treatment (* = p<0.05, PLSD vs vehicle). By the end of the treatment phase (day 17) human CRP was lowered by no less than 71% (ISIS 353512) and by as much as 94% (ISIS 353491). Number of animals per treatment is noted next to each treatment. Inset. In the same animals there was no reduction of endogenous mouse CRP levels, and IL-6 was significantly reduced only in mice treated with ISIS 329993.
Figure 4: Treatment of CRPTg mice with CRP-specific ASOs inhibits the production of human CRP mRNA and does not elevate serum transaminases. A. Each of the human CRP-lowering ASOs achieved CRP-lowering effect via reduction of hepatic human CRP mRNA by day 17. B. ASOs were well tolerated by CRPTg as judged by absence of elevation of plasma ALT levels.
Figure 5: Mouse and human CRP blood levels during collagen-induced arthritis in CRPTg mice. CIA was induced in CRPTg mice and the disease allowed to develop. One day after the clinical score reached ≥ 2.0 (day 0) mice began receiving treatment with placebo ISIS 141923 (white bars) or the human CRP targeting drug ISIS 329993 (black bars). Mice were randomly assigned to receive drug or placebo, and each was delivered by i.p. injection (25/mg/kg) twice weekly. Human CRP serum levels were measured during the ensuing symptomatic phase and are plotted here as a percentage of baseline values (obtained prior to immunization). A. For up to 20 days after arthritis was established, human CRP levels were significantly lowered in CRPTg mice receiving ISIS 329993. B. In the same animals mouse CRP levels were not significantly affected in either the drug treated nor placebo treated group. The number of mice is indicated and * signifies p<0.05 for Student’s t-tests.
Figure 6: The clinical signs of established CIA in CRPTg mice are reduced by pharmacological reduction of human CRP.

CRPTg mice (n=141) were immunized with type II collagen as described in the Materials and Methods and each animal was monitored for development of symptoms of CIA. Arthritis was allowed to develop unhindered (□) and the 48 mice with established arthritis were randomly assigned to one of the 2 treatment arms. ASOs were delivered by i.p. injection (25 mg/kg) twice weekly and treatment continued for 4 weeks. Of the 48 CRPTg mice with established disease 23 received the placebo ISIS 141923 (○) and 25 received the human CRP targeting drug ISIS 329993 (●). Compared to placebo treated animals, arthritis was attenuated in CRPTg given the human CRP targeting ASO. The inset excludes animals whose total clinical score at the time of initiation of therapy was > 5.0. Their CDI was significantly lower for mice receiving ASO 329993 than placebo (p=0.029). AUC, area under the curve. # and *", p < 0.01 and p < 0.05, respectively, for t-tests.
Figure 7: The percentage of CRPT g mice achieving 20%, 50%, and 70% improvement in arthritis clinical symptoms is increased by pharmacological lowering of human CRP. Amongst all mice with established CIA (i.e., regardless of their clinical score at recruitment into the treatment phase), the proportion of animals whose arthritis clinical score was improved by 20%, 50%, and 70% (analogous to the ACR20, ACR50, and ACR70 clinical outcome measures) was increased for the cohort that received ISIS 329993 compared to placebo (ISIS 141923). Mice that received ISIS 329993 achieved 50% and 70% improvement more rapidly than did the placebo treated group.
Supplementary Figure 1: **CRP lowering effect of ISIS 329993 in healthy subjects.** Individuals were treated for 3 weeks with ISIS 329993 at a dose of 600 mg/week or placebo, administered IV at indicated times (arrows).
CONCLUSIONS

CRP is an ancient and evolutionarily conserved molecule that modulates a diverse array of seemingly paradoxical actions. CRP is present in the circulation at low levels during health and at high levels during inflammatory events, two conditions wherein the activities of CRP have widely differing influences and outcomes (25). Consequently, CRP is implicated in diseases as varied as atherosclerosis (57–59), age-related macular degeneration (60), and autoimmune disease. Others have previously shown that CRP is protective in a mouse model of multiple sclerosis (61), and my current work herein details the participation of CRP in CIA, a mouse model of rheumatoid arthritis. In keeping with CRP’s complex biological actions, we show that CRP can have both protective and detrimental effects on CIA. To help clarify the mode of action for the former, we have produced new details on how CRP affects DCs.

CRP level in the blood has long been used as a surrogate marker of systemic inflammation and elevation of which is known to associate positively with increasing RA disease severity (62, 63). Despite CRP’s myriad of biological interactions with potential relevance to RA pathogenesis, few have directly examined the impact of CRP on arthritis. I have done so by using a robust model of rheumatoid arthritis in the mouse, i.e. collagen-induced arthritis. Thus I have shown that CRP deficiency affects a number of immunological responses and potentiates CIA development and progression. Additionally, I showed that transgenic overexpression of CRP inhibits CIA progression. This represents the first report showing that CRP likely participates directly in the
biology of RA in humans. Further this is the first study of CIA in mice whose CRP expression closely mimics the human condition. I have also examined a plausible mechanism by which CRP might exert its influence on the system. It is known that CRP interacts with FcγRs (32) expressed on a number of immune cells including DCs (35), and that DCs are the most potent regulators of T cells (47). Both cell types are thought to be important in the pathogenesis of arthritis (2, 64–66). Additionally, others previously showed that FcγRIIB is required for CRP’s protective effects in EAE (41). Based on the evidence that FcγRs, DCs, and T cells are important in the pathogenesis of arthritis (3), I posit that CRP might be exerting its effects on CIA by altering DC function. Indeed, I have provided evidence that deficiency of CRP increases the number of DCs and significantly alters their distribution amongst peripheral lymphoid tissues. I also showed that both long term and overnight exposure of developing BMDCs to CRP significantly upregulates surface expression of important activating costimulatory molecules including CD40, CD80, CD86, and OX40L, whereas it downregulates CD11b (shown to inhibit T cell proliferation (67)). With the exception of MHC-Class II, these effects were not FcγRIIB dependent. A significant potentiation of T cell proliferation was seen when CRP was added to cocultures of BMDCs and T cells.

It is well known that CRP level tracks positively with RA disease severity (27), yet we showed that transgenic overexpression of CRP actually limits CIA disease severity. The solution to this apparent paradox is a split paradigm in that CRP’s multiple biological functions and abilities are differentially influencial at distinct points in arthritic disease. The epidemiological data correlating CRP level and RA disease severity is taken from patients with active disease. In contrast, my study of CIA was designed to examine
the effect of CRP on development of arthritis. In established arthritis, CRP may be unable
to provide enough tolerizing signal to prevent progression of disease, and its other
biological interactions, such as forming proinflammatory immune complexes, likely
become more influencial. In line with this, I have shown that lowering CRP level in mice
with established disease is therapeutic. I showed that an antisense oligonucleotide
specific for human CRP safely and effectively lowered CRP levels in human CRP
transgenic mice. Lowering of CRP levels in huCRPTg mice with established CIA
significantly reduced the severity of disease. This novel CRP-lowering drug is now in
clinical trials.

We have thoroughly addressed a question of importance and curiosity and in
doing so have discovered important facts that will change the way that CRP, RA, and
disease management are considered. No longer can CRP be regarded as simply an
effective and accurate measurement of systemic inflammation, but it must be recognized
as a protein with a varied set of biological interactions and the potent ability to influence
the innate and adaptive immune systems, and by extension, the vast number of diseases
that have an immunological component. Outside of my own work, CRP’s biological
participation is beginning to be recognized for a number of diverse diseases. My work on
the topic of CRP’s role in arthritic disease has added to this body of knowledge and will
bring about a new attitude about CRP that should lead to continued utilization of the
unrecognized, but potent, activities of this protein. Our work has set the stage for further
examination of the relationship between CRP and RA and has contributed to the
development of a novel therapy for this disease.
GENERAL LIST OF REFERENCES


APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL LETTER
NOTICE OF RENEWAL

DATE: September 9, 2011

TO: ALEXANDER J SZALAI, B.S.
    SHEL-174 2162
    FAX (205) 996-6734

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: The Role of CRP in Arthritis Disease (Nicholas Jones)
         Sponsor: NIH
         Animal Project Number: 111008912

As of October 25, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

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Animal use must be renewed by October 7, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 111008912 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7602.