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Identification of two new arthritis severity loci that regulate levels of autoantibodies, IL-1β and joint damage

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Abstract

Objective—*Cia3* is a locus on rat chromosome 4 that regulates severity and joint damage in collagen and pristane-induced arthritis (CIA and PIA). This study aimed to refine the *Cia3* gene-containing interval towards gene identification and obtain insights into its mode of action.

Methods—Five DA.F344(Cia3) subcongenic strains were generated and studied in PIA and CIA. Levels of antibodies against type II collagen (both allo- and autoantibodies) were measured. Joints and synovial tissues were collected 32 days after the induction of PIA (chronic stage) for histology and qPCR for IL-1β and matrix metalloproteases (MMPs).

Results—Three subcongenics sharing the centromeric *Cia3d* interval were protected, while two subcongenics sharing the telomeric *Cia3g* interval, which did not overlap with *Cia3d*, were also protected, developing significantly less severe CIA and PIA. DA.F344(Cia3) and DA.F344(Cia3d) congenics with PIA preserved a normal joint architecture, while DA rats had pronounced synovial hyperplasia, angiogenesis, inflammatory infiltration, bone or cartilage erosions. DA.F344(Cia3d) and DA.F344(Cia3g) strains had significantly lower synovial levels of IL-1β (5-fold), MMP-1 (expressed predominantly in DA), MMP-3 (79-fold) and MMP-14 (21-fold) and reduced levels of pathogenic autoantibodies against type II collagen, compared with DA.

Conclusions—We have identified two new arthritis severity and articular damage loci within *Cia3*. These loci regulate pathogenic processes in two different models of RA, and the identification of these genes has the potential to generate new targets for therapies aimed at reducing disease severity and articular damage, and for prognostication in RA.

Keywords

Autoimmunity; Rheumatoid arthritis; animal model; erosion; IL-1β

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease with an overall prevalence of 0.5–1% in most populations. RA has a strong genetic component (1), and several MHC and non-MHC genes have been recently associated with disease susceptibility (2, 3). In contrast to susceptibility, very little is known about the genetic regulation of disease severity and joint damage in RA (4), and the available multi-institutional family-based or case-control cohorts used in genome-wide studies were not specifically designed to address this issue. We consider that the identification of genes specifically involved in the regulation of disease severity and joint damage have a greater potential to generate useful targets for the development of new therapies aimed at preserving joint architecture and function.

We have previously identified several disease severity and articular damage quantitative trait loci (QTL) in rat models of RA (5–7). One of the identified QTLs, *Cia3*, was mapped to rat chromosome 4 in an intercross between the arthritis-susceptible DA and arthritis-resistant F344 rat strains studied for collagen-induced arthritis (CIA). Studies in congenic rats where the F344-derived arthritis resistance alleles at *Cia3* were introduced into the arthritis-susceptible DA strain genome background, as in DA.F344(Cia3) congenic rats, determined that *Cia3* also regulates arthritis severity in pristane, oil (6) and adjuvant-induced arthritis (8) (PIA, OIA and AIA, respectively). *Cia3* co-localizes with QTLs involved in the regulation of arthritis in other rat intercrosses (9–11), and in other models of autoimmune diseases in rats and mice (12, 13). The *Cia3* syntenic regions in the human genome also contain loci regulating different forms of autoimmune diseases (12, 13), including RA (14), suggesting that it harbors genes relevant not only to RA, but possibly to other diseases as well.

In order to localize and reduce the interval containing the arthritis-regulatory gene, and to characterize its regulatory effects in arthritis severity, joint histology and synovial tissue cytokine gene expression, and production of autoantibodies against collagen, *Cia3* subcongenics were generated and studied for their susceptibility to and severity of PIA and CIA, two well-established models of autoimmune erosive arthritis. In the present study we describe the discovery that *Cia3* is accounted for by at least two different genes that operate independently to regulate disease severity.

MATERIAL AND METHODS

Rats

Specific pathogen-free DA (DA/BklArb) (arthritis-susceptible) and F344 (F344/Hsd, Harlan, Indianapolis, IN) (arthritis-resistant) inbred rat strains were used in the breeding of the congenic and subcongenic strains. DA rats were originally purchased from Bantin & Kingman, Inc. (Fremont, CA), maintained at the Arthritis and Rheumatism Branch, NIAMS, NIH, and then transferred to the Feinstein Institute for Medical Research (FIMR) (former North Shore-LIJ Research Institute) (DA/BklArbNsi) and used as controls. All the experiments involving animals were reviewed and approved by the FIMR Institutional Animal Care and Use Committee.

Construction of the genotype-guided *Cia3* QTL-congenic and subcongenic lines

A 75.47 Mb interval, containing the original 35 cM two logarithm of odds (LOD) support interval comprising *Cia3*, was introgressed from F344 into the DA rats through eight backcrosses (BC_8) followed by at least five intercrosses, as previously described (6). Subcongenic lines covering the *Cia3* interval (Figure 1) were generated for the present study. DA.F344(Cia3) congenics were backcrossed with DA rats to generate offspring heterozygous at the congenic interval. These heterozygous offspring were further

backcrossed with DA, and the offspring screened for new recombinants within the *Cia3* interval (see SSLP markers used on Figure 1). Offspring (BC_{10}) heterozygous at identical recombinant segments, based on SSLP markers, were brother-sister mated, and their offspring ($BC_{10}F_1$) genotyped to ensure homozygozity at the expected intervals.

Homozygous subcongenics were used to expand the subcongenic lines. Experiments were done with offspring from second to fifth intercrosses ($BC_{10}F_2$-F_7, and *Cia3d* experiments were further confirmed with $BC_{12}F_2$-F_5).

Genotyping

Tail tips were excised from 3–4 week-old rats, and DNA extracted with the DNeasy kit (Qiagen, Valencia, CA). PCR conditions have been previously reported, and were set up in 10μl reactions (15). GENESCAN 3.1 software (ABI) was used for fluorescent-labeled PCR products' data extraction and allele assignment. All genotypes were manually checked by two readers and questionable readings re-checked or repeated. For marker details, see the Rat Genetic Database (http://www.niams.nih.gov/rtbc/ratgbase/index.htm) and the Rat Genome Database (http://www.rgd.mcw.edu).

Induction of PIA

Eight to twelve week-old rats received 150 μl of pristane (2,6,10,14-tetramethylpentadecane, SIGMA-Aldrich Chemical Co., Milwaukee, WI) by intradermal injection (day zero) (6, 16, 17). The dose was divided in two injection sites at the base of the tail.

Induction of CIA

Bovine type II collagen (BII; Chondrex, Redmond, WA) was dissolved overnight in 0.1N acetic acid at 4°C (2mg/ml) and emulsified with incomplete Freund's adjuvant (IFA, Difco, Detroit, MI) to a final concentration of 1mg/ml. Eight to twelve-week old rats were injected intradermally at the base of the tail with 2mg/kg weight of BII divided into six injection sites (day zero), and a booster injection of 100μg BII/IFA administered on day seven (18). Serum was obtained on day 18 and stored at −80°C until tested.

Arthritis scoring

We used a previously described arthritis scoring system (5, 15, 18) that evaluates individual joints and measures arthritis severity according to joint size as follows: a) interphalangeal, metacarpophalangeal and metatarsophalangeal joints in each one of the four lateral digits were scored 0=no arthritis; 1=arthritis present; b) wrist, mid-forepaw, ankle and midfoot joints were scored 0=normal; 1=minimal swelling; 2=moderate swelling; 3=severe swelling; 4=severe swelling and non-weight bearing. The scores from all involved joints were added (maximum score per rat=80). The same observer obtained the arthritis scores on days 0, 14, 18, 21, 24, 28 and 31 following induction. The arthritis severity index (ASI), which is a measure of disease severity over time (area under the curve), was determined for each animal by adding the individual arthritis scores obtained over the course of the experiment. We have previously shown that the ASI correlates with histological changes and damage (7, 17).

Histology and histological scoring

At the end of the arthritis observation period (day 32), the right hind paw was fixed in 10% formaldehyde. Paws were then decalcified with a solution containing hydrochloric acid and 0.1M EDTA (Cal-Ex, Fisher Scientific, Fairlawn, NJ). Tissues were sectioned, embedded in paraffin, and slides prepared and stained with hematoxylin-eosin and safranin-O. Two slides

per rat were scored without knowledge of strain identity. We used a recently described comprehensive histological scoring system (17). Briefly, tibio-talar, talus-calcaneal and midfoot joints were histologically scored for the following parameters:

1. *Synovial inflammation.* Five high-power magnification fields (HMF) were scored for the percentage of infiltrating mononuclear cells as follows: 0=absent; 1=mild (1–10%); 2=moderate (11–50%); 3=severe (51–100%). The mean of the five HMF was used for analyses.
2. *Synovial hyperplasia.* 0=absent; 1=mild (5–10 layers); 2=moderate (11–20 layers); 3=severe (>20 layers).
3. *Extension of pannus formation based on the reader's impression.* 0=absent; 1=mild; 2=moderate; 3=severe.
4. *Synovial fibrosis.* 0=absent; 1=mild (1–10%); 2=moderate (11–50%); 3=severe (51–100%).
5. *Synovial vascularity (angiogenesis).* The number of vessels was counted in five HMF of synovial tissue, and the mean used for analyses.
6. *Cartilage erosion.* Percentage of the cartilage surface that was eroded: 0=absent; 1=mild (1–10%); 2=moderate (11–50%); 3=severe (51–100%).
7. *Cartilage degradation.* Based on safranin-O staining of proteoglycans, and described as the percentage of the cartilage that lost its staining: 0=none; 1=mild loss (1–10%); 2=moderate loss (11–50%); 3=severe loss (51–100%).
8. *Bone erosion.* 0=none; 1=minor erosion(s) observed only at HMF; 2=moderate erosion(s) observed at low magnification; 3=severe transcortical erosion(s).

Quantitative real-time PCR (qPCR)

Ankle synovial tissue obtained after the completion of the arthritis observation period (day 32) was immediately frozen in liquid nitrogen. Tissues were subsequently homogenized and total RNA isolated with the RNeasy Kit (Qiagen) and digested with DNase (Qiagen), according to the manufacturer's protocol. 200ng of total RNA from each sample were used for cDNA synthesis (SUPERSCRIPT II Kit, Invitrogen, Carlbad, CA). qPCR, methodology, primers and probes used for IL-1β, matrix metalloprotease-1 (MMP-1), MMP-3 and MMP-14, as well as GAPDH have been previously reported (7, 17, 19). Briefly, cDNA was optimized for relative gene expression by qPCR. TaqMan (ABI) 5′exonuclease assay and Roche Universal Probe Library (Roche) were used for qPCR. GAPDH was used as endogenous control. PCR reaction mixture contained 1X mastermix (Eurogentec, San Diego, CA), 200nM of forward and reverse primers, 100nM of gene-specific probes, and 150–200ng of cDNA. All samples were run in duplicates in an ABI 7700 Sequence Detection System (ABI), and the mean used for the analyses. *Ct* (threshold cycle) values were obtained and analyzed with the Sequence Detection System (SDS) software version 1.9.1 (ABI). The *Ct* value is inversely related to the starting template copy number. Relative expression in synovial tissues was adjusted for GAPDH in each sample (ΔCt). ΔCt values were compared using the t-test. Fold-change differences in gene expression between DA and subcongenics were compared with the $2^{-\Delta\Delta Ct}$ method (20).

Measurement of anti-collagen antibodies

Serum samples collected on day 18 after the induction of CIA were assayed for IgG antibodies against bovine and against rat type II collagen using commercially available ELISA Kits (Chondrex), according to the manufacturer's instructions, and results shown as IgG U/ml.

Statistical analyses

Males and females were initially studied separately for their arthritis severity, and in the absence of gender-specific effects were then combined for analysis. Non-normally distributed data were compared with ANOVA on ranks with a pairwise multiple comparison procedure (Dunn's method) for multiple groups, or with the Mann-Whitney test for two group comparisons. The t-test was used to compare normally distributed data (qPCRs). A p-value of 0.05 or less was regarded as significant. All statistical analyses were done with SigmaStat 3.0 (SPSS, Chicago, IL).

RESULTS

DA.F344(Cia3) congenic rats are protected and develop a significantly milder form of PIA and CIA

Both genders were similarly protected, and therefore male and female data were combined for analysis. The introgression of the F344-derived *Cia3* interval into the DA background, as in the DA.F344(Cia3) congenics (figure 1), was associated with a highly significant reduction of 81% in median PIA ASI, compared with DA rats (figure 2A and 3A) [median ASI: DA=95, DA.F344(Cia3)=18, p 0.001; table 1]. DA.F344(Cia3) congenics were also similarly protected in CIA (figure 2G), and had an 84.9% lower median ASI compared with DA rats [Median ASI: DA=265, DA.F344(Cia3)=40, p 0.001; table 1].

The protective effect was detected at day 14 (figures 2A and 2G), and persisted throughout the observation period, suggesting that the arthritis gene located within *Cia3* regulates both early and chronic stages during the course of disease pathogenesis.

Cia3 contains two arthritis severity genes: Identification of Cia3d

Rats subcongenics for the centromeric segments of *Cia3*, DA.F344(Cia3a), DA.F344(Cia3b) and DA.F344(Cia3d) (figure 1) were protected in PIA and had significantly lower arthritis scores (figure 2B, 2C, 2D and figure 3B and 3C), with a respective 51.6%, 49.7%, and 59.5% reduction in median ASI compared with DA [median ASI: DA=95, DA.F344(Cia3a)=46, p 0.001; DA.F344(Cia3b)=49.5, p=0.003; DA.F344(Cia3d)=38.5, p 0.001; all comparisons with DA; table 1]. These three subcongenics had a similar reduction in arthritis scores, compared with DA, and shared a 9.9 Mb region containing F344 alleles between *D4Uia2* and *D4Wox22* (figure 1).

Two centromeric subcongenics DA.F344(Cia3a) (figure 2H) and DA.F344(Cia3d) (figure 2I) were studied in CIA, and both were protected with significantly 78.9% and 75.7% lower median ASI, respectively, compared with DA [Median ASI: DA=265, DA.F344(Cia3a)=56, $p \leq 0.001$; DA.F344(Cia3d)=64, $p \leq 0.001$; table 1].

These observations suggest that the interval shared by *Cia3a, Cia3b and Cia3d*, which is the 9.9 Mb *Cia3d* locus itself, contains an arthritis gene that regulates both PIA and CIA.

Cia3d operates in a dominant manner to increase arthritis severity

In order to determine the mode of inheritance of *Cia3d*, homozygous DA.F344(Cia3d) rats were backcrossed with DA, and the offspring (BC_{11}) heterozygous at *Cia3d*, studied in PIA experiments. *Cia3d* heterozygous rats were not protected and had ASIs that were similar to DA (figure 2D), suggesting that the DA-derived arthritis severity alleles at this locus operate in a dominant manner.

Identification of Cia3g

The magnitude of the PIA protection detected in DA.F344(Cia3) congenics (81%) was more pronounced that in DA.F344(Cia3a), DA.F344(Cia3b) or DA.F344(Cia3d) subcongenics (49–59%). This observation suggested the existence of yet another arthritis gene located in the telomeric portion of *Cia3*. To address that possibility, DA.F344(Cia3f) and DA.F344(Cia3g) homozygous subcongenic strains (figure 1) were generated from DA.F344(Cia3), and studied in PIA. Both subcongenics were significantly protected in PIA, with 65.3% and 43.2% reduction in median ASI compared with DA (figure 2E and 2F, and figure 3D) [median ASI: DA=95, DA.F344(Cia3f)=33, p=0.002; DA.F344(Cia3g)=54, P=0.031; all comparisons versus DA; table 1].

DA.F344(Cia3g) was also significantly protected in CIA and had an 82.3% lower median ASI, compared with DA [Median ASI: DA=265, DA.F344(Cia3g)=47, p 0.001] (figure 2J).

DA.F344(Cia3g) is contained within DA.F344(Cia3f), and therefore its 34.5Mb interval between *D4Arb5* and *D4Arb4*, and its adjacent recombination regions, contain the arthritis gene.

Taken together, these results show that *Cia3* contains at least two distinct arthritis genes, and both genes regulate disease severity in two different models of autoimmune erosive arthritis, CIA and PIA. These observations suggest that these two genes regulate processes common and central to the pathogenesis of arthritis.

Histological studies in PIA synovial tissues

Males and females within each strain had similar histological findings and therefore, data from both genders were combined for analyses. DA rats with PIA had a highly abnormal joint histological architecture (table 1; figure 3E and F), with pronounced synovial hyperplasia and pannus formation, increased number of synovial vessels (angiogenesis), synovial infiltration with mononuclear cells, and extensive cartilage and bone erosive changes, and cartilage loss of proteoglycans.

Both DA.F344(Cia3) (figure 3G) and DA.F344(Cia3d) (figure 3H) preserved a nearly normal joint histological architecture with significantly lower histological scores compared with DA (table 2). These histological findings further support the reduced clinical arthritis severity observed in the congenics, and suggest that the disease genes contained within *Cia3* regulate the pathogenesis of synovial hyperplasia, pannus formation and joint destruction in arthritis.

Synovial tissue levels of IL-1βmRNA were reduced in DA.F344(Cia3d) and DA.F344(Cia3g) congenics compared with DA rats

qPCR analysis revealed that synovial levels of IL-1βmRNA were nearly five-fold higher in DA (figure 4A), compared with DA.F344(Cia3d) congenics [Δ*Ct*: DA=7.6, DA.F344(Cia3d)=9.54; p=0.001, Mann-Whitney test, figure 4A] (The Δ*Ct* is inversely correlated with the number of copies of mRNA in the tissue, and each *Ct* cycle difference is equivalent to a nearly two-fold difference in mRNA levels). The expression of IL-1β was also lower in DA.F344(Cia3g) compared with DA, [Δ*Ct:* DA.F344(Cia3g)=8.3], with a nearly 2-fold difference, but did not reach statistical significance (p=0.56; t-test), suggesting that the two loci regulate synovitis through difference mechanisms (figure 4A).

DA.F344(Cia3d) and DA.F344(Cia3g) have significantly lower synovial tissue levels of MMP-1, MMP-3 and MMP-14 mRNA compared with DA rats

MMP-1 was expressed in 86% (6/7) of DA synovial tissues, but only in 12.5% (1/8) of DA.F344(Cia3d) (P=0.01, Fisher's exact test) and in 33% (3/9) DA.F344(Cia3g) congenics (P=0.06, Fisher's exact test). Levels of MMP-3 and MMP-14 mRNA were 78-fold and 21-fold higher in DA, compared with DA.F344(Cia3d) [MMP-3 ΔCt: DA=27.65, DA.F344(Cia3d)=33.95; p= 0.00004; MMP-14 ΔCt: DA=28.26, DA.F344(Cia3d)=32.67; p= 0.004, t-test, figure 4A], and 8-fold and 1.4-fold when DA was compared with DA.F344(Cia3g) congenics [MMP-3 ΔCt: DA=27.65, DA.F344(Cia3g)=30.66; p= 0.00006; MMP-14 ΔCt: DA=27.65, DA.F344(Cia3d)=28.76; p= 0.007, t-test, figure 4A].

Reduced levels of allo antibodies against bovine and autoantibodies against rat type II collagen in *Cia3* congenic and subcongenic strains tested for CIA

High levels of IgG antibodies against bovine type II collagen (BII) were detected in all strains immunized with BII/IFA, confirming appropriate immunization (figure 4B). There was concordance between median levels of anti-BII and mean levels of autoantibodies anti-rat type II collagen (RII) in each strain (figure 4B and 4C), and levels of anti-BII (p=0.054) and anti-RII ($p \leq 0.05$) were lower in DA.F344(Cia3) congenics compared with DA.

The arthritis-protected subcongenic strains DA.F344(Cia3d) and DA.F344(Cia3g) also had significantly reduced levels of anti-BII and anti-RII ($p \leq 0.05$ and $p \leq 0.01$, respectively) compared with DA (figures 4B and 4C). DA.F344(Cia3g) had a more pronounced reduction in levels of antibodies anti-BII and anti-RII than DA.F344(Cia3d), again suggesting differences in gene-specific mode of action.

Cia3g is not explained by the variants in antigen-presenting lectin-like receptor gene complex (APLEC) previously associated with arthritis and the *Oia2* locus

We have sequenced the coding regions of the APLEC genes previously associated with arthritis severity in a DAxPVG intercross and congenics (Dcar1, Dcir2, Dcir1 and Mincle) (21). DA and F344, the parental strains used to discover *Cia3g*, had the same alleles at all of the amino-acid changing SNPs originally described between DA and PVG, suggesting that another new gene(s) account for this locus' effect in arthritis severity.

DISCUSSION

RA is associated with increased risk for disability and deformities, reduced survival and reduced income (22). Disease severity and articular damage are associated with increased risk for disability, joint deformities and reduced life expectancy in patients with RA (23–25). Therefore, genes implicated in the regulation of disease severity and articular damage are expected to generate important new targets for therapies aimed at preserving the joint architecture and function. Yet, little is known about severity or articular damage genes in arthritis.

In the present study, we report the generation of *Cia3* subcongenic strains and the identification and localization of two new arthritis severity and articular damage regulatory loci, *Cia3d*, the most centromeric 9.9 Mb interval of *Cia3*, and a second locus in the most telomeric segment contained within the 59.5 Mb *Cia3g* interval. *Cia3d* and *Cia3g* regulate disease severity of two different models of autoimmune erosive arthritis, CIA and PIA. The observation that the effect of these two loci is not limited to a single model suggests that they regulate processes that are central to arthritis pathogenesis, thus making them potentially even more relevant to human disease.

In addition to evaluating clinical arthritis severity during a 31-day observation period, a comprehensive histological analysis determined that the presence of F344 alleles at *Cia3* or only at *Cia3d* was associated with preservation of a normal joint architecture, with no synovial hyperplasia or pannus formation, no significant synovial inflammatory infiltration or angiogenesis, and no cartilage or bone erosive damage. Synovial angiogenesis is a critical event in the pathogenesis of autoimmune arthritis and in the development of synovial hyperplasia (26, 27). Similarly, synovial hyperplasia and pannus formation are typically associated with cartilage and bone erosions in arthritis (17, 28), and are partially dependent on the inflammatory mononuclear cellular infiltrate and the cytokines these infiltrating cells produce (29–32). Moreover, several of the cytokines produced by the synovial tissues and infiltrating mononuclear cells have angiogenic properties (33). Therefore, while we cannot determine at this point which specific cellular and molecular events the *Cia3d* and *Cia3g* genes regulate, our observations demonstrate that these genes control fundamental events required for the development of synovial inflammation, arthritis and articular damage.

Both DA.F344(Cia3d) and DA.F344(Cia3g) subcongenics had reduced synovial levels of IL-1β mRNA compared with DA, and synovial levels of IL-1β mRNA correlate with their respective protein levels in rats (34). The reduced levels of IL-1β, even at a later time-point during disease course (day 32), demonstrate that the regulatory effects of these two loci persist beyond the early stages of disease and into the chronic stages. IL-1β is a key pro-inflammatory cytokine involved in the pathogenesis of rodent arthritis (17, 35–37) and RA (38–40).

IL-1β is known to induce the expression of IL-1β itself, IL-6, and MMPs implicated in articular damage such as those expressed in significantly reduced levels in Cia3d and Cia3g congenics (41, 42). Furthermore, IL-1β induces both synovial hyperplasia and angiogenesis, features associated with erosive arthritis (41, 42). Therefore, the reduction in levels of IL-1β could explain the significantly reduced expression of MMP-1, MMP-3 and MMP-14 observed in Cia3d and Cia3g congenics. These MMPs have been implicated in cartilage and bone damage in RA and rodent models of RA, and therefore, the reduced expression of IL-1β and MMPs explains at least part of the histological joint protection observed in the subcongenics. Interestingly, while both subcongenics had reduced synovial levels of IL-1β mRNA, the reduction was more pronounced in DA.F344(Cia3d) than in DA.F344(Cia3g).

While levels of IL-1β were more significantly reduced in DA.F344(Cia3d), levels of antibodies anti-BII (immunogen) and pathogenic autoantibodies anti-RII were more significantly reduced in DA.F344(Cia3g) than in DA.F344(Cia3d). These observations suggest that *Cia3g* could be involved in B cell responses, or processes of the immune response required for B cell responses such as antigen presentation or T cell cognate interactions. B cells and antibody-producing plasma cells are present in arthritic synovial tissues (16, 43), and are required for disease development (44). Autoantibodies such as anti-CCP, rheumatoid factors and anti-type II collagen antibodies are commonly detected in RA patients and correlate with disease severity and joint damage (45, 46). Additionally, therapies targeting B cells have been highly effective in RA (47).

Therefore, our results show that *Cia3d* had a more significant effect on the expression of IL-1β, while *Cia3g* had a more significant effect on levels of autoantibodies. These observations suggest that the two genes have preferential effects on different pathogenic processes.

The *Cia3* syntenic region in the human genome contains loci regulating different forms of autoimmune diseases (12–14). *Cia3d* is syntenic to a region on human chromosome 7p not known to contain RA genes. *Cia3g* is syntenic to a RA susceptibility region on chromosome

7q (14), and co-localizes with five different arthritis severity loci previously identified in rats, including another CIA locus, *Cia13* (48), the oil-induced arthritis locus *Oia2* (21), *Aia3* (8), *Pia5* and with *Pia7* (49). Allelic variants in coding regions of four different APLEC genes were associated with the *Oia2* locus (21). However, DA and F344 had no non-synonimous coding region differences in the chromosome 4 APLEC genes implicated in *Oia2*. It is still possible that a non-coding polymorphism in one of the APLEC genes exists, and therefore we cannot completely exclude that one of the APLEC genes accounts for the *Cia3g* locus. However, since the original polymorphisms were in coding regions (21), as in the case of most disease-causing polymorphisms, and we used the same susceptible strain, we considered that it is more likely that *Cia3g* is a new arthritis severity gene and not the same as *Oia2*/APLEC.

In conclusion, the present study determined that *Cia3* contains two different and independent arthritis severity and articular damage regulatory genes. The *Cia3d* interval contains genes that regulate arthritis severity, synovial hyperplasia, pannus formation, synovial inflammation and angiogenesis and joint damage, processes central to disease pathogenesis underscoring the potential relevance of the gene identification. We are in the process of reducing the critical regions containing *Cia3d* and *Cia3g* to ≤1Mb for positional cloning of the specific genes. The identification of these genes will generate novel new targets for the development of new therapies aimed at reducing joint damage and disease severity, as well as potentially new tools for prognostication.

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Abbreviations

ASI	arthritis severity index
CIA	collagen-induced arthritis
PIA	pristane-induced arthritis
QTL	quantitative trait locus or loci
RA	rheumatoid arthritis

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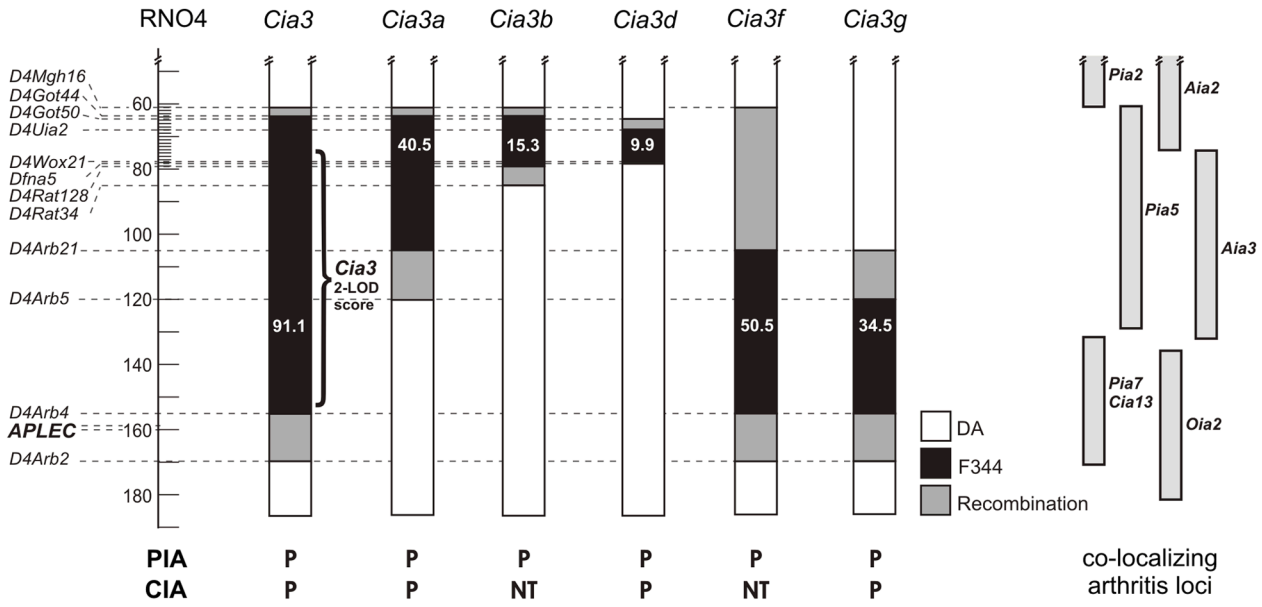

Figure 1. Markers used in the breeding of DA.F344(Cia3) congenic and subcongenics

Numbers indicate interval distance in megabases (Mb) (http://www.ensembl.org/Rattus_norvegicus/). Black filling indicates homozygous F344 alleles (F/F), white filling indicates homozygous DA alleles (D/D), and grey area indicates the region where recombination took place. Right side of the figure shows co-localizing arthritis loci. *PIA=pristane-induced arthritis; CIA= collagen-induced arthritis; AIA=adjuvant-induced arthritis; OIA=oil-induced arthritis. P=protected; NT=not tested.*

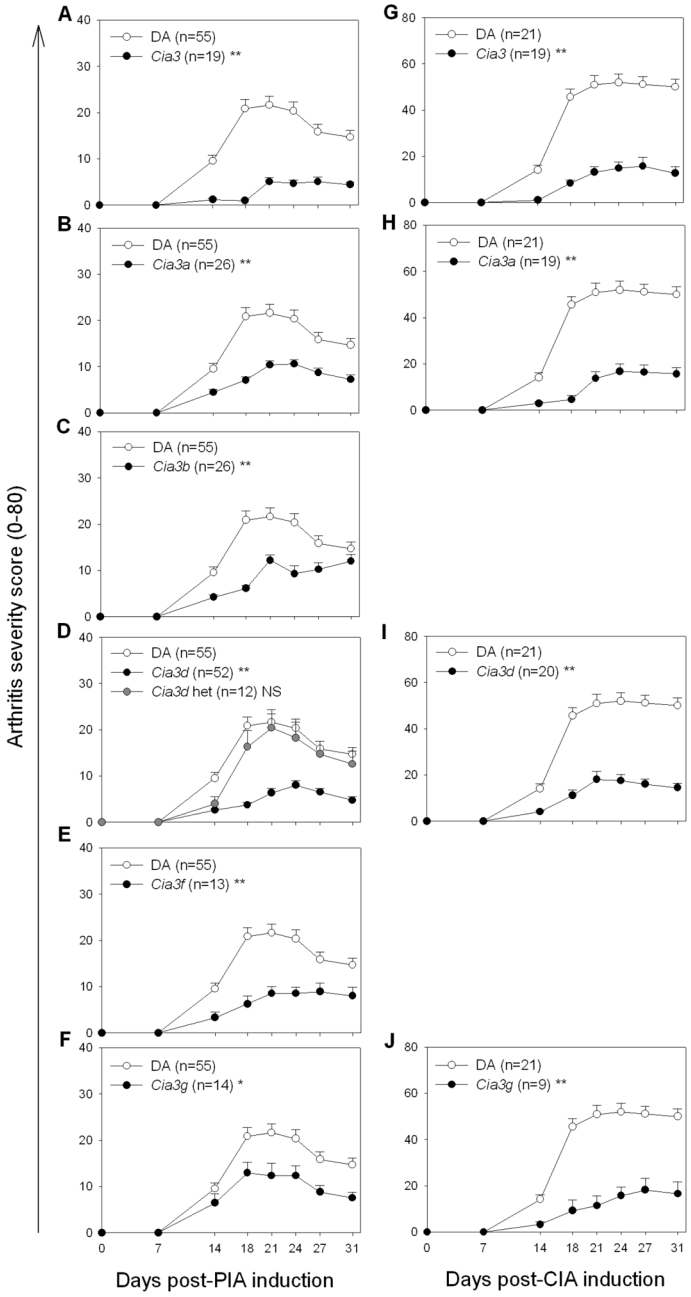

Figure 2. Arthritis severity clinical scores in DA and congenic/subcongenic rats during a 31-day observation period

Males and females had similar disease severity scores in each strain, and therefore were combined for analyses. **Left column:** ***Pristane-induced arthritis (PIA)***: **(A)** DA and DA.F344(Cia3); **(B)** DA and DA.F344(Cia3a); **(C)** DA and DA.F344(Cia3b); **(D)** DA and DA.F344(Cia3d), including rats heterozygous at the congenic interval; **(E)** DA and DA.F344(Cia3f); **(F)** DA and DA.F344(Cia3g); **Right column:** ***Collagen-induced arthritis (CIA)***: **(G)** DA and DA.F344(Cia3); **(H)** DA and DA.F344(Cia3a); **(I)** DA and DA.F344(Cia3d); **(J)** DA and DA.F344(Cia3g). *p<0.03; **p≤0.003, Mann-Whitney test; Data shown as mean ± SEM.

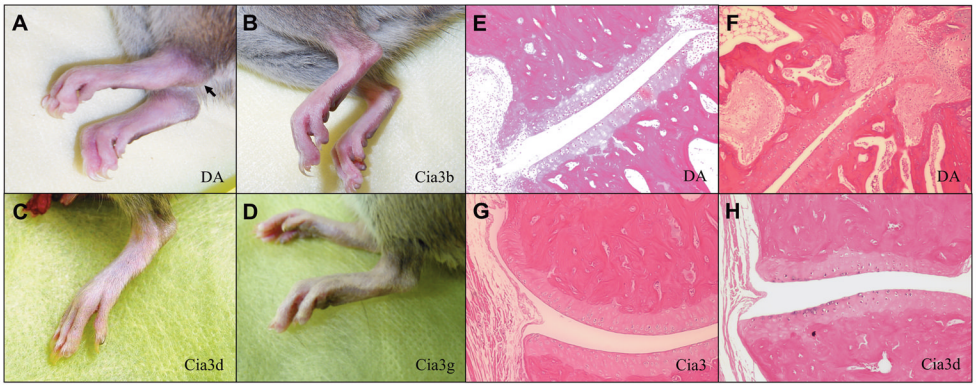

Figure 3. Clinical and histological characteristics of DA and DA.F344(Cia3) congenic and subcongenic strains

(A) DA rats developed severe arthritis with pronounced ankle and midfoot swelling and erythema (arrow), while **(B)** DA.F344(Cia3b), **(C)** DA.F344(Cia3d) and **(D)** DA.F344(Cia3g) developed very mild arthritis. **(E–F)** DA rats had extensive synovial hyperplasia, pannus formation, cartilage and bone invasion and erosion, contrasting with normal joint architecture in **(G)** DA.F344(Cia3) congenics, and **(H)** DA.F344(Cia3d) subcongenics. (Ankles were collected on day 32 post-pristane injection; hematoxylin-eosin staining; 100X magnification)

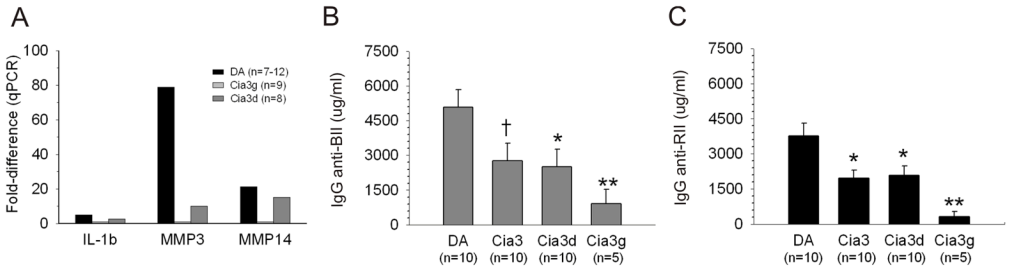

Figure 4. Synovial tissue levels of IL-1β, MMP-3 and MMP14 mRNA, allo and autoantibodies against type II collagen in DA and Cia3 strains

(A) qPCR analyses of synovial tissues from rats with PIA showing DA.F344(Cia3d) (n=8), and DA.F344(Cia3g) (n=9) rats with significantly lower levels of IL-1β, MMP-3 and MMP-14 mRNA compared with DA (n=12). Fold-differences were determined with the $2^{-\Delta\Delta Ct}$ method, and are shown compared with DA.F344(Cia3d) as reference. ΔCt results were used for comparisons with DA (all with p≤0.001, except for Cia3g IL-1β which had p=0.56; t-test). **(B)** Levels (measured at day 18 post-induction; μg IgG/mL) of antibodies against bovine type II collagen (anti-BII) and **(C)** autoantibodies against rat type II collagen (BII) were significantly lower in DA.F344(Cia3), DA.F344(Cia3d and in DA.F344(Cia3g) strains. (ANOVA on ranks with a pairwise multiple comparison procedure [Dunn's method]; *$p \leq 0.05$; ** $p \leq 0.01$; † $p=0.054$); results shown as mean±S.E.M..

Table 1

Arthritis Severity Scores for DA and Cia3 congenic and subcongenic rats.

Model	Strain	n	ASI [1]			*P*-value [2]	ASI reduction
			median	25%	75%		
PIA	DA	55	95.0	43.0	144.5		
	Cia3	19	18.0	16.0	26.0	≤ 0.001	81.1%
	Cia3a	26	46.0	32.3	57.8	≤ 0.001	51.6%
	Cia3b	26	49.5	36.3	66.5	0.003	47.9%
	Cia3d	46	38.5	23.5	54.8	≤ 0.001	59.5%
	Cia3f	13	33.0	31.0	43.0	0.002	65.3%
	Cia3g	14	54.0	35.5	70.3	0.031	43.2%
CIA	DA	21	265.0	210.0	342.0	---	
	Cia3	19	40.0	31.0	87.5	≤ 0.001	84.9%
	Cia3a	19	56.0	20.5	107.0	≤ 0.001	78.9%
	Cia3d	20	64.5	40.8	112.8	≤ 0.001	75.7%
	Cia3g	9	47.0	28.0	139.0	≤ 0.001	82.3%

[1] Arthritis Severity Index (sum of arthritis scores obtained over time).

[2] Mann-Whitney test (comparisons versus DA)

Table 2

Histology scoring of DA, DA.F344(Cia3) and DA.F344(Cia3d) congenics with pristane-Induced arthritis.

	DA (n=10)	Cia3 (n=8) [1]	Cia3d (n=13) [2]
Inflamm. infiltrate (0–3)	1.94 ± 0.17	0.40 ± 0.11	0.32 ± 0.08
Synovial hyperplasia (0–3)	3.00 ± 0.00	1.25 ± 0.37	0.92 ± 0.31
Pannus (0–3)	2.80 ± 0.13	0.63 ± 0.26	0.46 ± 0.14
Fibrosis (0–3)	2.30 ± 0.15	0.75 ± 0.41	0.15 ± 0.10
Vessels/HMF [3]	12.40 ± 1.27	6.20 ± 0.90	7.31 ± 0.55
Cartilage erosions (0–3)	2.90 ± 0.10	0.38 ± 0.26	1.08 ± 0.21
Bone erosions (0–3)	3.00 ± 0.00	0.75 ± 0.25	0.92 ± 0.34

[1] Means±SEM; DA vs. Cia3 $p \leq 0.0016$, (t-test).

[2] DA vs. Cia3d $p \leq 0.0006$ (t-test).

[3] High Magnification Field - 400X