EXTENDED REPORT

Cia25 on rat chromosome 12 regulates severity of autoimmune arthritis induced with pristane and with collagen

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Background: A genome-wide scan in a DA×ACI F₂ intercross studied for collagen-induced arthritis (CIA) identified the severity quantitative trait locus *Cia25* on rat chromosome 12. *Cia25* co-localises with loci regulating several forms of autoimmune diseases in rats, mice and humans, suggesting a common gene.

Objective: To characterise the effects of *Cia25* on severity of arthritis in congenic rats.

Methods: DA.AC(Cia25) congenic rats were constructed according to a genotype-guided strategy, and tested for pristane-induced arthritis (PIA) and CIA, induced with rat type II collagen (CII). A well-established scoring system previously shown to correlate with histological damage, including cartilage and bone erosions, synovial hyperplasia and synovial inflammation, was used.

Results: The introgression of ACI alleles at *Cia25* into DA background, as in DA.AC(Cia25) rats, was enough to significantly reduce arthritis severity by 60% in PIA and by 40% in CIA, both in males and females compared with DA rats of the same sex. Levels of IgG anti-CII in male DA.AC(Cia25) rats were 83% lower than in male DA. Levels of anti-CII in females were not affected by the congenic interval.

Conclusions: *Cia25* contains a gene that regulates disease severity in two distinct models of autoimmune arthritis. Although both genders were protected in arthritis studies, only male congenic rats had a dramatic reduction in levels of anti-CII, suggesting the possibility of a second arthritis gene in this interval that operates via the regulation of autoantibodies in a sex-specific manner. The identification of the gene(s) accounting for *Cia25* is expected to generate novel prognostic biomarkers and targets for therapy.

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects 0.5–1% of the population.^{1,2} Treatments targeting tumour necrosis factor α ,³ T cells⁴ and B cells⁵ have substantially improved disease control, yet sustained remission is rarely achieved. The strong genetic contribution to disease suggested by the heritability of 60%² makes the identification of genes regulating susceptibility to, and severity of, RA a promising strategy to generate novel targets for the development of more effective therapies.

Accordingly, several major histocompatibility complex (MHC) and non-MHC RA susceptibility loci have already been mapped,^{6–12} but only a few of their respective genes have been identified^{13–15} and most are yet to be replicated. Additionally, these studies were designed to identify susceptibility genes, some of which are likely to regulate early cognate immune interactions during preclinical stages, not necessarily influencing established and chronic stages of disease, or joint damage. Increased severity predicts and correlates with disability,¹⁶ joint damage¹⁷ and premature mortality,¹⁸ and, therefore, identifying severity genes is more likely to generate useful targets for the development of new therapies. However, very little is known about the genetic regulation of RA severity.

The study of rat models of autoimmune arthritis in inbred strains has identified several arthritis severity quantitative trait loci (QTLs),^{19–25} and a disease severity gene.²⁶ Genes identified in QTL regions will generate compelling candidates for focused RA case-control studies in well-characterised cohorts that include severity data, and for the development of new prognostic biomarkers and therapeutic strategies. We here report the construction of rats congenic for *Cia25*, a QTL that we have previously identified on rat chromosome 12,²⁰ and show evidence suggesting that this locus contains two different genes regulating the severity of arthritis induced with pristane and with collagen in both male and female rats.

METHODS

Rats

Specific pathogen-free ACI (ACI/Hsd) and DA (DA/Hsd) inbred rat strains were purchased from Harlan Sprague–Dawley (Indianapolis, Indiana, USA) and used in the breeding of the congenic strains. All the experiments involving animals were reviewed and approved by the Feinstein Institute for Medical Research Institutional Animal Care and Use Committee.

Genotype-guided construction of *Cia25*-congenic rats

A 22.9 Mb interval containing the 15.8 Mb two-logarithm of odds support interval comprising *Cia25*²⁰ was introgressed from the arthritis-resistant ACI strain into the arthritis-susceptible DA background using a genotype-guided breeding strategy to generate homozygotic DA.AC(Cia25) rats (fig 1). There was no evidence of genomic imprinting in the DA×ACI F₂ intercross genome-wide scan.^{19,20} Therefore, two DA×ACI and two ACI×DA mating pairs (using the convention of female × male) were used to produce F₁. The F₁ offspring were backcrossed with DA rats, and the progeny (and the following backcross generations) were genotyped with simple sequence length polymorphic markers on chromosomes 2 (*Cia7*: *D2Arb1*, *D2Mgh19*, *Cpb1*, *D2Rat38*; *Cia10*: *D2Mit12*, *D2Wox20*, *D2Rat55*, *D2Mgh29*), 10 (*Cia27*: *D10Rat19*, *D10Mit12*, *D10Wox7*, *D10Rat2*) and 12 (*Cia25*: *D12Wox12*, *D12Arb15*, *D12Mgh3*, *D12Arb3*, *D12Rat53*), which are known to contain QTLs regulating arthritis severity in the DA×ACI F₂ intercross.^{19,20} Offspring that maintained the *Cia25* interval in the heterozygotic state and had homozygotic DA alleles at the other three arthritis

Abbreviations: ASI, arthritis severity index; CIA, collagen-induced arthritis; CII, type II collagen; EAU, experimental autoimmune uveitis; IFA, incomplete Freund's adjuvant; MHC, major histocompatibility complex; PIA, pristane-induced arthritis; QTL, quantitative trait locus; RA, rheumatoid arthritis

QTLs were backcrossed again to the DA strain. After eight successive backcrosses, (BC₈) rats were intercrossed to generate homozygosity at the *Cia25* interval. These rats were brother-sister mated to establish and expand the DA.ACI(Cia25) congenic line. Offspring from the fifth and sixth intercrosses were used in the arthritis experiments. DA.ACI(Cia25) congenic rats were further backcrossed with DA to generate male DA.ACI(Cia25) rats heterozygous at the *Cia25* locus.

Genotyping

PCR conditions have been reported previously.^{20–30} The tail tips were excised at age 3–4 weeks and DNA extracted with the DNeasy kit (Qiagen, Valencia, California, USA). Fluorescent-labelled primers were used for PCR and products run along with TAMRA-labelled size standards in an ABI 310 capillary genotyper/sequencer (ABI, Foster City, California, USA). GENESCAN V.3.1 software (ABI) was used for data extraction and allele assignment. Two readers manually checked all genotypes, and questionable readings were re-checked or repeated. Marker details are available at the Rat Genetic database (<http://niams.nih.gov/rtbc/ratgbase>) and the Rat Genome database (<http://rgd.mcw.edu>).

Pristane-induced arthritis

The 8–12-week-old rats were anaesthetised and injected intradermally with 150 µl of pristane (2,6,10,14-tetramethylpentadecane) divided between two injection sites at the base of the tail (day 0).^{31–32} Pristane was initially purchased from ICN (Aurora, Ohio, USA), and then from Sigma-Aldrich (St Louis, Missouri, USA) owing to a discontinuation in supply. Pristane from these two brands induced arthritis of similar severity (data not shown).

Collagen-induced arthritis

Collagen-induced arthritis (CIA) was induced according to a previously described protocol.¹⁹ Purified homologous rat type II collagen (CII, Chondrex, Redmond, Washington, USA) was dissolved overnight in 0.1 N acetic acid (2 mg/ml) at 4°C and emulsified with incomplete Freund's adjuvant (IFA, Difco, Detroit, Michigan, USA) to a final concentration of 1 mg/ml. Anaesthetised 8–12-week-old rats were injected intradermally with 2 mg/kg of CII at the base of the tail, divided into six injection sites (day 0). A booster injection of 100 µg CII/IFA was administered on day 7.^{19–33}

Figure 1 The *Cia25* interval and colocalising autoimmunity loci. Numbers represent interval length and simple sequence length polymorphic marker positions relative to the centromere in megabases (Mb). The black segment identifies homozygous ACI alleles. The positions of *Ncf1* and the two-logarithm of odds (2-LOD) score support interval for *Cia25* are shown, and colocalising rat quantitative trait loci (QTLs, *Cia12*,²⁵ *Pia4*,²³ *Eau2*,²⁷ *Eae5*²⁸ and *Eae13*²⁹) are indicated in light grey.

Arthritis scoring

We used a previously described arthritis scoring system¹⁹ that evaluates individual joints and measures arthritis severity according to joint size as follows: (a) interphalangeal, metacarpophalangeal and metatarsophalangeal joints in the four lateral digits are scored: 0, no arthritis; 1, arthritis present and (b) wrist, mid-forepaw, ankle and midfoot joints are 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 per day = 80). The same observer obtained the arthritis scores on days 0, 14, 18, 21, 24, 28 and 31 after induction. The arthritis severity index (ASI), a comprehensive 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. The ASI correlates with the degree of histological damage, including synovial inflammation, synovial hyperplasia, and cartilage and bone erosive changes.^{32–34–35} To dissociate the severity and susceptibility components, we analysed only those animals that developed arthritis, defined as a daily score of ≥ 1 in at least two consecutive time points.

Measurement of anti-collagen (CII) autoantibodies

Serum samples collected on day 18 after CIA induction were diluted 1:40 000 and assayed in duplicates for anti-CII IgG, using a commercially available ELISA kit coated with highly purified CII (Chondrex), according to the manufacturer's instructions, and were reported in U/ml.

Statistical analyses

Based on previous observations of gender differences in the genetic regulation of CIA,²⁰ male and female rats were studied for arthritis severity both separately and together. Medians were compared using the Mann-Whitney test and correlations were calculated with the Pearson's correlation coefficient using SigmaStat V.3.0. A *p* value of ≤ 0.05 was considered significant.

RESULTS

Development of a significantly milder form of pristane-induced arthritis in DA.ACI(Cia25) rats

Male and female DA.ACI(Cia25) rats developed significantly milder pristane-induced arthritis (PIA) compared with DA rats of the same gender (median ASI (25th–75th centile): DA males 63 (49–95), DA.ACI(Cia25) males 15 (13–26); $p \leq 0.001$; DA females 57 (27–76); DA.ACI(Cia25) females 13 (8–18); $p = 0.003$; fig 2). ACI-derived alleles at the *Cia25* interval were associated with a 70% reduction in the median ASI of both male and female congenic rats with PIA.

Development of a significantly milder form of CIA in DA.ACI(Cia25) rats

Male and female congenic rats developed significantly milder CIA compared with DA rats of the same gender (median ASI (25th–75th centile): DA males 226 (209–259), DA.ACI(Cia25) males 126 (116–166); $p = 0.006$; DA females 292.5 (234–330); DA.ACI(Cia25) females 208 (173–231); $p \leq 0.001$; fig 3). The presence of ACI-derived alleles at the *Cia25* interval was associated with a 40% reduction in the median ASI of both male and female congenic rats with CIA.

Regulation of disease severity throughout the course of arthritis by *Cia25*

The arthritis-protective effect associated with ACI alleles at *Cia25* was detected as early as 14 days after induction for both PIA (males and females combined, median score at day 14 (25th–75th centile): DA 8 (4–13), DA.ACI(Cia25) 1 (1–2);

Figure 2 *Cia25* regulates the severity of pristane-induced arthritis (PIA). (A) Male and (B) female DA.AC1(*Cia25*) rats (white circles) had significantly milder PIA than DA (black circles). Scores are plotted as mean (SEM). (C) The cumulative arthritis severity scores (ASI), which are known to correlate with histology damage, were significantly higher in DA rats compared with homozygous DA.AC1(*Cia25*) congenic rats (median ASI (25th–75th centile): DA males 63 (49–95), DA.AC1(*Cia25*) males 15 (13–26); $p \leq 0.001$; DA females 57 (27–76); DA.AC1(*Cia25*) females 13 (8–18); $p = 0.003$, Mann–Whitney test). Boxes represent the 25th–75th centile, and error bars represent the 5th–95th centile.

$p \leq 0.001$) and CIA (males and females combined, median score at day 14 (25th–75th centile): DA 13 (3–18), DA.AC1(*Cia25*) 4 (2–6); $p = 0.004$), and persisted throughout the 31-day observation period.

Autoantibodies against type II collagen

Autoantibodies against CII were detectable in DA and DA.AC1(*Cia25*) animals immunised with CII, indicating appropriate immunisation (fig 4A). Levels of IgG against CII in male DA.AC1(*Cia25*) rats were 83% lower than those in male DA (median IgG, mg/ml (25th–75th centile): DA 32.2 (13.9–44.8); DA.AC1(*Cia25*) 5.3 (2.2–12.2); $p \leq 0.001$). Levels of IgG against CII in female DA.AC1(*Cia25*) rats were similar to those in DA (fig 4A). These observations suggest that *Cia25* also contains a gene that operates in a male-specific manner, where AC1 alleles significantly reduce levels of autoantibodies against CII. Levels

Figure 3 *Cia25* regulates the severity of collagen-induced arthritis (CIA). (A) Male and (B) female DA.AC1(*Cia25*) rats (white circles) had significantly milder CIA compared with DA rats (black circles). Scores are plotted as mean (SEM). (C) The cumulative arthritis severity scores (ASI), which are known to correlate with histology damage, were significantly higher in DA rats, compared with homozygous congenic rats (median ASI (25th–75th centile): DA males 211 (187–246), DA.AC1(*Cia25*) males 126 (116–166); $p = 0.006$; DA females 306 (263–340); DA.AC1(*Cia25*) females 208 (173–231); $p \leq 0.001$). Boxes represent 25th–75th centile, and error bars represent the 5th–95th centile.

of anti-collagen IgG significantly correlated with the ASI (Pearson's correlation coefficient = 0.365, $p = 0.03$, $n = 35$; fig 4B).

DISCUSSION

The study of intercrosses between rat inbred strains discordant for arthritis susceptibility and severity provides a powerful tool for the identification of loci regulating disease characteristics such as severity,^{20,33} chronicity^{20,23} and the production of rheumatoid factors.³⁶ The phenotypic effect of loci identified in linkage studies can be definitively confirmed in congenic strains^{30,32,35,37–39} such as the one described herein, thus providing the basis for the gene identification effort.²⁶ Findings from studies in rats can then be analysed in focused candidate gene RA case-control association studies.

Figure 4 *Cia25* influences the levels of anti-collagen autoantibodies in males. Levels of anti-collagen IgG were determined by ELISA in sera collected 18 days after immunisation with type II collagen (CII). (A) Levels of anti-collagen autoantibodies in male DA.ACI(Cia25) (shown as Cia25) rats were 80% lower than in male DA, but no difference was observed in females. (B) Levels of anti-collagen IgG correlated with the arthritis severity scores (ASI; Pearson's correlation coefficient = 0.365, $p = 0.03$, $n = 35$). * $p \leq 0.001$, Mann-Whitney test.

We have previously mapped five QTLs regulating CIA in an intercross between DA and ACI rats,^{19, 20} two strains that share the same arthritis-favouring MHC RT1^{av1} haplotype, yet differ in susceptibility to, and severity of, disease. In the present study, the *Cia25* locus was studied in DA.ACI(Cia25) congenic rats, which had significantly reduced severity of PIA and CIA compared with DA, confirming the presence of an arthritis-regulatory gene in this locus. The cumulative arthritis severity score (ASI) used here was previously shown to correlate with the degree of histological synovial inflammation, and cartilage and bone erosive changes.^{32–34, 35} Therefore, the protective effect of *Cia25* on clinical disease severity suggests that this gene is also relevant to joint damage.

The arthritis-protective effect associated with ACI alleles at *Cia25* was significant in both models, but more pronounced in PIA. The difference in the magnitude of protection between PIA and CIA suggests that the *Cia25* gene modulates pathways more directly relevant to PIA than to CIA.

Cia25 was originally identified as a female-specific QTL in the DA $\times$ ACI F₂ intercross studied for CIA.²⁰ However, both male and female congenic rats had reduced CIA severity. Although discrepancy between studies in the F₂ intercross and congenic rats remains incompletely understood, it has been described before.³⁹ It is considered that epistatic interactions between ACI alleles at *Cia25* and ACI alleles elsewhere in the genome of the F₂ offspring could have been a factor. The congenic data demonstrating protection in both genders has in fact broader relevance, as it suggests that more patients could benefit from therapies targeting the *Cia25* gene.

Both DA.ACI(Cia25) congenic rats and DA rats immunised with CII and IFA produced autoantibodies against CII, indicating adequate immunisation. Male DA.ACI(Cia25) congenic rats had a significant reduction of 83% in the levels of anti-CII autoantibodies, showing that the presence of ACI alleles at *Cia25* significantly suppresses levels of autoantibodies. This observation suggests that a gene within *Cia25* affects pathways leading to the activation of autoreactive B cells and/or the production of antibodies. Levels of anti-CII antibodies, particularly in males, correlated with disease severity, although in female DA.ACI(Cia25) congenic rats anti-CII levels were similar to those in DA rats, and did not correlate with disease severity. Anti-CII autoantibodies participate in the pathogenesis of CIA,^{40, 41} and their levels are genetically regulated and affect arthritis severity, as we have observed in DA.F344(Cia5a) rats congenic for a chromosome 10 QTL.³⁵ Therefore, these results raise the possibility that the *Cia25* interval contains two arthritis severity genes, one of which regulates arthritis and

autoantibodies in males, and the other operates via antibody-independent mechanisms to regulate disease in females, and perhaps in males as well. Studies in recombinant subcongenic rats should determine whether two independent gender-specific arthritis genes are in fact located within this interval.

Preliminary experiments suggest that *Cia25* operates in a dominant manner (data not shown). This knowledge will be important during the process of reducing the critical interval and positionally cloning the *Cia25* gene(s), since the arthritic phenotype will need to be screened in homozygotic recombinant subcongenic rats, and not in heterozygotic offspring.

The *Cia25* 22.3 Mb interval on rat chromosome 12 contains a number of potentially interesting candidate genes, such as *Ccl24*, *Nos1*, *Ptpn11* and *Ncf1*. *Ncf1* was previously found to account for *Pia4*.²⁶ The same disease-promoting and resistance *Ncf1* polymorphisms described by Olofsson *et al*²⁶ also segregate in the DA and ACI strains,²⁰ and these variants are capable of modulating the arthritic phenotype in DA rats.⁴² Although the *Ncf1* polymorphisms that we have previously reported²⁰ probably account for part of the *Cia25* effect detected in the congenic rats, we consider that there is yet a second arthritis severity gene within *Cia25*. Support for this hypothesis comes from two pieces of information. First, the difference in levels of anti-CII autoantibodies between male DA and DA.ACI(Cia25) rats, which was not detected in females, suggests two different pathogenic processes. Second, several loci regulating different autoimmune diseases colocalise with *Cia25*, and there is a strong possibility that there will be a common gene accounting for all these loci, and *Ncf1* does not seem to be the gene. Specifically, we have genotyped *Ncf1* in the strains used in the identification of the *Cia25* colocalising locus *Eau2*, which regulates experimental autoimmune uveitis (EAU).²⁷ Both F344 (EAU-resistant) and LEW (EAU-susceptible) have identical alleles at *Ncf1* codons 103 and 153 (Gulko *et al*, unpublished observations), and thus these arthritis regulatory single nucleotide polymorphisms do not explain the *Eau2* locus. Therefore, it can be conceived that the same as yet unknown gene accounting for *Eau2* will be the gene accounting for part of the *Cia25* effect. Studies of recombinant subcongenic rats that exclude *Ncf1* will be critical to confirm the existence of a second arthritis and autoimmunity gene within *Cia25*. We are in the process of generating these subcongenic rats.

Cia25 is of great interest, as it is in a region known to harbour several autoimmune disease-regulatory loci in rats,^{27–29} in mouse chromosome 5,^{43–45} and in the human syntenic regions on chromosomes 7q, 12q and 22q^{46–49} genomes, including the arthritis loci *Pia4*²³ and *Cia12*.²⁵ Taken together, these data

further support the concept that the same gene may regulate arthritis and other autoimmune diseases in rats, mice and humans. Although this clustering of non-MHC autoimmune regulatory loci does not prove that these loci are accounted for by the same gene, it certainly raises that possibility.⁵⁰ In fact, recent human studies have identified *CTLA4*^{51, 52} and *PTPN22*^{15, 53, 54} as two genes involved in the regulation of multiple autoimmune diseases, and *Cia25* could be the third one.

In conclusion, we have demonstrated that *Cia25* contains at least one gene that significantly influences arthritis severity. The identification of the *Cia25* gene(s) has the potential to generate an important novel prognostic biomarker, and candidate gene, or candidate pathway, for focused case-control analyses in RA cohorts designed to study disease severity. Ultimately, the identification of the *Cia25* gene(s) is expected to generate new targets for arthritis therapy.

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