Synovial expression of Th17-related and cancer-associated genes is regulated by the arthritis severity locus Cia10

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We have previously identified Cia10 as an arthritis severity and articular damage quantitative trait locus. In this study, we used Illumina RatRef-12 microarrays to analyze the expression of 21,922 genes in synovial tissues from arthritis-susceptible DA and arthritis-protected DA.ACI(Cia10) congenics with pristane-induced arthritis. 310 genes had significantly different expression. The genes upregulated in DA, and reciprocally downregulated in DA.ACI(Cia10) included IL-11, Ccl12 and Cxcl10, as well as genes implicated in Th17 responses such as IL-17A, IL-6, Ccr6, Cxcr3 and Stat4. Suppressors of immune responses Tgf and Vdr, and inhibitors of oxidative stress were upregulated in congenics. There was an over-representation of genes implicated in cancer and cancer-related phenotypes such as tumor growth and invasion among the differentially expressed genes. Cancer-favoring genes like Ctsd, Ikbke, and Kras were expressed in increased levels in DA, whereas inhibitors of cancer phenotypes such as Timp2, Reck and Tgfb3 were increased in DA.ACI(Cia10). These results suggest that Cia10 may control arthritis severity, synovial hyperplasia and joint damage via the regulation of the expression of cancer-related genes, inflammatory mediators and Th17-related markers. These new findings have the potential to generate new targets for therapies aimed at reducing arthritis severity and joint damage in rheumatoid arthritis.

RESULTS

DA.ACI(Cia10) congenics are protected, and developed significantly milder PIA compared with DA

DA.ACI(Cia10) congenic rats developed significantly milder arthritis compared with DA, and with a median arthritis severity index (ASI) of 12.3 compared with 30.4, respectively (P = 0.002 Mann-Whitney test) (Figure 1b). These observations were in agreement with our previous studies.

Gene expression analysis of synovial tissues differentiates DA from DA.ACI(Cia10) congenics

7593 (34.6%) of the 21,922 genes present in the array were expressed by all 18 synovium samples and were used for analysis. 310 of the 7,593 genes (4.08%) expressed by all synovial tissues met the criteria for differential expression. Of the 310 genes, 120 (38.7%) were expressed in significantly higher levels in DA.ACI(Cia10) compared with DA rats. One hundred ninety of the 310 genes (61.3%) were expressed in significantly increased levels in DA synovium, and were reciprocally reduced in DA.ACI(Cia10) (Table 1).

INTRODUCTION

Rheumatoid arthritis (RA) is a complex autoimmune disease characterized by chronic joint inflammation that commonly leads to joint destructive changes, disability and reduced quality of living. RA affects 1% of the population and is regulated by both genetic and environmental factors. Most of the genetic studies have focused in the identification of susceptibility genes. The HLA-DRB1 shared-epitope association with RA susceptibility has been known for over two decades, and, in recent years, several non-MHC susceptibility alleles have been identified, including PTPN22, CTLA4, TNFAIP3 and TRAF1. Yet, little is known about the genetic regulation of disease severity and joint damage, which are among the best predictors of disease outcome and disability.

We have identified the non-MHC arthritis severity and joint damage quantitative trait locus Cia10 on rat chromosome 2, studying an intercross between MHC-identical DA (severe arthritis) and ACI (arthritis-resistant) rats. DA.ACI(Cia10) congenic rats, which are identical to DA except for the presence of ACI alleles at the Cia10 interval, were generated using a genotype-guided strategy, and found to develop a significantly milder form of pristane-induced arthritis (PIA), and to preserve normal joint architecture compared with DA rats. DA.ACI(Cia10) congenics also had significantly reduced synovial tissue levels of mRNA of proinflammatory cytokines central to RA pathogenesis. During the refining of the congenic interval toward positional cloning of the Cia10 gene, we aimed to identify molecular pathways and potential candidate genes involved in protecting DA.ACI(Cia10) congenics, using genome-wide microarray gene expression profiling of synovial tissues. In the present study, we report that the presence of ACI alleles at the Cia10 interval correlates with reduced expression of genes implicated in Th17 responses, including IL-17A. Additionally, congenics had reduced expression of genes involved in the regulation of cancer growth and invasion, which may suggest new targets for therapies aimed at reducing synovial pannus growth and cartilage and bone invasion and destruction.
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The genes with the most significantly increased expression in the DA.ACI(Cia10) congenics and reduced expression in DA included Mup4 (4.88-fold, \( P = 0.0067 \)), Aldh1a1 (3.7-fold \( P = 0.0099 \)), Rpesp (3.56-fold, \( P = 0.0082 \)), Igfbp6 (3.56-fold, \( P = 0.0068 \)), Gstm1 (2.58-fold, \( P = 0.0029 \)), Timp2 (2.15-fold, \( P = 0.0024 \)) and Reck (2.1-fold, \( P = 0.005 \)) (Table 1).

Among the 190 genes with significantly reduced expression levels in the synovial tissues of DA.ACI(Cia10) congenics and increased expression in DA was Gp49b (5.39-fold, \( P = 0.0023 \)), Cd53 (3.28-fold, \( P = 0.0044 \)), Fcgr1a (2.94-fold, \( P = 0.007 \)), Emr1 (2.78-fold, \( P = 0.00002 \)) and Rbbp7 (2.68-fold, \( P = 0.0051 \)) (Table 1).

Seven of the differentially expressed genes were confirmed with qPCR (Figure 2).

The differentially expressed genes were grouped into specific 'disease categories', 'cellular functions' and 'gene networks' Analyses of the 310 differentially expressed genes showed an over-representation of ten disease categories and/or molecular and cellular functions, as detected by the IPA pathway analyses (Table 2). These categories included cancer (89 genes), cell death (68 genes), reproductive system disease (this category predominantly included endometriosis and genes implicated in cancer of the reproductive system or breast) (57 genes), inflammatory disease (53 genes), hematological disease (37 genes) and others (Table 2).

The IPA network/pathway detection analyses identified seven additional functional groups over-represented among the differentially expressed genes (Supplementary Table 1). These included 'Embryonic development, Tissue development, Cell death' (27 genes), 'Inflammatory response, Connective tissue disorders, Tissue morphology' (20 genes) and 'Cell cycle, connective tissue development and function organ morphology' (19 genes), all with significant scores (Supplementary Table 1).

Inflammatory cytokines, chemokines and related genes

We used three approaches to identify differentially expressed cytokines, chemokines and other known inflammatory mediators or receptors: a) genes expressed by all 18 synovial samples and meeting the criteria for significance described in the Methods section \( (P \leq 0.01, \text{p} \geq 1.5 \text{- fold difference}) \); b) genes expressed by only one of the strains and not the other; and c) genes predominantly expressed in one strain and not in the other. Using these combined parameters, we identify 21 cytokines, chemokines, receptors or related genes of relevance (Table 3).

Inflammatory mediators and receptors upregulated in DA IL-6 was preferentially expressed in 92% of DA (n = 11) and only in 33% (n = 2) congenics' synovial tissues (Table 3). IL-11 was expressed in significantly increased levels in DA, compared with congenics (1.66-fold, \( P = 0.007 \)). These two pleiotropic cytokines are also expressed in increased levels in RA synovium, and are known to activate NFκB and osteoclasts, and to increase cell tolerance to oxidative stress, both phenomena known to take place in the arthritic synovial tissues.\(^1\) - \(^11\)

Cxc110, Ccl12 and Ccl21b among others, were preferentially expressed, or expressed in significantly increased levels in DA, compared with congenics. Chemokine receptors Ccr6, Cx3cr1 and Cxcr3, as well as cytokine receptors, IL-1r11 (IL-33r) and IL-3ra were also upregulated in the synovial tissues from DA rats. Interestingly, Ccl21b, Ccr6 and Stat4 have been associated with RA susceptibility.\(^12\)

Additional important mediators of the inflammatory response upregulated in DA included Csk, a key signaling gene that regulates T and B cell proliferation, activation, and migration that interacts with the RA susceptibility gene Ptpn22.\(^2\)

Four of the above genes, including IL-6, Stat4, Ccr6 and Cxcl110, have been implicated in the differentiation, activity or chemotaxis of Th17 cells, a cell type central to autoimmune disease pathogenesis. We next looked for the three genes more specifically related to Th17 cells: IL-17A, IL-17F and Rorc (RORγt).

The Illumina RatRef microarray does not contain probes for IL-17A specifically related to Th17 cells: IL-17A, IL-17F and Rorc (RORγt). Therefore, we used qPCR to study the expression of these two genes. IL-17A was preferentially expressed in DA synovial tissues (93%), compared with DA.ACI(Cia10) congenics (43%), and this difference was statistically significant \( (P = 0.0025, \text{Fisher exact test}, \text{Table 3}) \). IL-17F was not expressed in either

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**Figure 1.** A map of the Cia10 interval in DA.ACI(Cia10) congenics and ASI scores. (a) Map of the DA.ACI(Cia10) congenic interval on rat chromosome 2, showing DA alleles (white), ACI alleles (black) and recombination interval (gray). Key SSLP markers used in the congenic breeding are shown (Mb = position in megabases on the rat genome assembly v.3.4). (b) Cumulative median ASI scores of DA (ASI = 30.4) compared with DA.ACI(Cia10) (ASI = 12.3) during 21 days following PIA induction \( (P = 0.002, \text{Mann–Whitney test}) \) (DA n = 12 and DA.ACI(Cia10) n = 6). Boxes represent the 25-75 percentile, and error bars are the 5-95 percentile.
Table 1. Most significantly differentially expressed genes between DA.ACI(Cia10) congenics and DA

| Gene symbol | Gene name                                         | Accession number | Fold difference | P-value |
|-------------|---------------------------------------------------|------------------|----------------|---------|
| LOC499078   | Similar to glycoprotein 49b                        | GI_6238899-S      | 5.39           | 0.0023  |
| Csd3        | Cluster of differentiation 53                     | NM_012533.1       | 3.28           | 0.0040  |
| RGD1566002  | Similar to RIKEN cDNA 3110001N18                   | XM_221003.3       | 3.09           | 0.0038  |
| Fcg1a       | Fc fragment of IgG, high-affinity la, receptor (CD64) | XM_001062370.1   | 2.94           | 0.0073  |
| ccc109b     | Coiled-coil domain containing 109B                | XM_001076863.1    | 2.89           | 0.0036  |
| LOC499136   | Hypothetical gene                                 | XM_574429.1       | 2.85           | 0.0014  |
| Rangap1     | RAN GTPase-activating protein 1                    | XM_576313.1       | 2.84           | 0.0051  |
| RGD1565520  | Similar to 60S ribosomal protein L7a               | XR_001551.8       | 2.81           | 0.0025  |
| Emr1        | Efg-like module containing mucin like hormone receptor | NM_001007557.1  | 2.78           | 0.0000  |
| LOC308350   | Similar to PIR81                                   | XM_218261.3       | 2.73           | 0.0074  |
| RGD1564866  | Similar to heterogeneous nuclear ribonucleoprotein A1 | XR_007647.1       | 2.72           | 0.0038  |
| LOC687849   | Hypothetical gene                                 | XM_001080339.1    | 2.69           | 0.0012  |
| Rbbp7       | Retinoblastoma-binding protein 7                   | NM_031816.1       | 2.68           | 0.0052  |
| RGD1563053  | Similar to ribosomal protein L6                    | XR_007388.1       | 2.67           | 0.0010  |
| RGD1563124  | Similar to 40S ribosomal protein S20               | XM_576204.2       | 2.66           | 0.0013  |
| RGD1559935  | Similar to Selenoprotein H                         | XR_008210.1       | 2.66           | 0.0027  |
| Fcgr1a      | Fc fragment of IgG, high-affinity la, receptor (CD64) | XM_001062370.1   | 2.94           | 0.0073  |
| Rbbp7       | RAN GTPase-activating protein 1                    | XM_576313.1       | 2.84           | 0.0051  |
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Inflammatory mediators and receptors upregulated in DA.ACI(Cia10) congenics

Tgfα, Tgfb2 and the receptor Tgfb2r3 were upregulated or predominantly expressed in DA.ACI(Cia10) congenics, compared with DA (Table 3). Tgfb and its receptors are known to suppress immune responses and to induce the differentiation of Treg cells. Several genes upregulated in DA.ACI(Cia10) congenics are known to interact with, or to be regulated by Tgfb, further suggesting a role for this gene in mediating protection in the congenics (Figure 3).

The Vdr gene was predominantly expressed in congenics (80%) compared with DA (33%) (Table 3). IPA pathway and gene interaction analyses showed that several of the most significantly differentially expressed genes were induced by or interacted with the Vdr, including Tgfb2 (Figure 3). The Vdr is known to inhibit the expression of cytokines like IL-6, which is in agreement with our observations of an inverse correlation between Vdr-Tgfb2 and IL-6.

Cc11 (Eotaxin) expression was increased in the protected DA.ACI(Cia10) congenics (1.93-fold, \( P = 0.003 \)). Interestingly, Cc11 levels have been shown to correlate with reduced radiographic damage in RA via yet unknown mechanisms. Cc11 is a chemoattractant to eosinophils and Th2 cells, and modulates monocyte activity.

Genes implicated in cancer and cancer-related phenotypes accounted for the greatest percentage of the differentially expressed genes.

Eighty-nine genes of the 310 (28.7%) differentially expressed genes are implicated in cancer and cancer-related phenotypes. These included oncogenes, cell-cycle regulators, tumor-suppressor genes, proteases and others. The 27 genes with the strongest literature support for a role in cancer are listed on Table 4. Among the cancer-favoring genes upregulated in DA, there were eight oncogenes or tumorigenesis genes, including Rbm3, Ikbke, Rcl, Kras, four cell proliferation enhancing genes (Mcts1, Aplnr, Ppap2a and Lcr3) and three invasion-associated genes (Ctsd, Cd53 and Loxl1). Two additional mediators of cancer invasion and metastasis, Cxcl10 and Cxcr3, were preferentially expressed by DA synovial tissues (\( DA = 12 \) (100%), DA.ACI(Cia10) = 6 (50%)) (Table 3). IL-11, which as described above was expressed in increased levels in DA (Table 3), has also been implicated in the pathogenesis of carcinomas. Histone deacetylases (Hdac1, Hdac2 and Rbbp7) had increased expression in DA, and are also commonly upregulated in cancers and considered to regulate cell differentiation and proliferation.

Nine of the genes upregulated in DA.ACI(Cia10) are known to suppress a cancer phenotype. Specifically, four of these genes (Pawr, Per1, Igfbp6 and Thbd) negatively regulate cell proliferation and growth, and three genes (Rgs4, Nov and Tgfb3r) negatively regulate cell invasion (Table 3). Matrix metalloproteinases (MMPs) are known to increase invasion of cancer and synovial cells and are key mediators of cartilage and joint destruction in RA. Timp2 (2.15-fold, \( P = 0.0024 \)) and Reck (2.14-fold, \( P = 0.005 \)) two MMP inhibitors, were also significantly upregulated in DA.ACI(Cia10). Four of the cancer-related genes (Cd53, Nov, Reck and Timp2) were confirmed with qPCR (Figure 2). Therefore, our results suggest a general increased expression of genes known to favor cell proliferation and invasion in DA, whereas the congenics has an increased expression of anti-proliferation and anti-invasion/anti-destruction (MMP inhibitors) genes. These observations provide new insight into the molecular processes regulating the reduced synovial hyperplasia and reduced

### Table 1 (Continued)

| Gene symbol | Gene name | Accession number | Fold difference | P-value |
|-------------|-----------|-----------------|-----------------|--------|
| Tmem117     | Transmembrane protein 117 | XM_576330.2 | 2.06 | 0.0023 |
| Nes         | Nestin    | NM_012987.1     | 2.06 | 0.0060 |
| Rgs4        | Regulator of G-protein signaling 4 | NM_017214.1 | 2.05 | 0.0079 |
| Hexim1      | Hexamethylene bis-acetamide inducible 1 | XM_573204.1 | 2.04 | 0.0022 |
| Axl         | AXL receptor tyrosine kinase | NM_001013147.1 | 2.03 | 0.0046 |
| Tspan2      | Tetraspanin-2 | NM_022589.1 | 2.02 | 0.0057 |

The Vdr gene was predominantly expressed in congenics (80%) compared with DA (33%) (Table 3). IPA pathway and gene interaction analyses showed that several of the most significantly differentially expressed genes were induced by or interacted with the Vdr, including Tgfb2 (Figure 3). The Vdr is known to inhibit the expression of cytokines like IL-6, which is in agreement with our observations of an inverse correlation between Vdr-Tgfb2 and IL-6.

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Genes upregulated in DA

| Gene symbol | Gene name                        | Accession number | Expressed in all samples | Preferential strain expression |
|-------------|----------------------------------|------------------|--------------------------|-------------------------------|
| Ccl2b       | Chemokine (C-C motif) ligand 21b | NM_001008513.1   | 1.61                     | 0.002                         |
| Csk         | c-src tyrosine kinase            | NM_236290.3      | 2.02                     | 0.002                         |
| IL-11       | Interleukin-11                   | NM_346531.2      | 1.66                     | 0.007                         |
| IL-3ra      | Interleukin-3 receptor subunit alpha | NM_139260.1   | 1.52                     | 0.009                         |

Genes predominantly expressed in DA

| Gene symbol | Gene name                        | Accession number | Expressed in all samples | Preferential strain expression |
|-------------|----------------------------------|------------------|--------------------------|-------------------------------|
| Ccl2        | Chemokine (C-C motif) ligand 12  | NM_213425.2      | 11 (92)                  | 2 (33) 0.021                 |
| Ccr6        | Chemokine (C-C motif) receptor   | XM_01013145.1    | 12 (100)                 | 3 (50) 0.024                 |
| Cx3r1       | Chemokine (C-X3-C motif) receptor| NM_133534.1      | 10 (83)                  | 1 (16) 0.012                 |
| Cxcl10      | Chemokine (C-X-C motif) ligand 10| NM_139089.1      | 12 (100)                 | 3 (50) 0.024                 |
| Cxcr3       | Chemokine (C-X-C motif) receptor 3 | NM_053415.1    | 12 (100)                 | 3 (50) 0.024                 |
| Ifn4        | Interferon gamma inducible protein 47 | NM_172019.1 | 9 (75)                   | 0 (0) 0.009                  |
| IL-10       | Interleukin 10                   | NM_012854.1      | 8 (67)                   | 0 (0) 0.012                  |
| IL-11I (IL-33R) | Interleukin 1 receptor-like 1 | NM_013037.1      | 12 (100)                 | 2 (33) 0.005                 |
| IL-6        | Interleukin-6                    | NM_012589.1      | 11 (92)                  | 2 (33) 0.021                 |
| IL-17A      | interleukin-17A                  | NM_001106897     | 13 (93)                  | 3 (43) 0.025                 |
| Stat4       | Signal transducer and activator of transcription 4 | NM_001012226.1 | 11 (92)                  | 2 (33) 0.021                 |

Genes upregulated in DA.ACI(Cia10)

| Gene symbol | Gene name                        | Accession number | Expressed in all samples | Preferential strain expression |
|-------------|----------------------------------|------------------|--------------------------|-------------------------------|
| Ccl11       | Chemokine (C-C motif) ligand 11 (eotaxin) | NM_019205.1 | 1.93                     | 0.003                         |
| Tgfa        | Transforming growth factor alpha  | NM_012671.1      | 4.20                     | 0.006                         |
| Tgf2b       | Transforming growth factor beta 2 | NM_031131.1      | 1.93                     | 0.0001                        |
| Trnsf12     | Tumor necrosis factor ligand superfamily member 12 (TWEAK) | NM_001001513.2 | 2.21                     | 0.003                         |
| Catc        | Catalase                         | NM_012520.1      | 2.07                     | 0.008                         |
| Gstm1c      | Glutathione S-transferase mu 1   | NM_017014.1      | 2.71                     | 0.003                         |
| Mt3c        | Metallothionein-3; growth inhibitory factor (GIF) | NM_053968.2 | 3.34                     | 0.009                         |
| Nfe2l1c     | Nuclear factor, erythroid derived 2-like | NM_340886.3 | 1.76                     | 0.009                         |
| Sqstm1c     | Sequestosome 1                   | NM_175894.3      | 1.66                     | 0.002                         |

Genes predominantly expressed in DA.ACI(Cia10)

| Gene symbol | Gene name                        | Accession number | Expressed in all samples | Preferential strain expression |
|-------------|----------------------------------|------------------|--------------------------|-------------------------------|
| Tgfr3       | Transforming growth factor, beta receptor III | NM_017256.1 | 6 (50)                   | 6 (100) 0.053                |
| Vdr         | Vitamin D (1,25-dihydroxvitamin D3) receptor | NM_017058.1 | 4 (33)                   | 4 (80) 0.321                 |

a Genes expressed in all samples were compared with t-test, and preferential strain expression with Fisher exact test. b Genes associated with genetic susceptibility in rheumatoid arthritis. c Inhibitors of oxidative stress. d Gene associated with disease severity in rheumatoid arthritis. Grey box: gene implicated in Th17 cell responses.

cartilage and joint damage that we had previously reported on DA.ACI(Cia10) congenics compared with DA.

Apoptosis and cell survival genes are over-represented among the differentially expressed genes. Sixty-eight cell death regulatory genes (Table 2), including 43 apoptosis genes (13.9% of all differentially expressed genes) had a significantly different expression between DA and DA.ACI(Cia10) (Supplementary Table 2). However, a similar number of pro-apoptosis (DA = 11 and DA.ACI(Cia10) = 8) and anti-apoptosis (DA = 12 and DA.ACI(Cia10) = 10) genes was upregulated in DA and in DA.ACI(Cia10). Therefore, although several apoptosis genes were differentially expressed, there was not a clear suggestion of an increased or reduced apoptotic gene expression pattern in either strain.

Anti-oxidant genes are increased in DA.ACI(Cia10)

Genes with anti-oxidant properties (Cat, Mt3, Nfe2l1 and Sqstm1), including Gstm1, which has been associated with disease severity and articular damage in RA,17 were among the most significantly upregulated genes in DA.ACI(Cia10) (Figure 3 and Table 3). NFκB pathway interacting genes are differentially regulated between DA and DA.ACI(Cia10)

Ten of the differentially expressed genes are known to interact with the NFκB pathway (Supplementary Table 3). These included three NFκB activators (Ikbb, Ccl21b and Ikbbkap) and one inhibitor (Ucp2) upregulated in DA. As described above, IL-6 and IL-11, which are known to activate NFκB, were also expressed in increased levels, or preferentially expressed in DA. Among the genes upregulated in DA.ACI(Cia10) congenics, there were those that reduce activation (Cat) and transcription (Pawr and Thbd) of NFκB. Additionally, several genes upregulated in DA.ACI(Cia10) congenics have been reported to interfere with the NFκB pathway (Supplementary Figure 1). Therefore, we looked for 244 NFκB/C-rel target genes previously reported by others18 and determined that 78 were expressed by all of our samples (32%). Only one of these genes (Acs14) was differentially expressed (2.6-fold upregulated in DA.ACI(Cia10), P = 0.003), suggesting that NFκB was not differentially activated between the two strains. An in vitro NFκB luciferase reporter assay, performed in cultured fibroblast-like synoviocyte (FLS) obtained from DA and DA.ACI(Cia10) rat synovial tissues and stimulated with IL-1β, did not detect any significant difference in NFκB activity between the two
strains, in agreement with the lack of an NFκB expression signature (Supplementary Figure 1).

Differentially expressed genes located within the DA.ACI(Cia10) interval on chromosome 2

Two hundred four of the 7593 genes expressed by all samples were contained within the Cia10 quantitative trait locus region on rat chromosome 2. Sixteen of these 204 (7.8%) genes were differentially expressed between DA and DA.ACI(Cia10) congenics, which was more than the 76 (1%) that would have been expected by chance \( (P = 0.018, \text{Chi-square test}) \). Seven of these sixteen genes were expressed in increased levels in DA.ACI(Cia10) and included Pde5a, Palmd, Tspan2, Nes, Adamtsl4 and two antioxidant genes Gstm1 (2.58-fold, \( P = 0.0029 \)) and Gstm2 (2.70-fold, \( P = 0.0028 \); Table 5).

Nine of the above 16 differentially expressed genes were upregulated in DA, and conversely downregulated in DA.ACI(Cia10) congenics, and included Cldn5, Fgfr1a, Shc1, Tnfaip8l2, gene similar to Gapdh, Sass6, Hist2h2ac, Ncu-g1 and gene similar to Nrf2 (Table 5). Cldn5 (3.28-fold, \( P = 0.004 \)) and Fgfr1a (2.94-fold, \( P = 0.007 \)) were among the most significantly differentially expressed genes.

The differential expression of Cldn5, Fgfr1a and Gstm1 was confirmed using qPCR (Figure 2). Thirteen of the differentially expressed genes located within the DA.ACI(Cia10) interval are located in the same cytogenetic band on chromosome 2 (2q34) suggesting the possibility that there could be a polymorphism within the region affecting mRNA transcription or stability of multiple genes.

None of the genes contained within the Cia10 interval were preferentially expressed in one strain versus the other.

microRNAs located within the Cia10 interval contain no polymorphisms

We hypothesized that part of the differential gene expression pattern could be explained by polymorphisms in a microRNA located within the Cia10 interval. The polymorphism could lead to an alteration of the microRNA expression or function and modulation of target genes. Eight microRNAs are predicted to reside within the Cia10 interval on rat chromosome 2 (miR9-1, miR15b, miR16, miR92b, miR137, miR186, miR190b and miR760). Although seven of these microRNAs had differentially expressed targets, none of them had more target genes than would have been expected by chance (miR9-1: \( n = 11 \), miR15b: \( n = 16 \), miR16: \( n = 16 \), miR92b: \( n = 9 \), miR137: \( n = 9 \), miR186: \( n = 5 \), miR760: \( n = 6 \)).

For further confirmation, we sequenced these seven microRNAs and their surrounding DNA (500-1000 bp), which contains regulatory regions. However, no polymorphisms were detected between DA and ACI.

DISCUSSION

Increased disease severity and articular damage are associated with increased risk for disability, deformities and reduced life expectancy in patients with RA. Yet, the genes that regulate disease severity and articular damage remain largely unknown. We have been interested in the identification of these genes and consider that they have great potential to generate new and perhaps better targets for therapies aimed at preserving joint integrity and function. In the present study, we used synovial tissues from DA rats, which develop severe arthritis (PIA) with pronounced synovial hyperplasia, and cartilage and bone destruction, and synovial tissues from arthritis-protected and non-erosive DA.ACI(Cia10) congenics. These two strains are genetically identical except for the presence of ACI alleles at the Cia10 interval, underscoring the magnitude of the effect of this single locus on clinical disease, histologic joint damage and gene expression.

DA.ACI(Cia10) and DA had significant differences in the expression of inflammatory mediators. That difference was not broad, but, instead, limited to a very specific set of genes, most of which have been involved in the regulation of cartilage and bone erosions, and articular damage. IL-6 and IL-11, which belong to the...
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Damage.21 Ccl21b is chemotactic for Ccr7-expressing T and B cells, in the development of synovial hyperplasia and joint erosions and Ccr2-expressing monocytes and neutrophils, which have a key role expressed in reduced levels in congenics. Ccl12 is chemotactic for and chemokine receptors Ccr6, Cx3cr1 and Cxcr3 were also arthritis and reduces articular damage.24 Cxcl10 and its receptor is expressed by myeloid cells, and its blockade ameliorates rodent arthritis pathogenesis and joint damage,8,9 the literature on IL-11 cytokines with significantly reduced expression in DA.ACI(Cia10) IL-6 family of genes and signal through gp130, were among the expression of Tgfb2 and Ccl11. Tgfbr3 was also preferentially expressed in congensics’ synovial tissues. Tgfβ is considered a suppressor of T-cell responses and has a central role in the differentiation of Treg cells. Furthermore, treatment with Tgfβ ameliorates autoimmune arthritis in rodents whereas anti-Tgfβ antibodies exacerbate disease.27 Ccl11, also known as Eotaxin-1, is an eosinophil chemoattractant expressed by T cells and fibroblasts. Increased serum levels of Ccl11 have been associated with a differentiation-inhibitory properties, 29 and Tgfb-inducing activities Ccl11 is considered a marker of proinflammatory and joint damage-favoring genes in DA, while damage, with increased levels or preferential expression of findings suggest that Cia10 directly or indirectly regulates the expression of genes implicated in arthritis severity and joint damage.4,6,14 Lastly, the Vdr was predominantly expressed in the synovial tissues of congenics. The Vdr has known IL-6-suppressive, 28 Th17 differentiation-inhibitory properties, and Tgfb-inducing activities (Figure 3), which matches the scenario identified in this study. Vdr agonists also reduced arthritis severity.30 Together, our findings suggest that Cia10 directly or indirectly regulates the expression of genes implicated in arthritis severity and joint damage, with increased levels or preferential expression of proinflammatory and joint damage-favoring genes in DA, while the opposite takes place in congenics.

Although IL-23, IL-17-F and RORgt (Rorc) were not differentially expressed in synovial tissues collected on day 21, four of the genes upregulated or preferentially expressed in DA, and conversely downregulated in DA.ACI(Cia10) congenics, suggesting an in-

## Table 4. Differentially expressed genes related to cancer

| Gene symbol | Accession number | Gene name | Fold difference | P-value |
|-------------|-----------------|-----------|-----------------|---------|
| Upregulated in DA | | | | |
| Oncogenes and tumorigenesis | | | | |
| Rcl (CEORF108) | NM_135325.1 | Nucleoside 2'-deoxyribosyltransferase domain containing protein (RGD620382) | 2.38 | 0.004 |
| Kras | NM_031515.1 | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog | 1.93 | 0.005 |
| Rbm3 | XM_001063211.1 | RNA-binding motif (RNP1, RRM) protein 3 | 1.57 | 0.009 |
| Ilkbk | XM_001058036.1 | Inhibitor of nuclear factor kappa-B kinase subunit epsilon | 1.51 | 0.007 |
| St14 | NM_053635.2 | Suppression of tumorigenicity 14 | 2.55 | 0.0004 |
| Cdc25a | NM_135571.1 | Cell division cycle 25 homolog A | 1.67 | 0.007 |
| Hdac1 | XM_216349.3 | Histone deacetylase 1 | 1.52 | 0.006 |
| Hdac2 | XM_342149.3 | Histone deacetylase 2 | 1.53 | 0.0007 |
| Cell proliferation | | | | |
| Mcts1 | XM_001064848.1 | Malignant T-cell amplified sequence 1 | 2.20 | 0.001 |
| Ppap2a | NM_022538.1 | Phosphatidic acid phosphatase type 2a | 2.12 | 0.002 |
| Ier3 | NM_212505.1 | Immediate early response 3 | 1.93 | 0.008 |
| Aplnr | NM_031499.2 | Apelin receptor | 1.80 | 0.009 |
| Cell invasion | | | | |
| Cd35 | NM_012523.1 | Cluster of differentiation 53 | 3.28 | 0.004 |
| Loxl1 | NM_001012125.1 | Lysyl oxidase-like 1 | 1.92 | 0.005 |
| Ctsd | NM_134334.2 | Cathepsin D | 2.27 | 0.001 |
| Histone deacetylation | | | | |
| Rbpb7 | NM_031816.1 | Retinoblastoma-binding protein 7 | 2.68 | 0.005 |
| Hdac2 | XM_342149.3 | Histone deacetylase 2 | 1.53 | 0.001 |
| Hdac1 | XM_216349.3 | Histone deacetylase 1 | 1.52 | 0.007 |
| Upregulated in DA.ACI(Cia10) | | | | |
| Inhibitors of cell proliferation and growth | | | | |
| Igfbp6 | NM_013104.2 | Insulin-like growth factor binding protein 6 | 3.56 | 0.007 |
| Per1 | XM_340822.2 | Period 1 | 2.78 | 0.001 |
| Thbd | NM_031771.2 | Thrombomodulin | 1.96 | 0.002 |
| Ppap2a | NM_034856.2 | PRKC, apoptosis, WT1, regulator | 1.54 | 0.005 |
| Inhibitors of invasion | | | | |
| Nov | NM_030868.2 | Nephroblastoma overexpressed gene | 2.86 | 0.007 |
| Rgs4 | NM_017214.1 | Regulator of G-protein signaling 4 | 2.05 | 0.008 |
| Tgfbr3 | NM_017256.1 | Transforming growth factor, beta receptor III | – | – |
| Inhibitors of MMPs | | | | |
| Timp2 | NM_021989.2 | Tissue metallopeptidase inhibitor 2 | 2.15 | 0.002 |
| Reck | XM_001070551.1 | Reversion-inducing-cysteine-rich protein with kazal motifs | 2.10 | 0.005 |

Some of these genes have more than a single cancer-related function. aTgfbr3 was expressed by 6 (50%) DA and 6 (100%) DA.ACI(Cia10) synovial tissues. Tgfbr3 is also a suppressor of cancer growth and cell proliferation.
increased presence of IL-17 producing or inducing cells: a) IL-17A was expressed in nearly all DA samples (93%), but only in 43% of congenics; b) Ccr6 is a cell surface marker of Th17 T cells,31 c) Stat4 mediates IL-23R signaling, which is required for the generation of Th17 cells, and d) Cxcr3 is expressed by mast cells, which have been indirectly controlling arthritis severity via the regulation of the NF-κB pathway.36 A subset of the differentially expressed genes in the present study, including IL-6, IL-11 and Ccl21b, are known activators of NF-κB activity. However, we were not able to detect a differentially expressed NF-κB-interacting gene. For further confirmation a NF-κB luciferase reporter assay was studied in FLS from DA and DA.ACI(Cia10) congenics; b) Ccr6 is a cell surface marker of Th17 T cells;31 c) Stat4 mediates IL-23R signaling, which is required for the generation of Th17 cells, and d) Cxcr3 is expressed by mast cells, which have been considered the main source of IL-17 in the synovial tissue.32 Furthermore, DA synovial tissues preferentially expressed IL-6, which is a key cytokine for differentiation of Th17 cells, but lacked Vdr expression, which is an inhibitor of Th17 differentiation. These observations raise the possibility that Cia10 could be a new gene regulating arthritis severity via the regulation of the differentiation and/or homing of Th17 cells to the joint.

Four of the genes with the most significantly reduced expression in DA.ACI(Cia10) congenics are either associated with genetic susceptibility to RA (Ccr6, Ccl21b, Stat4),12,33 or as in the case of Csk, directly interact and regulate the activity of a RA susceptibility gene (PTPN22).2 Furthermore, Gstm1 null alleles have been associated with RA severity and radiographic erosive damage,17 and DA synovial tissues did, in fact, have significantly reduced expression compared with DA.ACI(Cia10). However, sequencing of DA Gstm1 gene revealed no deletions, insertions or significant sequence variants that might explain the reduced expression (data not shown). Taken together, our results suggest that the Cia10 gene could interact with RA susceptibility genes to regulate their expression and perhaps function, raising the possibility of potential epistatic interactions.

NFκB is a central regulator of synovial hyperplasia and articular damage,34,35 and several RA susceptibility genes are involved in this pathway.36 A subset of the differentially expressed genes in the present study, including IL-6, IL-11 and Ccl21b, are known activators of NFκB, and NFκB-interacting genes were differentially expressed, raising the possibility that Cia10 might be involved in the regulation of NFκB activity. However, we were not able to detect a differentially expressed NFκB transcriptional signature.13 For further confirmation a NFκB luciferase reporter assay was studied in FLS from DA and DA.ACI(Cia10) congenics stimulated with IL-1β, and did not detect

### Table 6. Primers and probes used for microarray results validation with qPCR, and primers used for microRNA sequencing

| Gene symbol | Accession number | Forward primer | Reverse primer | Probe number |
|-------------|------------------|----------------|----------------|--------------|
| **qPCR**    |                  |                |                |              |
| Cds3        | NM_012523.1      | CTGGTCTCCATGTCACGAGAC | GCCCGGCCGCGACGAGAC | 4            |
| Fcgr1a      | NM_01010083.61   | CTTGTCAGTAGAATCTACGAGA | CTTAATTCCGTTGACGAGAC | 110          |
| Gapdh       | NM_017008        | GGAGGCTGTCATGTCGAGAC | GCCAGTGCCTGACAGAC | 106          |
| Gstm1       | NM_017014.1      | TGTACACGACGAGACGACGAGAC | TTCTCTCGTGGTCGAGAC | 80           |
| LOC499078b  | NM_017006.601    | GGAGACTGACGACGACGACGAGAC | GCCGTAACACGTTGTTCGGTCACAGAC | 38           |
| Nov         | NM_030868.2      | CGGCCGCGCGGCAGACGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | 12           |
| Reck        | NM_0110795.41    | AAAAGTGGGACACATGTTGAGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | 106          |
| Timp2       | NM_021989.2      | CTTGACCTGTCGAGACGACGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | 38           |
| Rorc        | RGD:1595785      | AGCCAGACTCCTCCCTAGTGTCGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | 12           |
| Il17a       | NM_010689.97     | CTCTCGGTGACGACGACGACGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | 106          |
| Il17f       | NM_001051.11     | GGCTGTCGACGACGACGACGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | 38           |
| **Sequencing** |             |                |                |              |
| mir9-1      | MIM000838        | CAGCTAGATTCGCGGACG | ATTCGTGAGGAGAGGAGGAGGAGGAGGAGGAG | -            |
| mir13b/16   | MIM000843/MIM000844 | TGGAGGCTGGGAGGAGGAGGAGGAGGAGGAG | -            |
| mir92b      | MIM0006167      | GAGGTAGGTGGGGAGGAGGAGGAGGAGGAGGAG | -            |
| mir137      | MIM000910       | AACACAGGGAAACCTACGGGAGGAGGAGGAGGAG | -            |
| mir186      | MIM000931       | TTCTAGGATTCGACGACGACGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | -            |
| mir190b     | MIM006135       | AGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG | -            |
| mir760      | MIM006164       | CTCTGTCGACGACGACGACGAC | TGGGATGGGAGGAGGAGGAGGAGGAGGAGGAG | -            |

*miR and its accession numbers were obtained from Ensembl and the miRBase. **Similar to Gp49b. ***miR15b and miR16 are adjacent to each other and sequenced with a pair of primers that covered both miRNAs.
any significant difference in transcriptional activity. Therefore, our results suggest that Cia10 does not have a major effect on the regulation of the NFκB pathway.

There are several similarities between the pathogenesis of RA synovial hyperplasia, its invasion and destruction of cartilage and bone, and the behavior of cancers. Previous studies from our group and others have identified increased expression of cancer-related genes in FLS from arthritic rats and from patients with RA. In the present study, one of the predominant differentially expressed groups was related to cancer and cancer phenotypes. Genes involved in oncogenesis (Kras, Ikkb, St14), cell proliferation (Cdc25a, Ier3, Ppap2a), cancer invasion (Cdx53, Ctc9, Cxcl10, Cxcr3, Loxl1), and histone deacetylation (Hdac1, Hdac2 and Rbbp7) were expressed in increased levels in DA and in reduced levels in congenics.

On the contrary of the cancer-favoring genes upregulated in DA, genes upregulated in DA.ACI(Cia10) were protective against cancer phenotypes. For instance, Nov, Rgs4 and Tgfbr3, which are inhibitors of cancer cell migration and invasion, were expressed in increased levels in DA.ACI(Cia10). Two MMP inhibitors (Timp2 and Reck) were upregulated in DA.ACI(Cia10) and downregulated in DA. Reck negatively regulates cancer cell invasion, metastasis and angiogenesis in cancer, and its expression is reduced in cancer cells. Like in cancers and in DA synovium, Reck is expressed in reduced levels in RA synovial tissues, compared with OA controls. Timp2 is a negative regulator of MMPs, angiogenesis, cancer cell growth, invasion and metastasis. Taken together, our results show that the presence of DA alleles at Cia10 increases the expression of genes favoring cancer development, proliferation and invasion, while the opposite is seen in the presence of ACI alleles. These observations suggest that these Cia10-regulated cancer genes could be involved in the regulation of synovial hyperplasia and pannus invasion and destruction of cartilage and bone.

Survival and apoptosis abnormalities have been described in cancer, in arthritic synovial tissues and in autoimmunity. We considered that the synovial hyperplasia in DA, and the lack of it in DA.ACI(Cia10) could be attributed to differences in expression of apoptosis genes. Although many apoptosis and cell survival genes were differentially expressed, there was not a clear bias toward having increased numbers of pro-apoptosis versus anti-apoptosis genes in either strain.

Thirteen of the sixteen differentially expressed genes located within the DA.ACI(Cia10) interval map to the same cytogenetic site polymorphisms have been a rare explanation for autoimmunity or other complex traits, it is certainly a possibility worth considering.

We also considered that a polymorphism in a microRNA located within the Cia10 interval might explain part of the differences in synovial gene expression. The Cia10 interval contains eight predicted microRNAs, but sequencing these microRNAs revealed no polymorphisms. Additionally, analyses of the expression of predicted microRNA targets revealed no significant differences between the strains, making the microRNA hypothesis unlikely to explain the Cia10 effect.

In conclusion, we have determined that Cia10 regulates the expression of a unique set of inflammatory mediators, including markers of Th17 cells, IL-6, IL-17A and IL-11, oxidative stress regulators and a cancer-associated gene expression signature. These observations suggest a mechanism of action for the Cia10 gene, and several new potential prognostic biomarkers and targets for therapies aimed at reducing disease severity and joint damage in RA.
cDNA synthesis and quantitative PCR (qPCR)
cDNA was synthesized from the same RNA samples hybridized to the RatRef-12 Expression BeadChip. 2 μl of total RNA was used for cDNA synthesis using the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). cDNA was diluted 1:10 for qPCR. Rat specific primers and probes were designed using the Universal ProbeLibrary (Roche, Indianapolis, IN, USA) (Table 6). Gapdh was used as an internal control and all samples were run in duplicate. The average threshold cycle (Ct) values were used to analyze relative gene expression of Th17 genes Cd53, FcgR1a, Gp49b, Timp2 Reck, Nov and Gstm1. Probes were used at a final concentration of 100 nM, 5’ ends were labeled with FAM and 3’ ends with TAMRA. Primers were used at 200 nM with Eurogentec qPCR mastermix (Eurogentec, San Diego, CA, USA). Reactions were run on an ABI 7700 qPCR thermocycler at 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 0.15 min and 60°C for 1 minute. Relative expression of genes was normalized to Gapdh in each sample (ΔCt), and ΔCt values were used for t-test analysis. qPCR fold-differences were calculated using the 2^-ΔΔCt method.

microRNA analyses
The miRBase.org database was used to identify the microRNAs located within the Cia10 interval, and www.targetScan.org was used to predict their targets. The eight microRNAs contained within the Cia10 and with differentially expressed predicted targets were sequenced as described below.

Sequencing
Splenic genomic DNA (gDNA) was extracted using DNeasy Blood and Tissue Kit (Qiagen). Primers were designed using the Whitehead Institute Primer3 website (http://frodo.wi.mit.edu/primer3/input.htm) to amplify 500–1000 bp regions surrounding eight known microRNA sequences located within the Cia10 interval (Table 6). PCR products were sequenced and analysed with the DNASTAR sequencing analysis software (DNASTAR, Madison, WI, USA).

NFκB luciferase reporter assay
Synovial tissues were collected from a different group of DA and from DAA(Cia10) joints 21 days after the induction of PIA for FLS isolation. FLS were isolated as previously described.41 Cells from passage four or greater were transfected with the NFκB luciferase reporter plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen). Control cells were transfected with a luciferase plasmid without a promoter. Following transfections, cells were cultured overnight on FBS-free medium, followed by stimulation with IL-1β 10 ng ml^-1 for 48 h. The NFκB reporter activity was measured with the TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Arbitrary units of the Renilla reporter were used for internal normalization.

Statistical analysis
The t-test was used to compare the expression of genes expressed by all samples. Chi-squared and Fisher exact tests were performed using SigmaStat 3.0 (SPSS, Chicago, IL, USA).

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CONFLICT OF INTEREST
The authors declare no conflict of interest.
Cia10 regulates synovial Th17 and cancer genes signature
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