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Wnt Signaling Genes of Murine Chromosome 15 are Involved in Gender-affected Pathways of Inflammatory Arthritis

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Abstract

Objective—Gender disparities in rheumatoid arthritis (RA) are well documented despite the lack of any known major RA susceptibility genes mapped to sex chromosomes. Murine chromosome 15 carries the sex-affected *Pgia8* locus that mediates proteoglycan-induced arthritis (PGIA); homologous human loci are associated with RA. This study uses a *Pgia8* congenic strain to identify genes/mechanisms implicated in gender disparities in arthritis.

Methods—Gene expression analysis was performed using RNA isolated from paws of congenic males and females with collagen antibody-induced arthritis (CAIA). Data were corroborated with RT-PCR and also studied in mice prior to disease onset. Ingenuity Pathways Analysis of the expression patterns and gene functions were used to discover locus-specific and sex-affected signature transcripts.

Results—We found that the *Pgia8* locus regulates antibody-mediated inflammatory arthritis differently in males and females. In *Pgia8* congenic males, arthritis severity was 30% less ($p < 0.005$) than in wild-type males, but the anti-inflammatory effect was similar in wild type and congenic females. Transcriptome analysis indicated that twelve genes within the locus were significantly deregulated in arthritic joints of congenic mice; expression of these genes was also gender specific. The genes that correlated the most with arthritis severity include collagen triple helix repeat containing-1 (*Cthrc1*), metalloproteinase *Adamts12*, R-spondyn (*Rspo2*) and Syndecan (*Sdc2*) ($r=0.87-0.91$). The level of *Cthrc1* message also correlated with that of pro-inflammatory cytokines IL-1 β and IL-6.

Conclusion—Gender-specific disparities in RA are linked to transcriptional regulation of genes involved in cartilage degradation (*Adamts12*) and canonical and non-canonical Wnt signaling (*Cthrc1*, *Rspo2*, *Sdc2*).

Rheumatoid arthritis (RA) is a progressive autoimmune inflammatory disease characterized by the complex interplay of genetics and environment (1–3). The influence of gender as non-genetic RA risk factor was recognized decades ago: women have worse RA symptoms than men, and represent the majority (approximately 75%) of RA patients (4, 5). Since the prognosis of arthritic patients has improved in the last few decades, gender disparities in RA were recently re-evaluated with the most up-to-date clinical parameters. The findings corroborated previous observations that women had worse arthritis than men for almost all clinical RA scores and also confirmed significant female to male preponderance in RA (6).

Early linkage studies demonstrated the involvement of sex chromosomes in RA patients (7, 8), but recent genome-wide association studies (GWAS) have not found RA susceptibility

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genes on sex chromosomes (1, 3, 9) suggesting that the mainstream mode of gender-dependent RA regulation is hormonal rather than linked directly to sex chromosomes. However, genetic linkage analysis of animal models for RA have provided examples of significant gender influences on autosomal modifier loci (10–14).

Murine chromosome 15 (ch15), particularly the *Pgia8* genomic interval (13, 14), carries genes implicated in numerous autoimmune and inflammatory diseases including experimental autoimmune encephalitis, Theiler's murine encephalomyelitis virus-induced demyelination, several types of rodent arthritides induced with either collagen type II (CIA) or aggrecan (PGIA) and *Borrelia burgdorferi* infection (14–16). In the human genome, ch15-syntenic region is associated with RA (9, 17), serum rheumatoid factor (18), and is associated with the efficacy of anti-TNF- α treatment in RA (19). Many of the murine ch15 loci were shown to be influenced by gender (14, 15, 20).

Multiple murine models of RA emphasize the fact that inflammation could be provoked by numerous stimuli such as genetic alterations or immunization with cartilage-related antigens but lead to similar pathology, including inflamed synovium, pannus formation and bone and cartilage deterioration at the effector phase of the disease. Once arthritis is initiated, it tends to be self-perpetuating and could be transferred to a naïve donor using serum or cells from an arthritic animal (21, 22), antibodies to type II collagen (CAIA), (23) or antibodies to glucose-6-phosphate isomerase (24). Antibody-mediated murine arthritides share similarities with the inflammatory effector phase of RA in humans. This phase is an important target for therapeutic interventions, such as anti-CD20 rituximab and anti-IL-1 β canakinumab therapies (25, 26).

We hypothesize that gender specific connective tissue remodeling might be a common point of convergence for the clustering of multiple inflammatory diseases within the *Pgia8* congenic interval. In this study, we investigated the susceptibility to CAIA of BALB/c.DBA/2-*Pgia8* (C.D2-*Pgia8*) congenic mice that carry a sex-affected arthritis-suppressive genomic interval previously found to be associated with PGIA (14). We used whole-genome transcriptome analysis of both congenic and wild-type males and females to address the major mechanisms/pathways that underlie the *Pgia8* locus-specific anti-inflammatory effect.

Methods

Mice and congenic strains breeding

Mice were housed in a specific pathogen-free environment in the Institute for Animal Studies at the Albert Einstein College of Medicine (Bronx, NY). Animals received a standard rodent diet #5854 and water *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee. Congenic C.D2-*Pgia8* and C.D2-*Pgia9* males (14) were backcrossed to BALB/c females to obtain an N2 generation. N2 male and female founders carrying similar sub-congenic genomic intervals were selected using genomic markers for establishing sub-congenic inbred strains.

Collagen Antibody-induced Arthritis (CAIA)

Inbred wild-type and congenic males and females were injected with a cocktail of four monoclonal antibodies (ArthritoMab™ Antibody Cocktail, MD Biosciences, St. Paul, MN), according to the manufacturer's protocol. Briefly, 4 month old mice were injected in a tail vein with 2 mg of antibody per mouse on day 0 followed by an intraperitoneal injection of 40 μ g of LPS on day 5. Mice were observed once or twice a day for paw swelling and redness. The scoring system was based on the number of inflamed joints in each paw, as described previously (27). Inflammation in the phalanges (1 point per digit), metacarpus or

metatarsus (0–5 points), and in wrist or ankle joints (range 0–5) were scored separately, and then added together to produce a total score of 0–60 per mouse.

RNA isolation and reverse transcription

Total RNA was isolated from both front and hind paws using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Paw tissue was taken from digits to the ankle or to the distal radius/ulna. RNA integrity and quality was tested on an Agilent 2100 bioanalyzer, and RNA concentration was measured using an Agilent NanoDrop 1000 spectrophotometer.

Oligonucleotide microarray for differential gene expression analysis

RNA was isolated from arthritic wild-type BALB/c male, congenic C.D2-*Pgia8* male and congenic C.D2-*Pgia8* female mice. Each replicate contained RNA combined from four mice with similar arthritis scores. Altogether, three replicates for three experimental conditions represented 21 mice. Fluorescent probe synthesis, hybridization to Affymetrix oligonucleotide chips and scanning was performed according to the standard protocol in the Albert Einstein College of Medicine Affymetrix Facility. We used a GeneChip® Mouse Gene 1.0 ST Array that offered whole-genome and whole-transcript coverage, with approximately 27 probes for each of the 28,853 genes represented on the array. The Affymetrix Gene Expression Console and the Robust Multichip Analysis algorithm were used to combine several chips after background adjustments and chip normalization.

Bioinformatic and statistical data analysis

Analysis of overlapping sets of genes (Venn diagram) was performed using Visual Basic for Microsoft® Office Excel (Microsoft Corporation, Redmond, WA). Hierarchical clustering of expression profiles/genes was performed with a DNA-Chip Analyzer using Pearson's correlation between the standardized expression values (28). The functional analysis of arthritis severity-associated genes was generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Functional analysis identified the biological functions and/or diseases that were most significant to the data set using the right-tailed Fisher's exact test. The resulting p-value indicated the probability that each biological function and/or disease assigned to that data set is due to chance alone. Data are presented as mean ± s.e.m. Statistical analysis of expression value correlation was performed using SPSS (Chicago, IL).

Gene information including genome position, names and description is based on the build 37 assembly from the National Center for Biotechnology Information and the Mouse Genome Sequencing Consortium.

Real-time PCR analysis

cDNA generated with SuperScript® First-Strand Synthesis kit (Invitrogen) was assayed using an Applied Biosystems 7900 HT Fast Real-Time PCR System with 384-well plates, followed by SDS software data analysis. Expression was measured individually for each gene in triplicate with at least two repeats using the Fast SYBR® Green system (Applied Biosystems, Foster City, CA). Each run and plate contained a *Gapdh* reference. Quantification of RT-PCR results was performed using a modification of the comparative C_t method. Relative RNA concentration was calculated using the following equation: $\text{power}(b_g, C_{tg})/\text{power}(b_r, C_{tr})$, where base b_g is PCR efficiency for the target gene; C_{tg} is the cycle number for the target gene signal above the detection threshold; base b_r is PCR efficiency for the reference gene; C_{tr} is the cycle number for the reference gene signal above

the detection threshold. Amplification efficiencies were established by building standard curves for template dilutions versus C_t using specific primer pairs for each gene.

Results

Arthritis severity in Ch15 congenic strains is gender-dependent

C.D2-*Pgia8* and C.D2-*Pgia9* congenic mice carry two different genomic intervals that were found previously to be genetically linked to PGIA (14). Chromosome Y was replaced from the original DBA/2 to a BALB/c genetic background. In this study, we narrowed the *Pgia9* congenic interval to approximately 20 Mb (new congenic interval *Pgia9t*) using two consecutive backcrosses of congenic males to BALB/c females performed under the control of genomic markers (Fig. 1B).

C.D2-*Pgia8* and C.D2-*Pgia9t* congenic males and females were injected with cocktail of four monoclonal antibodies specific to collagen type II epitopes to induce CAIA (Fig. 1A). Wild-type BALB/c mice showed high sensitivity to inflammatory polyarthritis that involved both hind and front paws. No significant difference in arthritis severity was observed between BALB/c males and females, and arthritis severity in C.D2-*Pgia9t* congenic males was similar to BALB/c. CAIA severity in *Pgia8* congenic females was slightly higher than in BALB/c females (20% stronger, $p < 0.03$), but the most pronounced effect was observed in *Pgia8* congenic males, with only 55–70% of the inflammation seen in the wild-type BALB/c males (Fig. 1A). The difference originated even before LPS injection, and was highly significant ($p < 0.005$) for the entire observation period. Similarly, congenic females showed two-fold stronger CAIA than congenic males ($p < 0.005$).

Thus, we reproduced the arthritis-suppressive genetic effect of the *Pgia8* locus using an animal model for inflammatory arthritis (CAIA). The genetic anti-inflammatory effect was male-specific.

Transcriptome analysis of genes whose expression was associated with both gender and arthritis

The *Pgia8* locus contains over two hundred protein-encoding and small RNA-coding genes (Fig. 1). To search for causative genes that are associated with primary events in arthritis pathophysiology, we performed gene expression analysis under three experimental conditions (Fig. 1C). Comparison of BALB/c males versus congenic males should reveal a set of genes whose expression is affected only by *Pgia8* genetic differences/alleles under inflammatory conditions. With a 1.5-fold change (FC) expression threshold, we found 293 genes whose expression was affected by both a congenic interval and inflammation (Fig. 1D, severity-associated genes).

Comparison of congenic males with congenic females produced a set of genes whose expression is under the complex control of gender, inflammation and other genes located on sex chromosomes. Comparison of gene expression in C.D2-*Pgia8* congenic males versus congenic females yielded 1059 sex-affected genes (Fig. 1D). Expression of the majority of severity genes ($n = 241$) was also gender-biased.

Expression of only 15 of 285 locus-specific genes was affected by gender, and only three genes were associated with CAIA severity when a 1.5-FC threshold was applied (Fig. 1D, locus genes). These three major genes included *Cthrc1*, *C1q* and tumor necrosis factor related protein 3 (*C1qtnf3*), and ADAM metalloproteinase with thrombospondin type 1 motif 12 (*Adamts12*). All three genes showed a similar pattern of differential expression; message quantity was lower in congenic males versus wild-type BALB/c males by approximately 2-FC, and upregulated in congenic females by 1.8–2.8-FC (Table 1, and Supplementary

Tables S1 and S2). When a more relaxed threshold of 1.25-FC was applied, only a dozen locus-specific genes were found to be associated with both arthritis severity and gender (Table 2, signature genes).

Functional and expression gene patterns in murine CAIA and human RA are similar

Since the CAIA genome-wide transcriptome was not well characterized, it was important to validate the model and compare it to a prototypical human inflammatory disease such as RA. To search for similarity in biological functions in RA and CAIA, we performed unsupervised hierarchical clustering for expression patterns of major arthritis severity genes ($n=25$) that demonstrated deregulation at least ± 2 -FC (Fig. 2A). These genes were among the 293 detected at 1.5-FC threshold upon comparison of C.D2-*Pgia8* congenic males and wild-type BALB/c males (Fig. 1D).

Hierarchical clustering was successful in linking the acute phase reaction protein messages of serum amyloid A3 (*Saa3*) and pentraxin 3 (*Ptx3*) into one cluster (Fig. 2A, cluster C). Cluster D combined cell adhesion molecules of endothelial and platelet selectins E and P and hyaluronan synthase (*Has1*). Interestingly, expression of *Serpine1* and *Mmp3*, both of which have proteolytic activity, coalesced in cluster B with expression of *Cxcl5* (chemokine ligand 5 or *Rantes*). In cluster E, tissue inhibitor of metalloproteinase (*Timp1*) clustered with matrix metalloproteinase *Mmp19* and with insufficiently studied *C1qtnf6* (Fig. 2A, cluster E).

Weaker arthritis in congenic males was associated with the strongest downregulation of *Saa3* (-3.25 -FC), *Ptx3* (-2.8 -FC), *Cxcl5* (-2.7 -FC), and hyaluronan-binding protein *Tnfrsf11b* (-4.08 -FC) gene expression. Interestingly that some messages were upregulated in weakly arthritic joints in *Pgia8* congenic males. For instance, cluster G included genes that are negatively associated with inflammation, including neutrophil-specific transcripts for cathepsin G and myeloperoxidase (Fig. 2A).

Of the major arthritis severity genes ($n=25$), only the *Cthrc1* gene was located within the congenic interval. It was 2.34-FC downregulated and clustered with mesoderm-specific transcript (*Mest*) and apelin receptor (*Aplnr*) (Fig. 2A, cluster F).

Similarity between RA and CAIA was investigated with Ingenuity Pathways Analysis, which identified a total of 66 significant functional clusters, $p < 0.05$. The top nine clusters with $p < 10^{-12}$ included “connective tissue disorders”, “genetic disorders”, and “inflammatory response.” Known RA-associated genes prevailed in two top CAIA-associated gene clusters with extremely significant grouping ($p < 10^{-21}$). Smaller clusters comprised genes of cellular movement, hematological system development and function, immune cell trafficking, cancer, and skeletal disorders (Fig. 2B).

Altogether, Ingenuity Pathways Analysis of biological functions and hierarchical clustering of expression patterns confirmed that CAIA in congenic mice is a pertinent animal model for identifying homologous RA-associated molecular pathways.

Sex chromosomes-biased gene expression

We observed a notable difference in CAIA severity between congenic male and congenic female mice, but similar severity of arthritis in wild-type BALB/c males and females (Fig. 1A). To find genes whose expression was affected by sex but not the *Pgia8* interval, we compared females and males from the same congenic strain. Despite the fact that both chromosome X and Y were of BALB/c origin, XX/XY chromosome disparity remained. Accordingly, we found many differentially expressed genes located on sex chromosomes. For instance, four chromosome Y genes were found to be expressed almost exclusively in

males, including translation factor *Eif2s3y*, DEAD box polypeptide, tetratricopeptide Uty and demethylase *Kdm5d* (Table 1). Expression of the *Xist* gene, which controls the inactivation of the X chromosome in females, was 11-fold higher in females than in males (Table 1).

The majority of differentially expressed genes were not located on sex chromosomes. This group included well-known biomarkers of inflammation, including IL-1 β , PTX3, chemokine ligand 5 and serum amyloid A3. Expression of many granulocyte-specific genes was also sex-affected, including elastase 2, myeloperoxidase and eosinophil-associated ribonuclease A (Table 1).

The function of several major sex-affected genes was clearly related to androgen and estrogen turnover. For example, estrogen sulfotransferase (*Sult1e1*) and estradiol 17-beta-dehydrogenase (*Hsd17b7*) were ~2.5-fold more abundant in males (Table 1). Both enzymes inactivate steroids by increasing their hydrophilicity (29).

Expression of a dozen of congenic interval-specific signature genes was associated with arthritis and affected by sex

To find causative genes that suppress CAIA in C.D2-*Pgia8* males, we selected genes that meet three criteria: (i) location within the congenic interval; (ii) expression disparity between males and females; and (iii) expression associated with inflammation. Defined by these criteria, the signature set included twelve genes (Table 2). Most of the signature transcripts were downregulated in males and thus linked to attenuated arthritis.

In the set of signature genes, biological functions and tissue localizations were enriched with bone remodeling, cartilage turnover, mesenchymal cell growth, cell adhesion and trafficking. Based on the biological functions, the following genes were considered to be relevant locus-specific gene-candidates: oncostatin M receptor *Osmr*, *C1qtnf3*, *Adamts12*, *Pdzd2*, Syndecan *Sdc2*, *Cthrc1*, *Rspo2*, and exostosin *Ext1* (Table 2).

Cthrc1 was the most significantly downregulated gene in the locus according to oligonucleotide chip gene expression: -2.34-FC lower in congenic males vs. BALB/c males ($p < 0.004$), while most prominently upregulated in congenic females when expression was compared to congenic males: 2.83-folds up, $p < 0.00002$.

RT-PCR confirmed Affymetrix chip differential expression patterns

We were challenged to reproduce the complex pattern of differential gene regulation and its association with arthritis using individual RNA samples and employing another approach such as RT-PCR. We addressed most of the signature genes and those enriched with known RA biomarkers IL-6 and Tnfaip6. RT-PCR was performed for 40 individual RNA samples from arthritic males and females of wild-type and congenic mice.

First, we calculated correlation between CAIA severity and relative *gene/Gapdh* expression. From the set of signature genes, *Cthrc1* demonstrated the best correlation with arthritis severity, with a coefficient of correlation $r = 0.91$ (Fig. 3). This successfully corroborated the results of the Affymetrix gene expression analysis for locus-specific genes, in which *Cthrc1* was on top (Fig. 2A). Two other genes whose expression correlated most strongly with arthritis were *Adamts12* and *C1qtnf3*, $r = 0.87$ – 0.89 .

Secondly, we found a strong correlation between signature gene messages. Particularly, mRNAs for *Cthrc1*, *Adamts12* and *C1qtnf3* were significantly correlated not only with arthritis, but also with each other, $r = 0.87$ – 0.93 (Fig. 3C). Interestingly, the correlation of signature transcripts with CAIA severity was stronger than the correlation with known

arthritis biomarkers *Tnfrsf10b* and IL-6, $r=0.58-0.69$. Concentration of the *Cthrc1* message most significantly correlated with Syndecan 2 transcript ($r=0.86$), which in turn was transcriptionally biased with R-spondyn 2 ($r=0.85$) (Fig. 3C).

Thus, the most relevant transcriptome plot explaining the gender-biased anti-inflammatory effect of the *Pgia8* locus is based on co-regulated expression of *Adamts12*, *C1qtnf3* and *Cthrc1* genes, which is further linked to Syndecan 2 and R-spondyn 2 expression.

Signature genes in naïve mice

Gender-biased disparities in gene expression and hormone levels exist prior to arthritis development and should be considered primary risk factors. Additionally, *Cthrc1* participates in the non-canonical planar cell polarity Wnt signaling pathway (PCP-Wnt) (30), which is implicated in chondrocyte maturation and cartilage formation during ontogenesis (31). Therefore, we examined signature gene expression levels in naïve mice of both sexes with the goal of finding alterations in transcriptome patterns, to see whether any of these genes carry a functional mutation/SNP. Total RNA was isolated from the paws of male and female wild-type BALB/c mice and male and female *Pgia8* congenic mice. Signature genes as well as known markers of inflammation and bone/cartilage remodeling were measured including cytokine messages of IL-1 β and IL-6, which was found earlier as PGIA biomarker for the locus (14).

Expression of most signature genes was much lower in the paws of naïve mice when compared to arthritic paws, implying that most signature genes were highly inducible in the inflammatory state. *Adamts12* was induced by approximately 150 fold, and *Rspo2* message leaped up by 2,000 fold (Fig. 4). However, expression of *C1qtnf6*, *Defb3* and *Sdc2* was comparable to *Gapdh* levels in naïve mice and in mice with CAIA (Figs. 4A).

We identified three patterns of gene expression in naïve mice. Type A showed higher expression in males than in females, but no difference between congenic and wild-type mice (Fig. 4A). Types B and C showed disparity between naïve congenic mice and naïve BALB/c. Expression of *Rspo2* and *Sdc2* was higher in BALB/c females compared to males, but congenic mice showed a flat pattern (Fig. 4B). In type C (*Cthrc1*, IL-6, IL-1 β), the difference between BALB/c males and females was most dramatic, but disappeared in congenic mice (Fig. 4C). Basically, pattern C is a mirror image of pattern B, implying that the *Cthrc1* message concentration was inversely related to R-spondyn 2 and Syndecan 2 mRNAs in naïve mice.

Cthrc1 was induced 20-fold on average, but its expression and inducibility was significantly dependent upon gender and genetic background. In naïve BALB/c mice, *Cthrc1* expression was 3-fold higher in males than in naïve wild-type females. However, in congenic mice, *Cthrc1* expression was indistinguishable between males and females (Fig. 4C). Furthermore, the *Cthrc1* expression pattern in naïve BALB/c mice corresponded exactly to transcriptome images for arthritis-associated cytokines IL-1 β and IL-6. The difference was highly significant: 3.7-fold higher in BALB/c males versus females, $p < 0.001$.

Therefore, links between *Cthrc1*, *Rspo2*, and *Sdc2* messages in inflammation were also found in naïve, non-inflamed tissues.

Discussion

The *Pgia8* locus was initially discovered to be linked to PGIA, but later the genetic association of the locus in *Borrelia burgdorferi*-induced arthritis and CIA was found as well (14–16). A homologous chromosome interval in humans carries genetic risk factors

associated with RA clinical scores, rheumatoid factor, and with anti-TNF- α treatment efficacy (9, 17, 18). Also, an RA-associated SNP was mapped to the middle of the cadherin-containing interval *Cdh12-Cdh10* in humans (9). Another SNP that was associated with reduced response to anti-TNF- α treatment in RA was mapped to the *Pdzd2* gene (19) which interacts with delta-catenin (32). The murine homologues of both of these genes are located within the *Pgia8* locus. In this study, we found that the *Pdzd2* message was linked to CAIA suppression in males (Table 2, signature genes) thus confirming *Pdzd2* involvement in inflammatory arthritis at the level of transcription.

Our results demonstrate that the *Adamts12*, *C1qtnf3* and *Cthrc1* genes were most significantly associated with attenuated arthritis severity in males. *Adamts12* is genetically associated with asthma, Crohn's disease and RA (33, 34). In arthritis, the ADAMTS12 enzyme is responsible for cartilage destruction via degradation of cartilage oligomeric matrix protein (COMP) (35). Data about the role of C1QTNF3 and CTHRC1 proteins in inflammation are scarce. Interestingly that these proteins are similar to each other in several ways. Both 26 kD secreted proteins have collagenous repeat-containing sequences, both are expressed in fibroblasts and in developing bone and cartilage and promote cell motility. They are also implicated in an arterial injury model and in the TGF- β 1 pathway (36–40). Therefore, the similarity between *C1qtnf3* and *Cthrc1* expression patterns that we identified in this CAIA model correlates well with the similarity of both proteins' biological functions.

Cthrc1 is strongly expressed during embryonic development in many organs, particularly in growth plate cartilage and in the bone matrix where it controls collagen deposition through the regulation of *Smad2/3* and TGF- β signaling (38, 39). Expression of *Cthrc1* in normal adult tissues is very low but surges in pathological states such as arterial injury or in skin wounds (38). The CTHRC1 protein positively regulates the PCP-Wnt pathway by promoting formation of the Wnt ligand and Frizzled receptor complex (30). Through the PCP-Wnt pathway, CTHRC1 regulates cell motility in embryogenesis and cancer cell migration and invasiveness in adults (41). In the development of skeletal tissues, the PCP-Wnt pathway regulates chondrocyte maturation and cartilage formation (31). Genetic alterations within the pathway are associated with chondroplasia, dysregulation of collagen deposition and changes in cartilage morphology (31, 42).

In this study we observed for the first time *Cthrc1* expression in inflamed paws of adult mice. Together with R-spondyn 2 and Syndecan 2 co-expression, this strongly suggests the involvement of Wnt pathways in inflammation. Our data corroborated an earlier report showing a blockade of Wnt5A/Frizzled 5 signaling through a non-canonical Wnt pathway inhibits rheumatoid synoviocyte activation via suppression of IL-6, IL-15 and RANKL (43). Together, these data suggest that CTHRC1, Wnt signaling and inflammatory arthritis might be related to inflammatory cell migration, chemotaxis and activation of fibroblast-like synoviocytes.

In inflammatory arthritis, the normal balance between new tissue formation and tissue degradation is shifted to the latter. *Adamts12* overexpression in arthritis apparently signifies cartilage degradation and catabolic activity. However, other proteins may be equally involved in cartilage/bone resorption and in tissue repair. Since *Cthrc1* is expressed in fibroblast-like cells, participates in non-canonical Wnt pathway that controls cell motility and taxis and the level of *Cthrc1* expression correlates directly with *Adamts12* mRNA levels in arthritis, *Cthrc1* is most likely involved in catabolic activity and cartilage degradation.

From this perspective, the function of CTHRC1 in the PCP-Wnt pathway is somewhat similar to the function of Diskopf (DKK1) in the canonical Wnt pathway. DKK1 is a key regulator of new tissue formation via canonical (anabolic) Wnt signaling (44, 45). Recently,

it was shown that DKK1 inhibition reverses the bone-destructive patterns in arthritis to the bone-forming events of osteoarthritis and is involved in bone growth and fusion (45, 46). Similarly, CTHRC1 is a positive regulator of non-canonical (catabolic) Wnt signaling, which simultaneously inhibits the canonical branch (30).

Gender disparities are characteristic of many autoimmune inflammatory diseases, including arthritis (47). Estrogens have been the focus of rationalization of higher female preponderance in RA for decades in part because of the easily observable association between pregnancy and RA remission, while the postpartum stage and breastfeeding are linked to higher disease severity (5, 6, 29, 48). Examples of the effects of estrogen on bone remodeling and on immune system are copious. Also, estrogen production is limited not only to females, but it is also produced by the adrenal cortex, adipose tissue and testicles via aromatization of testosterone (6, 29). For example, in total RNA isolated from inflamed paws of *Pgia8* congenic males, we found 2.5-fold higher expression of estrogen sulfotransferase *Sult1e1* and estradiol 17-beta-dehydrogenase *Hsd17b7*; both enzymes increase the solubility of steroid hormones and thus enhance their metabolic deactivation (29).

Estrogens also affect non-immune tissues, like synovium, suggesting that these tissues might also be affected by inflammation in a gender-biased manner. Recently, it was reported that rodent females and males respond differently to anti-inflammatory treatment with the glucocorticoid dexamethasone (49). Approximately half of differentially expressed genes in liver were gender-specific. The strongest gender bias was observed in signaling pathways connected to phosphatase and tensin homolog PTEN, apoptosis and IL-6 (49).

According to recent GWAS, the RA-specific genetic background seems to be significantly immune-biased, with T, B and antigen-presenting cell engagement (1–3). However, the rationale for gender disparities in arthritis is also connected to synovium pathways with apparent involvement of cartilage and bone remodeling genes in pro- and anti-inflammatory responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CAIA	collagen antibody-induced arthritis
CIA	collagen-induced arthritis
FC	fold changes of differential gene expression
PGIA	proteoglycan-induced arthritis
RA	rheumatoid arthritis

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Figure 1. Collagen antibody-induced arthritis in ch15 congenic strains

Panel A. Time course of CAIA in wild-type BALB/c (black triangles: males; open black circles: females), in *Pgia8* congenic mice (red triangles: males; red open circles: females), and in *Pgia9t* congenic mice (blue triangles: males). Anti-collagen antibodies were injected five days prior (day -5) to LPS injection that was performed at the day 0. *Pgia8* congenic males showed consistently weaker arthritis but 100% incidence in five independent experiments. The strongest statistical difference for arthritis severity was recorded for congenic males versus wild-type males, $p < 0.005$. Arthritis in *Pgia8* congenic females was more prominent than in congenic males, $p < 0.005$. **Panel B.** C.D2-*Pgia9t* congenic strain carried the telomeric part of ch15 of DBA/2 origin (closed boxes); C.D2-*Pgia8* mice represented centromeric part of ch15. Genomic markers that flank congenic intervals are shown. **Panel C.** For differential expression analysis, three experimental conditions were studied: *Pgia8* congenic males and females versus BALB/c males. **Panel D.** Venn diagram. Severity-associated genes were identified by comparing *Pgia8* males and BALB/c males (red circle). Sex-affected transcripts were targeted via comparison of congenic males with congenic females (blue circle). Altogether, 285 genes were localized within the *Pgia8* congenic interval (green circle).

Figure 2. Clustering of arthritis severity genes
Panel A. Hierarchical clustering of major arthritis severity genes (clustering tree is shown on left) with fold change (FC) 2x and greater. Expression profiles were studied in BALB/c males, congenic *Pgia8-m* males and in congenic *Pgia8-f* females. Scale for differential expression fold changes is presented below the image. Main gene clusters (A – G) are shown next to gene names. FC is a ratio of differential expression between *Pgia8-m* and BALB/-m. **Panel B.** Functional clustering of biological functions/diseases that were most significant in the set of major arthritis severity genes. P-value of Fisher’s exact test for clustering is shown along the y-axis (Log(1/P)). Clustering threshold p-value of 0.05 is shown with dashed line. Number of genes in each cluster is presented in parentheses next to cluster names.

Figure 3. Arthritis severity is reliant on gene co-expression network

Relative *Cthrc1* to *Gapdh* expression in individual mouse samples measured using RT-PCR was closely correlated with CAIA severity in wild-type BALB/c mice (A) and in *Pgia8* congenic mice (B) (triangles – males; circles - females). Standard deviations for RT-PCR triplicates are shown. Linear regression approximation is shown with dashed lines for males and with solid line for females. Pearson's coefficients of correlation $r=0.91$ was calculated for wild-type and congenic mice. Gene co-expression network for locus-specific signature genes (C). Connectors show positive correlations, and correlation coefficients are placed nearby. Three genes' expression was most tightly correlated with CAIA severity: *Cthrc1*, *Adams12* and *C1qtnf3*. Expression of these genes was cross-correlated with each other. *Cthrc1* was co-expressed together with Syndecan *Sdc2* and spondyn *Rspo2*.

Figure 4. Signature gene expression patterns in naïve mice

Three patterns of expression were discovered in naïve BALB/c and *Pgia8* congenic males (M) and females (F) (panels A, B and C). **Pattern A.** *C1qtnf6*, *Serpine 1*, *Defb3* messages were significantly more abundant in BALB/c males than in BALB/c females (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Congenic mice showed the expression patterns that were similar to BALB/c mice. **Pattern B.** *Rspo2* and *Sdc2* messages were higher in naïve BALB/c females, but congenic mice did not show difference. **Pattern C.** This pattern is reciprocal to the Pattern B: messages were higher in naïve BALB/c males, but congenic mice did not show significant difference. *Cthrc1* expression pattern was similar to IL-1 β and IL-6.

Table 1

Major gender-affected differentially expressed genes

Gene *	Ch	FC	P-val	Gene Description
<i>Eif2s3y</i>	Y	-66.0	0.003	eukaryotic translation initiation factor 2, subunit 3
<i>Ddx3y</i>	Y	-46.7	0.0007	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3
<i>Uty</i>	Y	-34.8	0.0002	tetratricopeptide repeat gene
<i>C7</i>	15	-26.4	0.001	complement component 7
<i>Kdm5d</i>	Y	-10.2	0.001	Lysine-specific demethylase 5D
<i>Inmt</i>	6	-5.0	0.001	indolethylamine N-methyltransferase
<i>Car1</i>	3	-4.1	0.028	carbonic anhydrase 1
<i>Rhag</i>	17	-3.9	0.006	Rhesus blood group-associated A glycoprotein
<i>Cyp2b23</i>	7	-3.8	0.00002	cytochrome P450, family 2, subfamily b, polypeptide 23
<i>Gypa</i>	8	-3.7	0.031	glycophorin A
<i>Prg2</i>	2	-3.6	0.0004	proteoglycan 2, bone marrow
<i>Slc4a1</i>	11	-3.5	0.019	solute carrier family 4 member 1
<i>Serpinb6c</i>	13	-3.5	0.0001	serine/cysteine peptidase inhibitor, clade B, member 6c
<i>Pbp</i>	5	-3.3	0.001	pro-platelet basic protein
<i>Cyp2e1</i>	7	-3.2	0.0003	cytochrome P450, family 2, subfamily e1
<i>Scd3</i>	19	-3.2	0.0006	stearoyl-coenzyme A desaturase 3
<i>Gpr165</i>	X	-3.1	0.015	G protein-coupled receptor 165
<i>Ly6g</i>	15	-3.0	0.0004	lymphocyte antigen 6 complex, locus G
<i>Dgat2l6</i>	X	-2.9	0.0001	diacylglycerol O-acyltransferase 2-like 6
<i>Cldn13</i>	5	-2.9	0.017	claudin 13
<i>Ear1</i>	14	-2.8	0.002	eosinophil-associated, ribonuclease A family, member 1
<i>Lee3c</i>	3	-2.8	0.009	late cornified envelope 3C
<i>Rptn</i>	3	-2.8	0.007	repetin
<i>Camp</i>	9	-2.8	0.008	cathelicidin antimicrobial peptide
<i>Cxrp3</i>	7	-2.7	0.002	cysteine and glycine-rich protein 3
<i>Ltf</i>	9	-2.7	0.0001	lactotransferrin
<i>Mpo</i>	11	-2.6	0.00004	myeloperoxidase

Gene *	Ch	FC	P-val	Gene Description
<i>Ela2</i>	10	-2.6	0.0007	elastase 2, neutrophil
<i>Sult1e1</i>	5	-2.6	0.0002	sulfotransferase family 1E, member 1
<i>Gsta3</i>	1	-2.6	0.016	glutathione S-transferase, alpha 3
<i>Pla2g2a</i>	4	-2.5	0.0001	phospholipase A2 group IIA, synovial
<i>Hsd17b7</i>	1	-2.5	0.00001	hydroxysteroid (17-beta) dehydrogenase 7
<i>Sbk2</i>	7	-2.5	0.00004	SH3-binding domain kinase family, member 2
<i>Myoz2</i>	3	-2.5	0.001	myozenin 2
<i>Aplnr</i>	2	2.5	0.002	apelin receptor
<i>Atp6v0d2</i>	4	2.5	0.0003	ATPase, H+ transporting, lysosomal V0 subunit D2
<i>Il1b</i>	2	2.5	0.001	interleukin 1 beta
<i>Sfrp2</i>	3	2.5	0.003	secreted frizzled-related protein 2
<i>Smoc1</i>	12	2.6	0.001	SPARC related modular calcium binding 1
<i>Slc16a4</i>	3	2.6	0.0005	solute carrier family 16 member 4
<i>Ptx3</i>	3	2.6	0.014	pentraxin related gene
<i>Tnfrsfip6</i>	2	2.8	0.013	tumor necrosis factor alpha induced protein 6
<i>Cthrc1</i>	15	2.8	0.00002	collagen triple helix repeat containing 1
<i>Cxcl5</i>	5	2.9	0.005	chemokine (C-X-C motif) ligand 5
<i>Cyp2g1</i>	7	3.0	0.007	cytochrome P450, family 2, subfamily g1
<i>Saa3</i>	7	3.2	0.018	serum amyloid A3
<i>Mest</i>	6	3.3	0.009	mesoderm specific transcript
<i>Prss35</i>	9	3.4	0.008	serine protease 35
<i>Xist</i>	X	10.9	0.006	inactive X specific transcripts

* FC, fold change is a ratio of hybridization signal in congenic females to the signal in congenic males; negative FC stands for 1/ratio values. Genes are arranged from the most male-specific (negative FC) to most female-specific (positive FC). Ch, chromosome location of the gene. Complete list of n=1059 gender-affected differentially expressed genes with FC ± 1.5 is shown in supplementary Table S2.

Table 2
Congenic interval-specific signature genes with expression affected by arthritis and gender

Gene ^S	Mb	Pgia8-M	Pgia8-F	BALB-M	Severity FC	Gender FC	Gene Description	Function, Pathway
<i>Osmr</i>	6.7	895.9	1263.3	1292.0	-1.44 **	1.40 ***	oncostatin M receptor	leukocyte trafficking, bone remodeling
<i>Slc1a3</i>	8.5	331.1	507.8	438.5	-1.32 *	1.53 **	glial high affinity glutamate transporter 3	glutamate transport
<i>Clqtnf3</i>	10.8	297.8	587.4	540.2	-1.81 **	1.97 **	Clq and TNF-related protein 3	mesenchymal stem cells growth, bone development
<i>Adams12</i>	10.9	397.6	716.4	714.8	-1.80 **	1.80 **	metallopeptidase with thrombospondin motif 1/12	cell adhesion, articular cartilage degradation
<i>Pdzd2</i>	12.2	756.7	603.5	581.7	1.30 *	-1.26 **	PDZ domain containing 2	initiation of tumorigenesis, cross-talk with TNF-alpha signaling in RA
<i>Baspl</i>	25.2	399.0	554.2	564.8	-1.42 **	1.38 **	brain abundant signal protein 1	neurite outgrowth and plasticity
<i>Cnbl</i>	31.5	421.9	258.4	302.3	1.40 *	-1.64 **	carboxymethylenebutane nolidase-like	cysteine hydrolase, e.g., for angiotensin receptor
<i>Sdc2</i>	32.8	1479.2	1898.4	1918.7	-1.30 **	1.27 **	syndecan 2	Wnt signaling, cell adhesion & migration
<i>Chre1</i>	38.9	843.4	2400.8	1974.5	-2.34 **	2.83 ****	collagen triple helix repeat containing 1	non-canonical Wnt signaling, bone remodeling
<i>Rspo2</i>	42.8	135.5	266.0	188.0	-1.39 ***	1.96 *	R-spondin 2 homolog	Wnt signaling
<i>Plchd11l</i>	44.2	177.5	152.2	125.9	1.41 **	-1.17	polycystic kidney & hepatic disease 1-like1	cellular immunity
<i>Ext1</i>	52.9	967.0	1636.6	1364.2	-1.41 **	1.68 ****	exostosin 1	new bone formation, osteochondroma

^SMb, genomic location of the gene on chr15 in million nucleotide base pairs; **Pgia8-M**, mean expression in congenic males; **Pgia8-F**, mean expression in congenic females; **BALB-M**, mean expression in wild-type BALB/c males; **Severity FC**, ratio of expression signals in congenic males versus BALB males. **Gender FC**, ratio of expression signals in congenic females versus congenic males. Statistical significance is shown with asterisks next to FC:

* , p<0.05,
 ** , p<0.01,
 *** p<0.001,
 **** p<0.0001.