Published in final edited form as:

Mol Immunol. 2013 December 31; 56(4): 531–539. doi:10.1016/j.molimm.2013.05.230.

Comparative antigen-induced gene expression profiles unveil novel aspects of susceptibility/resistance to adjuvant arthritis in rats

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Abstract

Lewis (LEW) and Wistar Kyoto (WKY) rats of the same major histocompatibility complex (MHC) haplotype (RT.1^b) display differential susceptibility to adjuvant-induced arthritis (AIA). LEW are susceptible while WKY are resistant to AIA. To gain insights into the mechanistic basis of these disparate outcomes, we compared the gene expression profiles of the draining lymph node cells (LNC) of these two rat strains early (day 7) following a potentially arthritogenic challenge. LNC were tested both ex vivo and after restimulation with the disease-related antigen, mycobacterial heat-shock protein 65. Biotin-labeled fragment cRNA was generated from RNA of LNC and then hybridized with an oligonucleotide-based DNA microarray chip. The differentially expressed genes (DEG) were compared by limiting the false discovery rate to <5% and fold change ≥ 2.0, and their association with quantitative trait loci (QTL) was analyzed. This analysis revealed a more active immune response overall in WKY than LEW rats. Important differences were observed in the association of DEG with QTL in LEW vs. WKY rats. Both the number of upregulated DEG associated with rat arthritis-QTL and their level of expression were relatively higher in LEW when compared to WKY rat; however, the number of downregulated DEG-associated with rat arthritis-QTL as well as AIA-QTL were found to be higher in WKY than in LEW rats. In conclusion, distinct gene expression profiles define arthritis-susceptible versus resistant phenotype of MHC-compatible inbred rats. These results would advance our understanding of the pathogenesis of autoimmune arthritis and might also offer potential novel targets for therapeutic purposes.

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1. Introduction

Rheumatoid arthritis (RA), one of the most common autoimmune diseases in humans, is associated with inflammation, pain, deformities and reduced quality of living. The precise etiology of RA remains unknown, but it is clear that the disease is multifactorial, resulting from complex interactions between genetic and environmental factors (Lipsky, 2008; Tobon et al., 2010). Furthermore, both the major histocompatibility complex (MHC) and non-MHC genes contribute to disease susceptibility (Brenner et al., 2005; MacGregor et al., 2000; Wandstrat and Wakeland, 2001). The non-MHC genes can mediate their influence by affecting a variety of cellular and molecular events including inflammation, immune responses, metabolic pathways, etc. (Babior, 1999; Brenner et al., 2005; Olofsson et al., 2003). Furthermore, several quantitative trait loci (QTLs) have been identified for experimentally-induced arthritis by conducting genetic linkage analyses of the crosses between arthritis-susceptible and arthritis-resistant rat strains (Griffiths et al., 2000; Kawahito et al., 1998; Rioja et al., 2005; Wester et al., 2003). About 107 QTLs associated with arthritis severity in the rat have been reported.

The LEW and WKY rat strains have the same MHC haplotype (RT-1¹) but differ in their susceptibility to adjuvant-induced arthritis (AIA) (Moudgil et al., 1997). These rat strains provide a powerful tool to study the role of immunobiochemical events controlled by the MHC and non-MHC genes in influencing disease severity. Several studies have revealed that RA patients as well as rats with AIA develop T cell and antibody responses to heat-shock protein 65 (Hsp65) (Huang et al., 2010; van Eden et al., 1988; Yu et al., 2011b). Furthermore, preventive or therapeutic interventions that suppress AIA also alter immune responses to mycobacterial hsp65 (Bhsp65) (Venkatesha et al., 2011; Yang et al., 2011). Thus, Bhsp65 represents one of the important disease-related antigens in AIA. The results of our previous study based on microarray analysis of the draining lymph node cells (LNC) of arthritic LEW rats showed that the incubation/preclinical phase (Inc) of AIA is a critical period characterized by most marked changes in gene expression *ex vivo* compared to the later phases of the disease (Yu et al., 2011a). Some of the AIA-susceptibility genes might influence and control very early processes in disease pathogenesis and not necessarily the late processes during established disease. We reasoned that the Inc phase of AIA might be an important period not only for intervention aimed at downregulation of arthritis in LEW rat but also for investigating the genes associated with arthritis resistance in WKY rats. Furthermore, the differences in gene expression influenced by Bhsp65 in LEW versus WKY rats might provide important insights into the nature of disease susceptibility/resistance (Wester et al., 2003).

In this study, we performed microarray analysis to examine the gene expression profiles of the draining LNC from arthritis-susceptible Lewis (LEW) rats and arthritis-resistant Wistar Kyoto (WKY) rats immunized with heat-killed *M. tuberculosis* H3Ra (Mtb), and compared their expression profiles. Furthermore, the genomic locations of specific differentially expressed genes (DEG) were mapped and compared with those of rat QTLs that have previously been reported by others to be associated with AIA and other types of experimental arthritis (e.g., Pristane-induced arthritis, PIA; collagen-induced arthritis, CIA; oil-induced arthritis, OIA; and streptococcal cell wall-induced arthritis, SCWIA). We believe that the results of our study would provide useful insights into both the pathogenic processes in autoimmune arthritis and the identification of potential new targets for therapeutic intervention for this disease.

2. Materials and Methods

2.1 Induction and evaluation of AIA

Male Lewis (LEW/SsNHsd) (RT-1¹) and Wistar Kyoto (WKY/NHsd) (RT-1¹) rats, 5 to 6-wk old, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed in an accredited animal facility at UMB. All animal handling and experimental work were carried out in accordance with the National Institutes of Health (NIH) guidelines for animal welfare, and the study was approved by the Institutional Animal Care and Use Committee (IACUC). Animals were acclimated to the holding room for at least 3 d before initiation of experimental work. AIA was induced in the LEW rats on d 0 by immunizing them subcutaneously (s.c.) at the base of the tail with 2 mg/rat of heat-killed *M. tuberculosis* H37Ra (Mtb) (Difco, Detroit, Michigan) emulsified in 200 μ l mineral oil (Sigma-Aldrich, St. Louis, MO). These animals were sacrificed on d 7 (Inc (Incubation) phase, before appearance of clinical arthritis) and their draining lymph nodes (LN) (superficial inguinal, para-aortic, and popliteal) were harvested for testing. WKY rats were subjected to the same schedule of Mtb injection and LN testing as LEW rats.

2.2 Lymph node cell (LNC) culture

A single suspension cells of LNC of LEW rats or WKY rats was cultured at 37°C for 24 h in a six-well plate (5×10^6 cells/well) in serum-free HL-1 medium (Lonza, Walkersville, MD) with or without Bhsp65 (5 μ g/ml) as described elsewhere (Yu et al., 2011a). Thereafter, the cells were processed for RNA isolation.

2.3 Total RNA extraction and Gene Chip hybridization

Total RNA was extracted from LNC using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA was purified with RNeasy Mini Kit (Qiagen Ltd, Crawley, UK). RNA concentration was determined spectrophotometrically (260/280 nm, 260/230 nm) using the NanoDrop ND-1000 (NanoDrop Technologies/Thermo Scientific, Wilmington, DE). The quality of RNA was further assessed on a RNA 6000 Nano LabChip kit (Agilent Technologies Inc., Palo Alto, CA) using Agilent 2100 Bioanalyzer. The RNA integrity number (RIN) (mean $\pm$ SD) of the RNA isolated from LNC cultured in vitro with or without Bhsp65 was 8.1 ± 0.36 with coefficient of variation (CV) of 4.5%.

Total RNA (100 ng) was used as the input for the amplification and generation of biotin-labeled fragment cRNA for expression analysis using the Affymetrix kit and the protocol supplied by the vendor (Affymetrix, Santa Clara, CA). Labeled cRNA was hybridized with an oligonucleotide-based DNA microarray, Rat GeneChip® Gene 1.0 ST Array System, for whole transcript coverage analysis. This microarray platform contains 700,000 unique 25-mer oligonucleotide features (spots) representing 27,342 Entrez Gene IDs. Hybridization on GeneChip® Fluidics Station 450, scanning and image processing on GeneChip® Scanner 3000 7G, and preliminary data management with Affymetrix MicroArraySuite software (MAS 5.0) were performed at the Genomics Core Facility at UMB in accordance with the manufacture's guidelines.

2.4 Microarray data analysis

The details are described in our previous paper (Yu et al., 2011a). Briefly, Affymetrix.cel files were uploaded to Affymetrix Expression Console™ 1.1, and normalized by the robust multichip average (RMA) method. SAM (Significance Analysis of Microarrays) was utilized to compare gene expression levels between two different rat strains (three independent experiments, i.e., 3 chips/group, biological replicates) by limiting the false discovery rate (FDR) to below 5%, which then was used as a cut-off to assess statistical significance and to identify differentially expressed genes (DEG) (Tusher et al., 2001).

Bhsp65-induced DEG were identified by comparison of the gene expression of LNC stimulated with Bhsp65 versus that of LNC cultured in medium alone. A heatmap showing changes in the expression level (fold change) of representative genes was generated in 'R' with the package 'gplots'. Further analysis was carried out to identify the biological processes of the DEG using Uniprot KB databases. Enrichment analysis was performed on different features using the Gene Ontology (GO) and KEGG databases (Ashburner et al., 2000; Falcon and Gentleman, 2007; Kanehisa et al., 2008), which revealed themes indicative of inflammation, immune response, antigen processing and presentation, etc. The experimental plan and data analysis of microarray in this study are in accordance with MIAME guidelines (Brazma et al., 2001).

2.5 Quantitative real-time PCR (qPCR)

The same RNA samples that were used for microarray analysis were tested in qPCR to validate the microarray data. Column-purified total RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad) with oligo (dT) primers as directed by the manufacturer. The cDNA templates for qPCR were prepared and then amplified using specific primers (Sigma) in SYBR Green PCR Master Mix (AB Applied Biosystems, Warrington UK) on a LightCycler Instrument (Roche Applied Science, Indianapolis, IN). The specific primers were designed to amplify a set of selected genes including Ifng, Il1, Il10, Il17, Il33, Irf, Irf1, Ccr5, Cxcl10, Nos2, Socs1 and Socs3. The levels of mRNA were normalized to HPRT. Then, the relative mRNA expression was calculated using the 2^{-Ct} method (Yu et al., 2011b).

2.6 Quantitative trait locus (QTL)-associated DEG

The QTLs for arthritis in the rat were investigated using QTLs-RGD-Rat Genome Database. The genomic location of the DEG was determined using gene search in the same database. The genomic positions of QTLs for arthritis were compared with their reported positions using "Rat-QTL".

3. RESULTS

An outline of various test and control groups is given in Table 1.

3.1 Bhsp65-induced gene expression profile of LEW rats in the preclinical phase of AIA

In our previously reported study (Yu et al., 2011a), we examined the expression profile of Bhsp65-induced genes in LEW rats at the preclinical phase of AIA, (i.e., on d 7 d after Mtb immunization before the appearance of clinical AIA). The results are given in Figure 1A and 1C. A total of 61 DEG were identified, of which 41 were upregulated and 20 were downregulated. Of 61, 20 DEG were shared with WKY rats, 14 upregulated and 6 downregulated.

3.2 Bhsp65-induced gene expression profile of WKY rats after Mtb injection

58 DEG (26 upregulated and 32 downregulated) were identified in Bhsp65-restimulated LNC of WKY rats compared to baseline (LNC cultured in medium only) (Figure 1B and 1C). As mentioned above, 20 of 58 DEG were shared between WKY and LEW rats.

3.3 Comparison of the gene expression profiles of AIA-susceptible LEW and AIA-resistant WKY rats

3.3.1 Comparison of Bhsp65-induced gene expression profile of LEW vs. WKY rats—Significant differences were observed in the gene expression profiles of Bhsp65-restimulated LNC of LEW and WKY rats (Figures 1C, 2, 3A(a), Table 2). For WKY rats, 26

of 58 DEG showed increased expression, whereas the remaining 32 revealed decreased expression level. Comparatively, in LEW rats, 41 of 61 DEG were upregulated compared to 20 genes that were downregulated. Among these 61 DEG, 20 genes were shared by both rat strains. All of the 20 genes (33%, 20 in 61, LEW rat; 35%, 20 in 58, WKY rat) had a similar trend (upregulation or downregulation) of expression, but different level of expression (fold change) (Figure 2). In WKY rats, the relative expression level of genes related to innate immunity (Il1b, Socs1, Ifi47, Ifih1, Ifit2, Ifit3, Irgm), Th1 response (Irf1, Il12rb2, Tbx21, Stat1; except Ifng which is higher), humoral immunity (Bst2, Nampt), chemokine/receptor (Ccl6, Ccl17, Cxcl10, Ccr5; except Cxcl2 and Cxcr7 which are higher), cell proliferation (Gpmb), and angiogenesis were lower, but that of Th2 (IL-33) and Th17 (LOC301289 (Interleukin-17 precursor), IL-17f, RGD1561292 (Interleukin-22 precursor)) response were higher than that in the LEW rats.

3.3.2 Comparison of ex vivo gene expression profile of LEW vs. WKY rats—

Marked differences were observed in ex vivo (LNC cultured in medium alone) gene expression profiles of LNC from the two rat strains. We identified 161 upregulated and 108 downregulated DEG (a total of 279 DEG) when comparing WKY to LEW rats (Figure 3A(b)). Among the upregulated DEG, the major group (33%, 52 of 161 genes) was represented by the genes which were associated with immune activity, such as Cd24, Il18, Tlrs, Cxcl10, Cxcl17, Mhc, Tap1, etc. Another major group (33%, 53 of 161 genes) included the genes without a defined function. In regard to the downregulated DEG, 26% (28 in 108 genes) each was associated with cell proliferation and “function-undefined”, respectively. Although the downregulated genes such as Klre1, Il7r, Ccr4, and Anxa1 that are related to immune activation, inflammation, and cell migration represented a relatively small proportion (13.8%, 15 of 108 genes) of the total, they may have an impact on the pathogenesis of arthritis. For example, Ccr4 is an important mediator of lymphocyte migration into and aggregation within the joint (Flytlie et al., 2010; Norii et al., 2006). The marked difference in profile of gene expression between the two rat strains was resulted of different response to Mtb stimulation by LEW and WKY rat.

3.4 Correlation analysis

To validate our microarray findings, q-PCR was performed on the selected 12 genes with the same RNA samples used in microarray analysis. A correlation analysis (Figure 3B) showed that the gene expression levels of the genes tested by microarray and qPCR analyses correlated quite well.

3.5 DEG located within the QTL linked with rat experimental arthritis

To further investigate the possibility that the DEG induced by Bhs65 play a role in arthritis pathogenesis, we identified the rat chromosomal locations for these DEG and compared their location to that of the rat arthritis-related QTLs (Figure 4). Interestingly, in LEW rats, 50.8% (31 of 61 genes) DEG were situated within rat arthritis-related QTL regions, whereas 16.4% (10 of 61 genes) were within QTL associated with AIA severity (rat AIA-QTL) (Table 3). In comparison, in WKY rats, 55% (32 out of 58 genes) of DEG were linked to rat arthritis-QTL, whereas 22.5% (13 of 58 genes) were linked with rat AIA-QTL (Table 3). However, analysis of the relationship of individual sub-group (upregulated DEG/downregulated DEG) of DEG with QTL showed important differences between LEW and WKY rats. Among the upregulated DEG associated with arthritis-QTL, the percentage was higher for LEW rats (37.7%) than that for WKY rats (27.6%) (Table 3). Most interestingly, the expression level (fold change) of most of the QTL-related genes (overlapping genes) was lower in WKY than that in LEW rats (Table 4). In regard to the downregulated DEG linked to arthritis-QTL, it was 13.1 % for LEW compared to 27.6% for WKY rats. However, for downregulated DEG linked to AIA-QTL, 7% were found in WKY but none in LEW rats.

To further analyze the likely contribution of these DEG to the pathogenesis of arthritis susceptibility/resistance, we categorized the DEG into 2 groups: those associated with QTLs (Group A) and others that are not associated with QTLs (Group B) (Table 4). Each group was further subgrouped into 3 categories according to the expression level. Group A1 is comprised of the DEG that co-localized with arthritis-QTLs and showed relatively higher expression in LEW than in WKY rats. This group contains some novel genes that have previously not been associated with arthritis activity such as *Slc7a2*, *Acat2*, *Ubd*, etc. Group A2 contains the DEG that also map to arthritis-QTL, but their expression level is reverse of DEG in Group 1. The mRNA expression level is higher in WKY rats compared to LEW rats. Apparently, some of these genes are related to arthritis severity but not to arthritis-resistance in WKY rats. Group A3 included the DEG that co-localized with arthritis-QTLs, but showed equivalent mRNA expression in LEW and WKY rats (Table 4). The DEG in Group B do not map to the known arthritis-QTLs.

4. Discussion

Studies in various experimental models of human autoimmune diseases have revealed that the MHC alone is not sufficient to determine susceptibility to autoimmunity, and that the non-MHC genes also influence disease susceptibility. Therefore, we took advantage of the two rat strains (LEW and WKY) that have the same MHC haplotype (RT-1¹), but display differential susceptibility to AIA. We examined the gene expression profiles of LNC harvested from LEW rats during the preclinical phase (d 7) of AIA and to compare them with that of WKY rats.

LNC harvested from LEW and WKY rats after 7 days of *Mtb* immunization and then cultured in medium (*ex vivo*) showed very different gene expression profiles. These results suggest that the two rat strains responded to *Mtb* immunization differently, resulting in arthritis development in the LEW rat but not in the WKY rats. Compared to LEW rats, the largest group of DEG in WKY rats was related to immune response, with a relatively higher expression level in WKY rats compared to LEW rats. These genes associated with innate immune response, Th1 response, B cell-related molecules, and humoral immunity are of special interest in regard to inflammatory arthritis. Similarly, the antigen processing and presentation-associated genes (*Psmb9*, *Tap1*, *H2-T24*, *RT1-N1/N2*) showed significant upregulated expression in response to *Mtb* stimulation in WKY compared to LEW rats. Interestingly, however the gene for *Ccr4* showed lower expression in the WKY rat. Perhaps, this might contribute to the reduced likelihood of developing arthritis in these rats, which otherwise have the ability to raise a strong immune response after *Mtb* immunization. This suggestion is based on the finding that recently, MDC/CCL22, the ligand for chemokine receptor CCR4, was shown to be present within the synovial membrane and synovial fluid of patients with RA, and that it could help recruit CCR4-expressing memory T cells into the joints (Flytlie et al., 2010). We also observed differential regulation of genes associated with cell proliferation and apoptosis in LEW vs. WKY rats. Most genes (80%) related to cell proliferation were downregulated, but those related to apoptosis (75%) were upregulated in WKY rats compared to LEW rats. We suggest that these genes might favor LNC apoptosis over proliferation during the incubation period of AIA, and thereby contribute to the disease-resistance of WKY rats.

Similar to the gene expression profile of *Mtb*-treated rats (*ex vivo*), the largest group of DEG in LNC restimulated *in vitro* with *Bhsp65* are related to immune response. With restimulation, *Bhsp65* differentially induced some important immune activation molecules, especially the cytokines. However, the expression level of most of these genes (fold change compared to baseline) was higher in LEW rats compared to WKY rats, except the genes encoding for IFN- and Th17 cytokines (Figure 2 and Table 4). Other immunity-associated

genes involved in the pathogenesis of arthritis such as Nampt (which promotes B cell maturation) (Evans et al., 2011) and Bst2 (involved in pre-B-cell growth) (Ishikawa et al., 1995), as well as Cbf (involved in complement pathway) were expressed at a relatively higher level in LEW compared to WKY rats. Taken together, the genes related to humoral and cellular immunity as well as innate immunity might be among the major contributors to arthritis severity. An imbalance between pathogenic and protective cytokines is believed to influence the severity of arthritis in different animal models. The ratio of fold change (of DEG in LEW vs. WKY rats) of Ifng, Il17f, and Il22 precursor varied from 0.8 to 0.824 (0.8, 0.824, 0.823), and that of Il17 precursor was 0.7. Therefore, no major shift from Th1 to Th17 was found in LEW rats compared to WKY rats. Also, there was no significant IL-4 or IL-10 response in either strain. Overall, the comparative gene expression profile of *in vitro* Bhsp65-restimulated LNC between WKY and LEW rat was markedly different from that of *ex vivo*. One likely reason for this difference in the two strains might be the relatively higher baseline level of gene expression in WKY rats, which apparently was not altered much after Bhsp65 restimulation. The high baseline level (*ex vivo*) in turn could be because of other components of Mtb besides Bhsp65 that might contribute to the induction of immune response during arthritis. The resolution of this issue would require further investigations.

Finally, we analyzed the association between the identified candidate genes (DEG) and QTLs for rat arthritis to investigate the differential arthritis susceptibility of LEW and WKY rats. Linkage analysis based on five different rat arthritis models (CIA, OIA, PIA, AIA, and SCWIA) has revealed 107 QTLs that might regulate experimental arthritis. Among them, 27 QTLs that are implicated in regulating arthritis severity in rats with AIA are spread over 9 chromosomes, especially chromosomes 10, 13 and 17. We observed that the distribution of genes within and outside rat arthritis-QTLs was almost identical in LEW and WKY rats. Our findings of the linkage of approximate 53% (50.8% in LEW, 55% in WKY) DEG linked with rat arthritis-QTL support the conclusion drawn by other investigators (Andersson and Stahl, 2010) that the DEG responsible for rat CIA susceptibility are evenly distributed between QTL and non-QTL regions. Although we do not have additional information to suggest that these genes directly contribute to arthritis induction, or the lack of it, we suggest that the difference in the expression of those genes might explain differential susceptibility to AIA of LEW and WKY rats. The above-mentioned DEG were found to be located within 60 of the known (which total is 107) rat-arthritis QTL (Figure 4).

Most of the genes that mapped to genomic regions previously reported to contain rat arthritis-QTLs showed higher fold change (upregulated expression) in LEW rats, but comparatively lower fold change (downregulated expression) in WKY rats. Thus, combining the gene expression level and information about QTLs gave additional insight into AIA susceptibility. We categorized the DEG based on QTL association and gene expression level. Genes under Group A1 and A2 might be considered to be the candidates responsible for AIA-susceptibility in LEW rats and AIA-resistance in WKY rats. However, this proposition would need additional experimental support. These results suggest, albeit indirectly, that Bhsp65 is an important disease-related component of Mtb. Though the genes in Group A3 are associated with arthritis-QTL, the expression level in LEW and WKY rats are comparable. In regard to the genes under Group B1 and B2 (differentially expressed between LEW and WKY rats, but not associated with arthritis QTL), we suggest that this group of DEG should also be considered for further evaluation for its likely contribution to disease susceptibility in AIA (Brenner et al., 2006; Remmers et al., 1996; Xiong et al., 2008).

Our previous results show that Mtb-immunized WKY rats are as capable of developing a proliferative T cell response to Bhsp65 as LEW rats (Mia et al., 2008). In fact, many examples of a rodent strain raising a strong systemic immune response to the immunizing

antigen but failing to develop an active disease have been documented by others. For example, the Fas mutant mice (B6-lpr/lpr) mount a strong immune response to the immunizing antigen (MOG peptide, myelin oligodendrocyte glycoprotein) as reflected by the production of autoantibodies and Th1 cytokines, however, they are highly resistant to EAE (Waldner et al., 1997). Similarly, EAE-resistant mice (C57B1/10.S, B10) developed a systemic immune response as did the EAE-susceptible mice (C57B1/6, B6) (Mix et al., 2004). However, the mechanisms that prevent the disease induction in resistant mice remain to be fully defined.

In conclusion, the present study described the comparative gene expression profiles (ex vivo and in vitro after Bhsp65 restimulation) of LEW and WKY rats, and the possibility of these DEG to contribute to disease susceptibility or resistance. The following genes were considered as promising candidate genes contributing to AIA-susceptibility/resistance on the basis of their level of gene expression, gene function and arthritis-QTL association. These candidates are Ifi47, Irgm, Cfb, Tap1, Il12rb2, CXCL10, Nos2, RGD1305184, Fam46a, Cyp11b2, Nqo1, Gzmb, Socs1, Bst2, Slc7a3, Il33, and Lpl. We believe that this study has unraveled novel candidate genes and pathways involved in susceptibility or resistance of AIA. We propose that these genes be examined further for exploring new therapeutic targets for experimental arthritis as well as human RA.

Acknowledgments

This work was supported by grants R01AT004321 and R03AI076942 from the National Institutes of Health (Bethesda, MD, USA). We thank Biomed Central for permission to use Figure 3A data and its derivatives from our previous publication in Arthritis Research Therapy (Yu et al, 2011a). We thank Shivaprasad H. Venkatesha and Siddaraju M. Nanjundaiah for help with qPCR assay.

Abbreviations

AIA	adjuvant-induced arthritis
Bhsp65	mycobacterial heat-shock protein 65
DEG	differentially expressed gene
QTL	Quantitative trait locus
IFN	interferon
Inc	incubation
IL	interleukin
LEW	Lewis
WKY	Wistar Kyoto
LNC	lymph node cells
Med	medium
Mtb	<i>Mycobacterium tuberculosis</i> H37Ra
qPCR	quantitative real-time PCR
RA	rheumatoid arthritis
CIA	collagen-induced arthritis
OIA	oil-induced arthritis
PIA	Pristane-induced arthritis

SCWIA streptococcal cell wall-induced arthritis

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Highlights

- Lewis and WKY rats display differential susceptibility to adjuvant arthritis.
- RNA from lymph node cells of rats in preclinical phase was tested in microarrays.
- Draining lymph node cells were tested ex vivo and after restimulation with Hsp65.
- Specific gene sets were differentially associated with rat arthritis-QTL in LEW/WKY.
- Distinct gene expression profiles define arthritis susceptible/resistant phenotype.

Figure 1. Gene expression profiles of Bhsp65-induced DEG of AIA-susceptible LEW and AIA-resistant WKY rats

Heat maps showing the expression profiles of Bhsp65-induced DEG of LEW (A) and WKY rats (B). Venn diagram showing the numbers of genes that are up/down-regulated (FDR<0.05), including shared DEG as well as individual DEG of LEW or WKY rats (C).

Figure 2. Comparison of the Bhsp65-induced DEG of AIA-susceptible LEW and AIA-resistant WKY rats at d 7 post-Mtb immunization

DEG were categorized into subgroups according to their biological function as follows: (A) general immune activity; (B) cytokines and receptors (a), and chemokines and receptors (b); (C) cell proliferation (a), adhesion molecules (b), angiogenesis (c), and bone damage (d); (D) cellular response to oxygen and oxidation-reduction (a), signal transduction and pathways (b); and (E) Metabolism, protein metabolic process-related (a) and lipid and cholesterol metabolic process-related (b). (*, significant DEG of LEW rats; #, significant DEG of WKY rats)

Figure 3. Comparison of the gene expression patterns of AIA-susceptible LEW and AIA-resistant WKY rats during the incubation (pre-clinical) phase of AIA

(A) Gene expression by microarray. (a) Numbers of Bhs65-induced DEG in LNC of LEW (L) and WKY (W) rats are shown. Also shown are DEGs that are shared (S) between the two groups. (b) DEG number of *ex vivo* (LNC cultured in med alone) gene expression of WKY versus LEW rats. **(B)** Correlation of the levels of gene expression obtained by microarray and qPCR. X axis, gene expression determined by Rat GeneChip®Gene 1.0 ST Array (Affymetrix) hybridization; Y axis, gene expression determined by qPCR. Data is represented as “Fold over medium” after normalization. ($r^2=0.8068$, $p<0.0001$) (DEG: differentially-expressed gene)

Figure 4. Locations of rat experimental arthritis QTLs and identified DEG

The figure shows QTLs on the same chromosome as the DEG (rat arthritis-QTL associated DEG). The information of the position of the flanks of the QTLs and the genomic location of these DEG were searched using QTLs-RGD-Rat Genome Database (<http://rgd.mcw.edu/>). Pia, pristane-induced arthritis; Cia, collagen-induced arthritis; Oia, oil-induced arthritis; and Scwia streptococcal cell wall-induced arthritis.

Table 1

Outline of various test and control groups examined in this study

Bhsp65-induced gene expression profile of LEW rats (using ex vivo gene expression as the baseline)	Figure 1A, 1C
Bhsp65-induced gene expression profile of WKY rats (using ex vivo gene expression as the baseline)	Figure 1B, 1C
Comparison of Bhsp65-induced gene expression profile of LEW vs. WKY rats (using the respective ex vivo gene expression as the baseline)	Figure 1C, 2, 3A(a) and Table 2
Comparison of ex vivo gene expression profile of LEW vs. WKY rats.	Figure 3A(b)
Correlation analysis of microarray and qPCR	Figure 3B
DEG located within the QTL linked with rat experimental arthritis.	Figure 4, Table 3 and 4

Table 2

Expression of Bhsp65-induced differentially-expressed genes in LEW and WKY rats.

Gene symbol	Gene name	Fold change (compared to baseline)	
		LEW rat	WKY rat
A ¹ Lif	Leukemia inhibitory factor	+2.73 *	+1.92 *
A ¹ Ifi47	Interferon gamma-inducible protein 47	+2.45 *	+1.21
A ¹ Ifih1	Interferon induced with helicase C domain 1	-1.14	-2.18 *
A ¹ Irgm	Immunity-related GTPase family, M	+2.82 *	+1.07
A ¹ Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58	+1.33	-1.96 *
A ¹ Cfb	Complement factor B	+2.79 *	+1.66
A ¹ Tap1	Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	+2.11 *	+1.18
A ¹ Dpp4	Dipeptidylpeptidase 4	-1.58	-1.83 *
A ¹ Irf1	Interferon regulator factor 1	+2.32 *	+1.28
A ¹ Il12rb2	Interleukin 12 receptor, beta 2	+4.08 *	+2.51 *
A ¹ Tbx21	T-box 21	+2.61 *	+1.92
A ¹ Ccl6	Chemokine (C-C motif) ligand 6	-1.51	-2.51 *
A ¹ Cxcl10	Chemokine (C-X-C motif) ligand 10	+8.02 *	+2.83
A ¹ Nampt	Nicotinamide phosphoribosyltransferase	+2.06 *	+1.04
A ¹ Wars	Tryptophanyl-RNA synthetase	+2.29 *	+1.04
A ¹ Nos2	Nitric oxide synthase 2, inducible	+7.83 *	+4.52 *
A ¹ Slc7a2	Solute carrier family 7 (cationic amino acid transport)	+4.98 *	+3.14 *
A ¹ Slc1a4	Solute carrier family 1 (cationic amino acid transport)	+2.52 *	+1.46
A ¹ Acat2	Acetyl-Coenzyme A acetyltransferase 2	+2.00 *	+1.54
A ¹ Ubd	Ubiquitin D	+4.11 *	+2.24 *
A ¹ Asns	Asparagine synthetase	+2.16 *	+1.73
A ¹ Psat1	Phosphoserine aminotransferase 1	+2.00 *	+1.70
A ¹ RGD1305184	Similar to Interferon inducible GTPase	+15.30 *	+3.18
A ¹ Fam46a	Family with sequence similarity 46, member A	-1.11	-2.70 *
A ¹ Kcnj5	Potassium inwardly-rectifying channel, subfamily	-1.86	-2.12 *
A ¹ Gpmb	Glycoprotein (transmembrane) nmb	-2.63	-2.79 *
A ² Gzma	Granzyme A	+2.85 *	+5.40 *
A ² Lyz2	Lysozyme 2	-3.16 *	-1.90 *
A ² Ifng	Interferon gamma	+10.00 *	+12.49 *
A ² Il17f	Interleukin-17F	+7.86 *	+9.72 *
A ² LOC301289	Similar to Interleukin-17 precursor	+16.74 *	+24.40 *
A ² Ezh2	Enhancer of zeste homolog 2 (Drosophila)	+1.64	+1.85 *

Gene symbol	Gene name	Fold change (compared to baseline)	
		LEW rat	WKY rat
A ² Cxcl2	Chemokine (C-X-C motif) ligand 2	+1.79	+2.78 *
A ² Cyp11b2	Cytochrome P450, subfamily 11B, polypeptide 2	-3.45 *	-1.94
A ² Nqo1	NAD(P)H dehydrogenase, quinone 1	+1.57	+2.29 *
A ² Socs3	Suppressor of cytokine signaling 3	+1.19	+2.11 *
A ² Axl	Axl receptor tyrosine kinase	-2.33 *	-1.28
A ² Txnrd1	Thioredoxin reductase 1	+1.44	+1.61 *
A ² Apol11a	Apolipoprotein L 11a	+2.34	+2.70 *
A ² Klre1	Killer cell lectin-like receptor, family E, member 1	-4.60 *	-3.19 *
A ² Clec4a3	C-type lectin domain family 4, member a3	-3.88 *	-2.94 *
A ³ Pla2g7	Phospholipase A2, group VII (platelet-activating	-3.04 *	-2.63 *
A ³ Inhba	Inhibin beta-A	-2.61	-2.63 *
A ³ Txnip	Thioredoxin interacting protein	-1.82	-1.87 *
A ³ Pls1	Plastin 1 (I isoform)	-2.22	-2.34 *
A ³ Ly49si1	Immunoreceptor Ly49si1	-2.29	-2.21 *
A ³ Csf1r	Colony stimulating factor 1 receptor	-1.88	-1.88 *
A ³ Plscr2	Phospholipid scramblase 2	-2.38 *	-2.76 *
A ² RGD1563110	similar to immunoreceptor Ly49si3	-2.54 *	-2.06
B ¹ Gzmb	Granzyme B	+4.48 *	+3.61 *
B ¹ Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	-1.11	-2.90 *
B ¹ Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	-1.01	-3.12 *
B ¹ Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-1.07	-2.47 *
B ¹ Il1b	Interleukin 1 beta	+2.96 *	+2.09
B ¹ Socs1	Suppressor of cytokine signaling 1	+3.66 *	+1.84
B ¹ Stat1	Signal transducer and activator of transcription 1	+1.79 *	+1.05
B ¹ LOC684383	Similar to C28H8.3	-1.01	-2.52 *
B ¹ Ccr5	Chemokine (C-C motif) receptor 5	+2.22 *	+1.33
B ¹ Bst2	Bone marrow stromal cell antigen 2	+2.90 *	+1.08
B ¹ Slc7a3	Solute carrier family 7 (cationic amino acid transp	+4.58 *	+1.69
B ² Il33	Interleukin 33	-2.50 *	-1.28
B ² Lpl	Lipoprotein lipase	-6.37 *	-3.61 *

Expression level of DEG associated with rat arthritis-QTL and other important DEG are shown. "+", upregulation; "-", downregulation;

* significant when compared to the corresponding baseline expression (FDR <0.05); "A1", "A2", "A3", "B1", "B1" before the "Gene Symbol" are similar to that in Table 2 in the main paper.

Table 3

The percentage of DEG associated with arthritis-related QLT in the rat.

	DEG number	DEG unrelated to rat arthritis-QTL	DEG within rat arthritis-QTL [#]	DEG within AIA-QTLs
LEW	Upregulated	41	18 (29.5%)	23 (37.7%)
	Downregulated	20	12 (19.7%)	8 (13.1%)
	Total	61	30 (49.2%)	31 (50.8%)
WKY	Upregulated	26	10 (17.2%)	16 (27.6%)
	Downregulated	32	16 (27.6%)	16 (27.6%)
	Total	58	26 (44.8%)	32 (55.2%)

Rat arthritis-QTL include QTL of rat CIA, OIA, PIA, AIA, and SCWIA.

DEG, differentially-expressed gene; CIA, collagen-induced arthritis; OIA, oil-induced arthritis; PIA, Pristane-induced arthritis; AIA, adjuvant-induced arthritis; SCWIA, streptococcal cell wall-induced arthritis

DEG marked with “[#]” and “*” are further discussed in Table 4

Table 4

Category of DEG based on their expression level and association with QTL.

Groups	QTL association	Expression level (fold change)		Gene Symbol	
		LEW	WKY		
A	1	+	H	L	Lif, Ifi47, Ifih1, Irgm, Dhx58, Cfb, Tap1, Dpp4, Irf1, Il12rb2, Tbx21, Ccl6, Cxcl10, Nampt, Wars, Nos2, Slc7a2, Slc1a4, Acat2, Ubd, Asns, Psat1, RGD1305184, Fam46a, Kenj5, Gpnmb
	2	+	L	H	Gzma, Lyz2, Ifng, Il-17f, LOC301289, Ezh2, Cxcl2, Cyp11b2, Nqo1, Soes3, Axl, Txnrd1, Apol11a, Klre1, Clec4a3, RGD1563110
	3	+	E	E	Csflr, Inhba, Txnip, Plscr2, Pla2g7, Pls1, Ly49sil
B	1	-	H	L	Gzmb, Ifit2, Ifit3, Ddx58, Il1b, Soes1, Stat1, LOC684383, Cer5, Bst2, Ass1, Slc7a3, Slc7a5, Cmpk2, Gbp5
	2	-	L	H	Gzmf, Il33, Cxcr7, Plau, RGD1561292, Lpl
	3	-	E	E	RGD1561819, Il1a, Ccl17, LOC686661, Tm7sf4, Xkrx, Tm4sf19, RGD1565355

Group A and B are detailed analysis of DEG shown in Table 3. Gene in bold face shows association with AIA-QTL. H=High; L=Low; E=Equal