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

Hua Yu[a], Changwan Lu[b,c], Ming T. Tan[c], Kamal D. Moudgil[a,d,*]

[a] Department of Microbiology and Immunology, University of Maryland School of Medicine, 685 West Baltimore Street, HSF-1, Suite 380, Baltimore, MD 21201, USA
[b] Department of Medicine, University of Maryland School of Medicine, 685 West Baltimore Street, MSTF-314, Baltimore, MD 21201, USA
[c] Division of Biostatistics and Bioinformatics, Department of Epidemiology and Public Health, University of Maryland School of Medicine, 685 West Baltimore Street, MSTF-261, Baltimore, MD 21201, USA
[d] Division of Rheumatology, Department of Medicine, University of Maryland School of Medicine, 685 West Baltimore Street, Baltimore, MD 21201, USA

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ABSTRACT

Lewis (LEW) and Wistar Kyoto (WKY) rats of the same major histocompatibility complex (MHC) haplotype (RT.1[l]) 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 overall a more active immune response 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.

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; Mattapallil et al., 2008; 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. (Brenner et al., 2005; Mattapallil et al., 2008; 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 Lewis (LEW) and Wistar Kyoto (WKY) rat strains have the same MHC haplotype (RT-1[l]) 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

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, *Mycobacterium tuberculosis* 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.

* Corresponding author at: Department of Microbiology and Immunology, University of Maryland School of Medicine, 685 West Baltimore Street, HSF-1, Suite-380, Baltimore, MD 21201, USA. Tel.: +1 410 706 7804; fax: +1 410 706 2129.
E-mail address: kmoud001@umaryland.edu (K.D. Moudgil).

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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 rather than 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 LEW rats and arthritis-resistant WKY rats immunized with heat-killed *Mycobacterium tuberculosis* $H_{37}R_a$ (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-1^1) and Wistar Kyoto (WKY/NHsd) (RT-1^1) 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 μl 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-cell suspension of LNC of LEW/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 μg/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 ± 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 Biopolymer-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