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Differentially Expressed Genes in MHC-Compatible Rat Strains That Are Susceptible or Resistant to Experimental Autoimmune Uveitis

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Abstract

Purpose—Experimental autoimmune uveitis (EAU) is an established model for immune-mediated human uveitis. Although several genes from major histocompatibility complex (MHC) loci have been shown to play a role in uveitis, little is known about the role of non-MHC genes in the pathogenesis of EAU. Several non-MHC genes have been implicated in the pathogenesis of various autoimmune diseases. The primary objective of this study was to identify the non-MHC genes involved in the pathogenesis of EAU, to identify potential drug targets and possibly to target their protein products for immunotherapy.

Methods—EAU was induced in the susceptible (Lewis; LEW) or resistant (Fischer 344; F344) rats that have identical MHC class II haplotype. Draining lymph node cells were obtained during the innate and adaptive phase of the immune response, and the pattern of gene expression was evaluated using microarray technology. Differentially expressed genes were validated at mRNA and protein levels using various methods.

Results—Susceptibility to EAU was associated with an increased expression of numerous non-MHC genes such as Th1-type cytokines and chemokines, antiapoptotic factors, hormones, and neurotransmitters and a downregulation of selected adhesion molecules. In this study a combined genetic–genomic approach was used to identify different patterns of gene expression associated with the sensitization and effector phase of EAU pathogenesis.

Conclusions—The data demonstrate that the differential expression of several non-MHC genes is associated with the mechanism of uveitis.

Experimental autoimmune uveitis (EAU) presents a histopathologic picture strikingly similar to that of human uveitis and serves as a model of autoimmune inflammatory disease of the neural retina.¹ EAU can be induced in susceptible animals by immunization with retinal

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autoantigens such as interphotoreceptor retinol binding protein (IRBP) or soluble antigen (S-Ag) and by adoptive transfer of antigen-specific CD4⁺ T cells.

The susceptibility and severity of uveitis is determined by the interplay between multiple genetic and environmental factors that can trigger the onset of an inflammatory process. The major genetic determinants of clinical and experimental uveitis as in any other autoimmune disease are the major histocompatibility complex (MHC) genes. However numerous non-MHC genes, such as those that determine the expression of several cytokines and their receptors, hormones of the hypothalamic–pituitary–adrenal (HPA) axis, regulatory factors for vascular effects and cell motility, and factors affecting the T cell repertoire and T-cell receptor (TCR) complex, have also been shown to play a role in determining the EAU disease phenotype.^{2,3} We have demonstrated that three major quantitative trait loci (QTL) on rat chromosomes 4 (*Eau1*), 12 (*Eau2*), and 10 (*Eau3*) are associated with susceptibility to EAU.² These QTL regions harbor numerous non-MHC genes, including genes encoding for the TCR, lymphokines, hormones, and neuro-transmitters. Since then, we have identified several additional rat chromosomal regions that modulate EAU susceptibility and colocalize with known QTLs of other autoimmune and inflammatory diseases in animal models and in humans.^{4,5} However little is known about the exact role these factors play in the onset of EAU.

We used a combination of QTL and microarray analyses to identify and correlate the expression pattern of non-MHC genes with the disease phenotype of EAU. This combination approach has been successfully used to understand the genetic basis of other autoimmune diseases.⁶ We integrated the QTL mapping data from an F2 generation of EAU-susceptible Lewis (LEW) and -resistant Fischer (F344) rats with the microarray gene expression data obtained from their parental inbred strains. Both these strains share a common MHC-class II haplotype and a compatible MHC-class I haplotype, (RT1-*I^v*), yet differ in their susceptibility to disease,³ thereby providing us with a valuable model to investigate the role of non-MHC genes that are associated with either susceptibility or resistance to disease phenotype.

We used a cDNA array to evaluate the expression of various immunologically relevant non-MHC genes in explanted lymph node (LN) cells collected from LEW and F344 rats at days 0, 3, 10, 14, and 28 postimmunization (pi) with R16 peptide. R16 peptide is an immunodominant and uveitogenic epitope of IRBP in LEW rats and also induces EAU in several strains of other haplotypes.⁷ Since the cells isolated from these LNs were evaluated ex vivo without any in vitro manipulation, they largely mirror responses in vivo. As all the animals were naïve to R16 peptide, the early time point (day 3) represents the innate immune response phase of the disease. Further, no R16 peptide-specific responses were detected at day 3 (data not shown), suggesting that responses observed during this early phase represent innate immune responses. On the other hand, R16 peptide-specific responses were easily detected after day 10, suggesting that, during days 10 to 28, the disease was mediated by adaptive immune responses. In addition to the ex vivo analysis of LN cells, we also evaluated the changes in gene expression profiles of LN cells (day 14 pi) after in vitro stimulation with the R16 peptide.

Our results demonstrate major differences in gene expression profiles between resistant (F344) and susceptible (LEW) strains of rats during the two phases of EAU induction. These data, in combination with the results from QTL analysis, allowed us to identify and validate several immunologically relevant non-MHC genes that are associated with the pathogenesis of EAU.

Materials and Methods

Experimental Animals

Female rats (6–8 weeks old) belonging to the Fischer 344/NHsd (F344) and Lewis/SsNHsd (LEW) inbred strains (MHC haplotype RT1-*I^v*) were purchased from Harlan Sprague-Dawley

(Indianapolis, IN). The animals were maintained in a specific pathogen-free environment and were fed standard laboratory chow ad libitum. All procedures conformed to institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of EAU

The rats were immunized with 30 μ g/rat R16 peptide of bovine interphotoreceptor retinoid-binding protein (IRBP; sequence ADGSSWEGVGVPDV, residues 1177-1191), which constitutes a major pathogenic epitope for the MHC haplotype RT1-*l^v*.⁸ R16 peptide was synthesized by conventional solid-phase chemistry on a peptide synthesizer (Applied Biosystems, Inc. [ABI], Foster City, CA), as described earlier.⁹ The antigen was emulsified 1:1 (vol/vol) with CFA (Sigma-Aldrich, St. Louis, MO), containing 2.5 mg/mL of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI), and injected subcutaneously at three sites, at the base of the tail (100 μ L) and in the hind limbs (each, 50 μ L).

The rats were observed daily for uveitis manifestations and killed at days 3, 10, 14, and 28 pi. The eyes of each animal were enucleated, fixed for 1 hour in 4% phosphate-buffered glutaraldehyde, and transferred into 10% phosphate-buffered formaldehyde. Fixed and dehydrated tissue was embedded in methacrylate. Sections of 4 to 6 μ m were cut through the pupillary–optic nerve plane and stained with standard hematoxylin and eosin. Quantitation of disease was performed in a masked fashion according to criteria described previously.¹⁰ Briefly, the eyes were assigned a score ranging from 0 to 4, depending on the extent of inflammation and tissue damage. The minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the anterior chamber, iris, ciliary body, choroid, vitreous, or retina (EAU grade 0.5). Progressively higher grades were assigned for the presence of discrete lesions in the tissue, such as vasculitis, granuloma formation, retinal folding and/or detachment, hemorrhages, and loss of photoreceptors. The maximum grade of 4.0 reflects extensive retinal damage, with complete destruction of the photoreceptor cell layer and gliosis. Statistical significance of differences in disease scores was calculated using Snedecor and Cochran's test for linear trend in proportions.

Draining LNs, inguinal and iliac, were collected for RNA isolation, in vitro proliferation assay, and cytokine analysis.

Antigen-Specific Lymphocyte Proliferation

Antigen-primed LN cells were collected from draining LNs 14 days pi. The cells were isolated, washed in RPMI 1640 medium containing 1.5% syngeneic rat serum and 0.5×10^6 cells/mL, and cultured with or without antigen (2 μ g/mL R16 peptide) for 48 hours at 37°C and 5% CO₂. The cells from individual animals were used for RNA isolation or flow cytometry, and the culture supernatants were pooled within the strains and used for cytokine and chemokine protein assays.

Isolation of RNA and cDNA Probe Preparation

Total RNA from LN cells of individual rats, naïve or killed at days 3, 10, 14, or 28 pi was extracted by phenol/chloroform (Clontech Laboratories, Palo Alto, CA) followed by DNase treatment according to the manufacturer's instructions. Total RNA was isolated from in vitro cultured LN cells of individual rats, according to the protocol for RNA isolation from cytoplasm of cells using the RNeasy kit (Qiagen Inc., Valencia, CA). The concentration and quality of the RNA were assessed by spectrophotometry and by agarose gel electrophoreses. Equal amounts of RNA were pooled from individual rats within each strain.

Radiolabeling of the total RNA with ^{33}P -dCTP (GE Healthcare, Piscataway, NJ) is described at <http://www.grc.nia.nih.gov/branches/rrb/dna.htm> (provided in the public domain by the Intramural Research Program, National Institute on Aging). Briefly, 5 μg total RNA was radiolabeled in a reverse-transcription reaction. The labeled cDNAs were incubated at 65°C for 30 minutes after adding 10 μL of 0.1 M NaOH to hydrolyze and remove RNA. The samples were pH neutralized by the addition of 45 μL of 0.5 M Tris (pH 8.0) and purified (Micro Bio-Spin P-30 Tris chromatography columns; Bio-Rad Laboratories). Each RNA sample was hybridized in triplicate, and each half of the membrane was a replicate of the other.

cDNA Microarray Hybridization and Imaging

cDNA microarray on nylon membranes containing a focused collection of 1152 immune-response-related genes produced by the DNA array unit at the National Institute on Aging (NIA) were used for the experiment. An entire list of the genes printed on the immunomicroarray can be found at <http://www.grc.nia.nih.gov/branches/rrb/dna/dna.htm>. The hybridization and wash conditions, imaging technique, and data acquisition were as described in detail elsewhere without any modification.^{11,12}

Microarray Data Analysis

Raw signal intensity data for each experiment were normalized by z transformation.¹³ Briefly, intensity data are first $\log_{10}$ transformed, followed by the calculation of z -scores. Raw gene expression data, $\log$ values, and z -scores were averaged using the mean $\pm$ SD. Comparisons between experiments were conducted by regression analysis. Note that z -score comparisons and z -ratios generally represent greater differences than traditional differences expressed as multiples of change (x -fold), as the underlying data have been compressed by $\log_{10}$ transformation. Tables of expression profiles were compiled by sorting z -score intensity values. Positive z -ratios indicate an increase in expression and negative z -ratios a decrease in expression. Clustering of changes in gene expression was determined by using public domain hierarchical cluster analysis (http://rana.lbl.gov/eisen/?page_id=42).¹⁴

Validation of Genes by RT-PCR Analysis

The expression rate of selected genes analyzed by microarray was validated by real-time PCR (*TaqMan*; ABI) and by quantitative RT-PCR analysis using a genetic analyzer (model 3100; ABI).

Gene-specific primers and probes for the real-time PCR were designed on computer (Primer Express 1.5a; ABI) and are listed in Table 1. Semiquantitative RT-PCR analysis was performed in a sequence detector (Prism 7700; ABI), using 15 μM each of forward and reverse primer, 5 μM of 6Fam-labeled probe, and template cDNA diluted 1:2 to 1:10 in 12.5 μL of a final volume of reaction. Run conditions consisted of 2 minutes at 50°C, followed by 10 minutes at 95°C, then 40 to 50 cycles of 15 seconds at 95°C, and 1 minute at 60°C. The concentration of cDNA template and the number of cycles were determined empirically for each gene.

The expression rate of each gene in triplicate wells was calculated according to the manufacturer's instructions. Briefly, threshold cycles (C_t), representing the PCR cycle at which the fluorescent signal can be detected above a baseline signal, were normalized after construction of a standard curve for each gene examined. For the endogenous reference control rodent *Gapdh* (*TaqMan* VIC Probe; ABI) was used. The ratio of the single gene expression value divided by the expression value of the internal *Gapdh* control gives the normalized expression level of the target gene. The statistical significance of observed differences between the expression rates of genes in different groups was verified by two-tailed t -test.

The mRNA levels of selected genes were also validated by relative quantitative RT-PCR (QuantumRNA 18S internal standards kit; Ambion, Austin, TX). Briefly, gene-specific primers were designed based on the curated Refseq mRNA sequence for each gene (designed with Primer Express software; ABI). Multiplex PCR was performed by using fluorescently labeled, gene-specific primer pairs (Table 2) and universal primer (QuantumRNA 18S; Ambion) as internal standards. Appropriate conditions to determine the annealing temperature, linear range of amplification and concentration of the 18S primer for multiplexing the reaction were determined for each gene separately. The fragment separation of the PCR products was performed on a genetic analyzer (model 3100; ABI) and analyzed (Genotyper 3.7 software; ABI) for the quantities of amplified products in terms of peak heights and peak areas normalizing the peak heights using scale factors. Gene-specific amplification was normalized to the amplification of 18S rRNA gene, and the ratio of treated sample over control sample was calculated.

Flow Cytometry Analysis for Cell Surface Marker Expression

Monoclonal antibodies to rat cell surface markers such as anti-CD3 (FITC), anti-CD4 (R-PE), anti-CD8 α (PerCP), anti-CD11b/c (FITC), and anti-CD53 (FITC) were purchased from BD-PharMingen (San Diego, CA) and anti-OX-62 (R-PE) antibody was purchased from Serotec Inc. (Raleigh, NC). Naïve rat serum 1% was used in the blocking of Fc receptors. Statistical significance of differences between observed values were determined with the two-tailed *t*-test.

Cytokine and Chemokine Assays

Cytokines and chemokines levels in the lymphocyte cell culture supernatants were determined using multiplex array technology (Search-Light Arrays; Endogen Chemical Co., Minneapolis, MN). Statistical significance of differences between observed values was analyzed with the two-tailed *t*-test.

Results

EAU Phenotype

To determine the phenotype of EAU in resistant (F344) versus susceptible (LEW) strains of rats, we immunized R16 peptide naïve rats from both of these strains with IRBP-derived uveitogenic R16 peptide. Our results demonstrate that a susceptible LEW strain developed typical EAU lesions in the retina as early as day 10 pi (Fig. 1A) and exhibited disease progression. In contrast, the resistant F344 strain of rats remained relatively free of disease. No R16 peptide-specific immune responses were detected at day 3, whereas R16-specific immune responses were detected at day 10 and later (data not shown). Eyes from individual rats were evaluated for histopathological changes at day 14 pi. The disease score in susceptible strains of rats ranged from 0.5 to 3.25, with an incidence rate of 100% (Fig. 1A). By day 28 pi, the disease scores decreased and ranged from 0.75 to 2.0, indicating a resolution of the active inflammatory process in eyes (Fig. 1A).

Microarray Analysis of Gene Expression Profiles in Susceptible versus Resistant Strains of Rats during the Course of EAU

Changes in gene expression profiles were evaluated after the induction EAU, using a focused human cDNA array. Previous studies have shown that gene sequences are highly conserved between species, thereby allowing the generation of reliable data using cross-species microarray analysis.¹⁵ The gene-expression profiles were evaluated in freshly isolated cells (days 3, 10, 14, and 28 pi) obtained from draining LNs of susceptible and resistant strains of

rats that were immunized with R16 peptide and compared with preimmunization samples. The cells were evaluated ex vivo, without any in vitro manipulation.

Hierarchical clustering of the gene expression profiles showed differential expression of several genes between the susceptible and resistant strains during the course of disease (Fig. 2). These differences were apparent, even in preimmunization samples. However, R16 peptide immunization was associated with differential expression of numerous genes as shown in Figure 2. Major changes were observed in the signature patterns on days 3 and 14 pi in resistant F344 strain of rats (Fig. 2) corresponding to the activation of innate immunity, peak of inflammation, and tissue damage in the eye, respectively. On day 3 pi, a total of 52 genes were found to be upregulated (z -ratio ≥ 1.5), whereas 82 genes were downregulated (z -ratio ≤ -1.5) in the susceptible strain of rats compared with the resistant strain. And on day 14, the expression levels of 61 genes were found to be higher (z -ratio ≥ 1.5), whereas 83 genes were lower (z -ratio ≤ -1.5) in the susceptible strain of rats compared with the resistant strain. The differentially expressed genes belonged to several broad categories including genes that are involved in apoptosis; cell cycle, oncogenes, and nuclear factors; intracellular signaling; lymphokines, chemokines, and their receptors; adhesion molecules and coreceptors; growth factors, hormones, and their receptors; and genes involved in the organ metabolism (Table 3).

Validation of Microarray Data by Other Methods

Genes were selected for validation based on one or more of the following criteria: presence in the EAU QTL regions,² rank in the z -score analysis, major fluctuation in expression during the course of disease as observed from the hierarchical clustering, known functional roles in the susceptibility to EAU or other T-cell-mediated autoimmune diseases. The genes selected based on these criteria and showing changes in expression at days 3 and 14 pi were validated at the transcription and/or translation level (Table 3). A result was considered validated when the expression profile of a single gene followed a similar pattern or trend when tested by both microarray and at least one of the other methods used for validation. We used a stringent approach to validate the selected genes. However, given the highly complex nature of microarray analysis and subsequent validation occasionally discrepancies may arise between data generated by microarrays and the methods used for validation, as has been demonstrated in several studies.¹⁶

Among the 42 genes listed in Table 3 that were subjected to validation either by cDNA quantitation or flow cytometry analysis, 28 (65.1%) were found to agree with the same trend in the differential expression (Figs. 3, 4) as that observed by microarray analysis (Table 3). The *Stat5b* and *Npy* genes, previously identified as candidate genes by QTL analysis,² were also included in our selected gene list for validation, even though they were absent from the microarray.

Several genes encoding for proteins involved in chemotaxis and migration of cells demonstrated differential expression during the innate phase of disease. A statistically significant increase in the levels of expression of the chemokine receptor *Ccr2* (1.00 ± 0.13 vs. 0.36 ± 0.14 , $P = 0.04$) and its ligand *Scya2* (monocyte chemotactic protein 1; MCP-1/CCL2; 16.07 ± 6.31 vs. 2.58 ± 0.98 , $P = 0.01$) was observed in the susceptible LEW rats compared with the expression levels in resistant F344 rats on day 3 pi (Fig. 3). Adhesion molecules appeared to be downregulated during the innate phase of disease induction (Figs. 3, 4). In particular, the expression of adhesion molecules mediating the interactions between leukocytes and endothelial cells, such as selectin E (*Sele*), selectin L (*Sell*; Fig. 3), and integrin $\beta 1$ (*Itgb1*; Fig. 4), decreased on day 3 pi, in the susceptible LEW strain compared with the resistant F344 strain. Other molecules involved in cell trafficking such as *Cd9* (Table 3, Fig. 4), *Cd53* (Fig. 3) and *Alcam* (Table 3, Fig. 4) were also downregulated at day 3 pi in the susceptible LEW rats compared with their expression in the F344 rats.

At the cellular level, susceptible rats appeared to have a higher frequency of CD3⁺CD8 α ⁺CD53⁺ T cells at baseline (day 0; $P = 0.04$) than resistant rats (Fig. 5). Although both strains showed an expansion of CD3⁺CD8 α ⁺CD53⁺ T cells at day 14 pi, the susceptible LEW strain had a higher frequency of CD53 expressing CD3⁺CD8 α ⁺ T cells than the resistant F344 strain. In contrast to CD3⁺CD8 α ⁺ T cells, the frequency of CD53-expressing CD3⁻CD8 α ⁺ cells was higher in the resistant F344 strain both at day 0 ($P = 0.04$) and day 14 ($P = 0.0001$) pi, than in the susceptible strain (Fig. 5).

Hormones and neurotransmitters play a role in susceptibility to autoimmune diseases.¹⁷ Variations in expression levels of genes encoding these molecules or their receptors were observed in the microarray analysis. In particular, neuropeptide Y (*Npy*), one of the candidate QTL genes, was expressed at significantly ($P = 0.05$) lower levels in LEW rats on day 3 pi (2.47 ± 0.99), compared with its expression in F344 rats (6.27 ± 3.37 ; Fig. 3). The rate of gene expression increased proportionately in both the strains from their basal level (Fig. 3), giving an equal day 3:day 0 ratio (Fig. 4). In parallel, the expression of *Npy* receptor 1 (*NpyR1*) was found to be lower in susceptible LEW rats than in resistant F344 rats (Fig. 4), in line with the microarray data (Table 3). Although the differential expression of leptin (*LEP*) did not attain statistical significance in the microarray data (Table 3) and was detected in lower amounts by real-time PCR (Fig. 3), by functional analysis (EAU scores), *Lep* KO mice were completely protected from disease (data not shown), suggesting a role for Leptin in EAU susceptibility, as reported in other autoimmune diseases.¹⁸

Signal transduction and transcription factors appeared to be generally upregulated during cell activation after immunization (Fig. 4), especially the FYN-related kinase (*Frk*) in the resistant F344 strain. On the other hand, RUNT-related transcription factor 1 (*Runx1*) was upregulated in susceptible LEW rats at day 3 pi, whereas it was downregulated in resistant F344 rats at the same time point (Fig. 4).

Genes involved in apoptosis, such as Bcl-2 associated X protein (*Bax*), baculoviral IAP repeat-containing protein 5 (*Birc5*), also called survivin and caspase 3 (*Casp3*), did not show any major differences in their expression patterns between the two strains either at day 3 or day 14 pi (Figs. 3, 4).

Microarray Gene Expression Profiles of In Vitro Antigen–Stimulated LN Cells

To evaluate the antigen-specific patterns of gene expression profiles induced in susceptible versus resistant strains of rats, freshly isolated LN cells (day 14 pi) from both the strains were stimulated in vitro with R16 peptide and used for microarray analysis (Fig. 6). The magnitude of differentially expressed genes between the susceptible LEW and resistant F344 strains was found to be amplified in response to in vitro antigenic restimulation (Fig. 7). After the statistical standards for screening, 61 genes had significantly higher expression levels (z -ratio ≥ 1.5), and 83 genes showed significantly lower gene expression levels (z -ratio ≤ -1.5) in susceptible LEW rats compared with LN cells from resistant F344 rats.

Genes Validated for Differential Expression during the Adaptive Immune Response after In Vitro Antigen-Specific Activation of Lymphocytes

Based on the position in EAU QTLs and functional evidence for involvement in the pathogenesis of uveitis, we identified 26 genes for validation from the list of differentially expressed genes of microarray data (Table 4). Gene expressions were validated at a transcriptional level by real-time RT-PCR and quantitative RT-PCR relative to 18s ribosomal RNA expression. Protein expression of the secreted cytokines and chemokines were quantitated using rat protein array. As previously specified, data were considered validated when the expression profile of a single gene followed a similar pattern of the microarray data,

at least by one of the validation methods (Table 4). We were able to confirm the data for 19 (67.8%) of the 28 genes, as well as for *Npy* and *Stat5b*, which were not present in the array but are included in the previously identified EAU QTL regions (Table 4).

Signature genes of the antigen-specific adaptive response (Fig. 6) differed from the signature genes characteristic of the innate phase of the response (Fig. 3). This general observation was reinforced at the single gene analysis level. Compared with the differential expression of various cell adhesion molecules during the innate immune response of the disease process, only *Sell* showed a significant difference ($P = 0.0435$) in the expression level between susceptible LEW rats (1.11 ± 0.49) and resistant F344 rats (1.81 ± 0.07), in response to the in vitro antigenic stimulation (Fig. 7).

Expression of different chemokines was proportionately induced in lymphocytes of both strains on antigen-specific stimulation (Fig. 8). Of interest, significantly higher levels of RANTES (CCL5) was secreted from unstimulated ($P = 0.0171$) and activated ($P = 0.0071$) lymphocytes of the resistant F344 strain (unstimulated: 70.9 ± 14.9 pg/mL; activated: 140 ± 44.6 pg/mL, respectively) than the susceptible LEW strain (unstimulated: 43.5 ± 7.7 pg/mL; activated: 77.1 ± 25.6 pg/mL, respectively) suggesting a potential protective role for RANTES in EAU (Fig. 8). Increased secretion of MCP-1 (also named CCL2, encoded by the *Scya2* gene) from its basal level was observed in antigen stimulated cells from both strains (Fig. 8), but was significant only in susceptible LEW strain (240.8 ± 44.9 pg/mL vs. 588.3 ± 143.7 pg/mL, $P = 0.0013$). This increase was not manifested by an increased level of cDNA expression specific for its receptor CCR2 in either strain (Fig. 7). Activated lymphocytes from the susceptible LEW rats showed significantly high expression levels of lymphokines such as interferon (IFN)- γ ($P = 0.0045$ comparing IFN γ produced by unstimulated: 36.1 ± 10.8 pg/mL and antigen-stimulated cells: 1189 ± 599 pg/mL from LEW rats, and $P = 0.0014$ comparing IFN γ produced by antigen-stimulated cells between both strains: 1189.3 ± 599 pg/mL and 214.2 ± 184.4 pg/mL, respectively), a cytokine associated with susceptibility to EAU,³ tumor necrosis factor α (TNF α ; $P = 0.0118$ comparing TNF α produced by unstimulated: 27 ± 10.4 pg/mL and antigen-stimulated cells: 71.9 ± 26.9 pg/mL, from LEW rats), and interleukin (IL)-10 ($P = 0.0067$, comparing unstimulated, 13.7 ± 6.4 pg/mL, and antigen-stimulated, 70 pg/mL ± 31.1 , cells from LEW rats; Fig. 8).

Signal transduction and transcription factors were proportionally expressed by antigen-activated lymphocytes of both strains (Fig. 7), but proteins involved in apoptosis showed a difference in the pattern of expression. In particular, although the expression of the proapoptotic factor Bax remained at the same levels before and after antigenic stimulation in the susceptible LEW rats (Fig. 7), the expression of the antiapoptotic protein Birc5 seemed to be upregulated (both by microarray and *TaqMan* analysis; ABI). Although the expression levels of these proteins were comparatively higher in the unstimulated cells of resistant F344 rats compared with the susceptible LEW rats, their levels tended to decrease on antigenic stimulation (Fig. 7).

Among the genes involved in metabolism and endocrine functions, *LEP* and *SOD1* expression levels were found to be upregulated after the antigenic stimulation in susceptible LEW rats compared with the resistant F344 strain of rats, as revealed by microarray analysis. However, we did not find major differences in these genes when we used the RT-PCR technique (Table 4, Fig. 7). Expression of *Npy* was increased after antigen stimulation in lymphocytes irrespective of their strain differences (Fig. 7).

Discussion

Functional genomics has been applied to understand the genetic basis of autoimmunity. More specifically to detect QTLs that harbor susceptibility or resistance genes in disparate strains of animals or to measure the levels of expression of thousands of genes using microarrays or oligonucleotide chips.⁶ We used this approach to depict a possible scenario in which a complex network of genes differentially expressed specifically contribute either to the mechanisms involved in the early phases of EAU induction or to the mechanisms of the antigen-specific cellular responses leading to the tissue damage in the eyes.

The time course analysis showed that the major changes in gene expression levels occurred in vivo in LN cells during the interval from days 0 to 3 pi, a period coinciding with activation of the innate immune response in reaction to the mycobacteria in complete Freund's adjuvant, required for EAU induction. It should be taken into account that the two phases may overlap, even as the adaptive response develops, due to the persisting antigen–adjuvant depot. In the present system under study, the key features observed in the LN cells of the susceptible LEW strain during this early part of the disease-induction process was the significantly high expression of the chemokine CCL2/MCP-1 (encoded by the gene *Scya2*) and adhesion molecule *Sell*, potentially increase the mobility and accumulation of mononuclear cells and lymphocytes at the sites of priming and activation suggesting an increased trafficking of leukocytes in the susceptible LEW strain during the early activation phase. Compatible with this scenario is the observation of a significantly reduced frequency of CD3⁺CD8 α ⁺CD53⁺ leukocytes in the LNs of susceptible LEW rats. This CD8⁺ non-T-cell population could be monocytes, dendritic cells, or NK cells.¹⁹ Attenuation of CD53⁺ monocytes has been described during infectious diseases in humans,²⁰ and its deficiency has a clinical phenotype similar to those of inherited defects of adhesion molecule that causes a syndrome of recurrent heterogeneous infectious diseases.²¹ NK cells have been shown to play both a protective and disease promoting activity in autoimmune disorders depending on the type of cytokine production.^{22,23} Increased expression of CD53 (OX-44) on CD8⁺ T cells from susceptible LEW rats is suggestive of prolonged survival of these cells, as CD53 had been shown to trigger survival response and protection of T cells from apoptosis.^{24,25} Also, attenuated expression of CD9, another adhesion molecules belonging to the tetraspanin family,²⁶ has been described during human infections²⁰ and was observed by us during the early phases of EAU induction (Fig. 4).

Cell trafficking through tissues depends on cell–cell and cell–matrix contacts mediated by integrins and other specialized adhesion molecules. The observed downregulation of molecules mediating the interactions between leukocytes and endothelial cells in the LN tissues of susceptible LEW rats, specifically *Sele* and *Sell*, could facilitate recruitment of effector cells into the retina. Cell recruitment is reported to be guided by chemotactic signals through the CCL2/CCR2 axis.²⁷ In our study, important variations of gene expression rates between strains and during the course of time was observed for the *Scya2* gene coding for CCL2 (MCP-1), its ligand CCR2, and VTN. It is noteworthy that the *Scya2* and *Vtn* genes are located within the same QTL region (*Eau3*) on rat chromosome 10.² The role of the CCL2/CCR2 axis in regulating the development of a Th1 response has been suggested.²⁸ In a pulmonary *Cryptococcus neoformans* infection model, neutralization of CCL2 or deletion of CCR2 resulted in comparable macrophage and T-cell recruitment deficits, as well as a switch from Th1- to Th2-type cytokine production within the infected lung. However, *Scya2* neutralization does not result in pulmonary eosinophilia, nor does it produce an IFN γ defect within the draining LNs, as does CCR2 deletion, suggesting that the adaptive phase of cell-mediated immunity (mononuclear cell recruitment) uses the CCL2/CCR2 signaling axis, whereas the innate phase (Th1 polarization) involves a *Scya2* (CCL2)-independent, CCR2 signaling pathway.²⁸ It has been proposed that, if antigen-presenting cells (APCs) promoting Th1

differentiation express CCR2, the loss of CCR2 may prevent Th1 polarization because Th1-APCs do not appear in the draining LNs. A similar scenario may apply in our case, in which the absence of CCR2⁺ APCs in the draining LNs, suggested by the significant downregulation of CCR2 gene expression at day 3 pi in the resistant F344 strain might fail the activation of Th1-polarized T cells to be able to trigger the adaptive response to the retinal antigen(s) responsible for the disease.²⁹ Furthermore, upregulation of *SCYA2* was shown in patients with rheumatoid arthritis,³⁰ and in a relapsing autoimmune encephalomyelitis model³¹ and its receptor gene *Ccr2* is located on rat chromosome 8q32, which has been found to regulate susceptibility to EAU.⁵

It is known that the major driving force in generation of autoimmunity is interactions among various genes involved in innate immune response. However, based on the differences in the expression profile and the QTL analysis in this uveitogenic model of LEW and F344 rats, major changes were noticed in the levels of MCP-1, the selectins, and CD53.

A completely different scenario is envisioned in the expression profiles of genes involved in the antigen-specific response of activated lymphocytes. The LN cells downregulated the expression of adhesion molecules (Sele and Sell), produced high levels of Th-1 type cytokines, and increased the expression of antiapoptotic factor Birc5. These events occurred in an orderly fashion in susceptible LEW rats. The most noticeable observation was that antigen-stimulated lymphocytes from the resistant F344 rats produced significantly higher levels of RANTES, a chemokine known to contribute in the recruitment of lymphocytes of Th1 phenotype into eyes in the mouse model of EAU.²⁷ However, our data suggest a role for RANTES in protection from EAU. In the previous studies using LEW rats as models for EAE³² and EAU,²⁷ RANTES was not associated with either of these Th1-mediated autoimmune diseases. It has already been described that RANTES neutralization may exacerbate EAU in mice by modulating the type of T cell subsets recruited to the eye,³³ and that RANTES produced by NK T cells, stimulated by either CD1d-transfected fibroblasts in vitro or CD1d⁺ tolerogenic APCs, contributed to induction of tolerance in immune-privileged sites, such as the anterior chamber of the eye.³⁴ This finding together with the fact that the RANTES gene (*Scya5*) is located in the *Eau3* QTL region on rat chromosome 10, strongly support the evidence that RANTES may play an important role during the adaptive immune response to the retinal antigen.

Evidence of interactions between the hormones, neuro-transmitters, and autoimmunity are observed in several models of autoimmune diseases and in the clinical practice. The overall expression of *Npy*, one of the candidate genes in the chromosome 4 *Eau1* QTL region, was found to increase from the basal level in both the strains during both innate and adaptive phase of EAU induction. The significant upregulation of the expression of *Npy* in the resistant F344 rats suggests a protective role for this neurotransmitter in the induction of EAU. It has been shown that *Npy* expression is induced in the brain after intraperitoneal injection of LPS together with proinflammatory cytokines,³⁵ and that NPY affects the natural killer activity and lymphocyte proliferation in mice,^{36,37} probably modulating the cytokine production.^{38,39} A direct involvement of *Npy* in the pathogenesis of a mouse model of systemic lupus erythematosus has been suggested,⁴⁰ but no mechanisms of action have been proposed. From our earlier experiments using knockout mice (data not shown), we found that an *Npy*-associated hormone Lep (leptin, also present in *Eau1* QTL) could be associated with the pathogenesis of EAU. The expression of leptin was upregulated in the susceptible LEW strain (Table 4) and *Lep*-knockout mice did not develop EAU, but moderate disease was induced in the wild-type C57BL/6J strain (data not shown). Leptin is an adipokine that communicates information on energy availability to the cells. As a cytokine, leptin also affects thymic homeostasis and promotes Th1 cell differentiation and cytokine production.⁴¹ The importance of leptin in the pathogenesis of murine models of autoimmune diabetes⁴² and multiple sclerosis^{43,44} and its

association with uveitis in patients^{45,46} had been described. In this context, our observation constitutes a stimulus to further investigations.

In the present study we used the LEW:F344 model of EAU to identify several candidate genes that may play a role in the pathogenesis of uveitis. The list of validated genes presented herein are those that were identified as candidate genes in the QTL regions of the LEW versus F344 EAU model and were also differentially expressed during the course of EAU. It should be noted that these are likely to be only a few among many causative factors that determine susceptibility to a multifactorial disease like uveitis. The differential gene expression with respect to susceptibility and resistance to uveitis reported here is based only on the existing genetic variability between the LEW and F344 strains of inbred rats, such that any shared susceptibility genes would not have been revealed. Additional causative factors may be revealed using a different combination of strains. Additional genes identified by microarray might have been validated by other quantitative or functional approaches, but these alternative approaches were not attempted because of practical limitations.

In conclusion, the results of the functional genomics analysis applied to the pathogenic mechanism of EAU depicted a complex scenario in which (1) lymphocytes and other inflammatory cells of the susceptible LEW strain are maintained in active proliferation by higher levels of proinflammatory cytokines, (2) these cells are actively recruited and moved to the sites of priming by the action of chemokines and adhesion molecules expressed by susceptible LEW rats more efficiently than by resistant F344 rats, and (3) once the immune response is activated in susceptible LEW rats, it is more difficult to stop it, since the genes for some inhibitory cytokines are less actively expressed. Together with these cellular and molecular mechanisms, hormones, and neurotransmitters also contribute to modulate the disease mechanisms of EAU. In the current work, we demonstrate the potential of the genetic-genomic approach, by combining the microarray and QTL data, in the understanding of complex diseases such as autoimmune uveitis, where there is clustering or linkage of susceptibility genes on chromosomal regions, incomplete penetrance, complex gene-gene interaction, and modest effect for each gene.

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References

1. Caspi R. Immunogenetic aspects of clinical and experimental uveitis. *Reg Immunol* 1992;4:321–330. [PubMed: 1290750]
2. Sun SH, Silver PB, Caspi RR, et al. Identification of genomic regions controlling experimental autoimmune uveoretinitis in rats. *Int Immunol* 1999;11:529–534. [PubMed: 10323205]
3. Caspi RR, Silver PB, Chan CC, et al. Genetic susceptibility to experimental autoimmune uveoretinitis in the rat is associated with an elevated Th1 response. *J Immunol* 1996;157:2668–2675. [PubMed: 8805672]
4. Becker KG, Simon EM, Bailey-Wilson JE, et al. Clustering of non-major histocompatibility complex susceptibility candidate loci in human autoimmune diseases. *Proc Natl Acad Sci USA* 1998;95:9979–9984. [PubMed: 9707586]
5. Mattapallil MJ, Sahin A, Silver PB, et al. Common genetic determinants of uveitis shared with other autoimmune disorders. *J Immunol*. In press
6. de Koning DJ, Carlborg O, Haley CS. The genetic dissection of immune response using gene-expression studies and genome mapping. *Vet Immunol Immunopathol* 2005;105:343–352. [PubMed: 15808311]

7. Sasamoto Y, Kotake S, Yoshikawa K, Wiggert B, Gery I, Matsuda H. Interphotoreceptor retinoid-binding protein derived peptide can induce experimental autoimmune uveoretinitis in various rat strains. *Curr Eye Res* 1994;13:845–849. [PubMed: 7851121]
8. Sanui H, Redmond T, Kotake S, et al. Identification of an immunodominant and highly immunopathogenic determinant in the retinal interphotoreceptor retinoid-binding protein (IRBP). *J Exp Med* 1989;169:1947–1960. [PubMed: 2471769]
9. Silver PB, Rizzo LV, Chan CC, Donoso LA, Wiggert B, Caspi RR. Identification of a major pathogenic epitope in the human IRBP molecule recognized by mice of the H-2r haplotype. *Invest Ophthalmol Vis Sci* 1995;36:946–954. [PubMed: 7706044]
10. Caspi, RR. Experimental autoimmune uveoretinitis (EAU): mouse and rat. In: Coligan, JE.; Kruisbeek, AM.; Margulies, DH., editors. *Current Protocols in Immunology*. Wiley & Sons; New York: 1997. p. 15-6-1-15-6-16.
11. Barrett T, Cheadle C, Wood WB, et al. Assembly and use of a broadly applicable neural cDNA microarray. *Restor Neurol Neurosci* 2001;18:127–135. [PubMed: 11847435]
12. Vawter MP, Barrett T, Cheadle C, et al. Application of cDNA microarrays to examine gene expression differences in schizophrenia. *Brain Res Bull* 2001;55:641–650. [PubMed: 11576761]
13. Cheadle C, Vawter M, Freed W, Becker K. Analysis of microarray data using Z score transformation. *J Mol Diagn* 2003;5:73–81. [PubMed: 12707371]
14. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998;95:14863–14868. [PubMed: 9843981]
15. Chisnar J, Mondala T, Fox H, et al. Analysis of result variability from high-density oligonucleotide arrays comparing same-species and cross-species hybridizations. *Biotechniques* 2002;33:516–518,520,522. [PubMed: 12238761]
16. Morey JS, Ryan JC, Van Dolah FM. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. *Biol Proced Online* 2006;8:175–193. [PubMed: 17242735]
17. Bedoui S, Miyake S, Lin Y, et al. Neuropeptide Y (Npy) suppresses experimental autoimmune encephalomyelitis: Npy1 receptor-specific inhibition of autoreactive Th1 responses in vivo. *J Immunol* 2003;171:3451–3458. [PubMed: 14500640]
18. Prod'homme T, Weber MS, Steinman L, Zamvil SS. A neuropeptide in immune-mediated inflammation, Y? *Trends Immunol* 2006;27:164–167. [PubMed: 16530483]
19. Taguchi T, Bellacosa A, Zhou JY, et al. Chromosomal localization of the Ox-44 (CD53) leukocyte antigen gene in man and rodents. *Cytogenet Cell Genet* 1993;64:217–221. [PubMed: 8404042]
20. Tohami T, Drucker L, Radnay J, Shapira H, Lishner M. Expression of tetraspanins in peripheral blood leukocytes: a comparison between normal and infectious conditions. *Tissue Antigens* 2004;64:235–242. [PubMed: 15304003]
21. Mollinedo F, Fontan G, Barasoain I, Lazo PA. Recurrent infectious diseases in human CD53 deficiency. *Clin Diagn Lab Immunol* 1997;4:229–231. [PubMed: 9067662]
22. Johansson S, Hall H, Berg L, Hoglund P. NK cells in autoimmune disease. *Curr Top Microbiol Immunol* 2006;298:259–277. [PubMed: 16323418]
23. Johansson S, Berg L, Hall H, Hoglund P. NK cells: elusive players in autoimmunity. *Trends Immunol* 2005;26:613–618. [PubMed: 16118064]
24. Yunta M, Lazo PA. Apoptosis protection and survival signal by the CD53 tetraspanin antigen. *Oncogene* 2003;22:1219–1224. [PubMed: 12606948]
25. Pedersen-Lane JH, Zurier RB, Lawrence DA. Analysis of the thiol status of peripheral blood leukocytes in rheumatoid arthritis patients. *J Leukoc Biol* 2007;81:934–941. [PubMed: 17210617]
26. Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. *FASEB J* 1997;11:428–442. [PubMed: 9194523]
27. Crane IJ, McKillop-Smith S, Wallace CA, Lamont GR, Forrester JV. Expression of the chemokines MIP-1alpha, MCP-1, and RANTES in experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci* 2001;42:1547–1552. [PubMed: 11381059]
28. Traynor T, Herring A, Dorf M, Kuziel W, Toews G, Huffnagle G. Differential roles of CC chemokine ligand 2/monocyte chemoattractant protein-1 and CCR2 in the development of T1 immunity. *J Immunol* 2002;168:4659–4666. [PubMed: 11971015]

29. Sun B, Sun SH, Chan CC, Caspi RR. Evaluation of in vivo cytokine expression in EAU-susceptible and resistant rats: a role for IL-10 in resistance? *Exp Eye Res* 2000;70:493–502. [PubMed: 10865998]
30. Koch AE, Kunkel SL, Harlow LA, et al. Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J Clin Invest* 1992;90:772–779. [PubMed: 1522232]
31. Jee Y, Yoon WK, Okura Y, Tanuma N, Matsumoto Y. Upregulation of monocyte chemotactic protein-1 and CC chemokine receptor 2 in the central nervous system is closely associated with relapse of autoimmune encephalomyelitis in Lewis rats. *J Neuroimmunol* 2002;128:49–57. [PubMed: 12098510]
32. Youssef S, Wildbaum G, Maor G, et al. Long-lasting protective immunity to experimental autoimmune encephalomyelitis following vaccination with naked DNA encoding C-C chemokines. *J Immunol* 1998;161:3870–3879. [PubMed: 9780152]
33. Sonoda KH, Sasa Y, Qiao H, et al. Immunoregulatory role of ocular macrophages: the macrophages produce RANTES to suppress experimental autoimmune uveitis. *J Immunol* 2003;171:2652–2659. [PubMed: 12928419]
34. Faunce DE, Stein-Streilein J. NKT cell-derived RANTES recruits APCs and CD8⁺ T cells to the spleen during the generation of regulatory T cells in tolerance. *J Immunol* 2002;169:31–38. [PubMed: 12077225]
35. Turrin N, Gayle D, Ilyin S, et al. Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal administration of bacterial lipopolysaccharide. *Brain Res Bull* 2001;54:443–453. [PubMed: 11306198]
36. De la Fuente M, Del Rio M, Victor V, Medina S. Neuropeptide Y effects on murine natural killer activity: changes with ageing and cAMP involvement. *Regul Pept* 2001;101:73–79. [PubMed: 11495681]
37. Medina S, Rio M, Cuadra B, Guayerbas N, Fuente M. Age-related changes in the modulatory action of gastrin-releasing peptide, neuropeptide Y and sulfated cholecystokinin octapeptide in the proliferation of murine lymphocytes. *Neuropeptides* 1999;33:173–179. [PubMed: 10657488]
38. Kawamura N, Tamura H, Obana S, et al. Differential effects of neuropeptides on cytokine production by mouse helper T cell subsets. *Neuroimmunomodulation* 1998;5:9–15. [PubMed: 9698253]
39. Medina S, Del Rio M, Hernanz A, De la Fuente M. Age-related changes in the neuropeptide Y effects on murine lymphoproliferation and interleukin-2 production. *Peptides* 2000;21:1403–1409. [PubMed: 11072128]
40. Bracci-Laudiero L, Aloe L, Stenfors C, Theodorsson E, Lundeberg T. Development of systemic lupus erythematosus in mice is associated with alteration of neuropeptide concentrations in inflamed kidneys and immunoregulatory organs. *Neurosci Lett* 1998;248:97–100. [PubMed: 9654351]
41. Matarese G, Moschos S, Mantzoros CS. Leptin in immunology. *J Immunol* 2005;174:3137–3142. [PubMed: 15749839]
42. Lee CH, Reifsnyder PC, Naggert JK, et al. Novel leptin receptor mutation in NOD/LtJ mice suppresses type 1 diabetes progression: I. Pathophysiological analysis. *Diabetes* 2005;54:2525–2532. [PubMed: 16123339]
43. Matarese G, Di Giacomo A, Sanna V, et al. Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. *J Immunol* 2001;166:5909–16. [PubMed: 11342605]
44. De Rosa V, Procaccini C, La Cava A, et al. Leptin neutralization interferes with pathogenic T cell autoreactivity in autoimmune encephalomyelitis. *J Clin Invest* 2006;116:447–455. [PubMed: 16410832]
45. Kavuncu S, Koc F, Kurt M, et al. Evaluation of serum leptin concentration in Behçet's disease with ocular involvement. *Graefes Arch Clin Exp Ophthalmol* 2005;243:1158–1160. [PubMed: 15906063]
46. Evereklioglu C, Inaloz HS, Kirtak N, et al. Serum leptin concentration is increased in patients with Behçet's syndrome and is correlated with disease activity. *Br J Dermatol* 2002;147:331–336. [PubMed: 12174107]

Figure 1.

EAU histopathology and scoring. **(A)** Average scores of posterior uveitis in both eyes of individual susceptible LEW (day 0, $n = 2$; day 3, $n = 3$; day 10, $n = 3$; day 14, $n = 25$; day 28, $n = 4$) and resistant F344 (day 0, $n = 2$; day 3, $n = 4$; day 10, $n = 4$; day 14, $n = 25$; day 28, $n = 4$) rats after immunization are shown. For the day 14 time point, data from 25 rats per strain from multiple experiments are included. Mean EAU score at day 14 in LEW rats is marked. **(B)** Histopathologic lesions of EAU in LEW and F344 rats. Histologic damage in the retina of LEW (*top*) and F344 (*bottom*) rats at day 10 (representing the onset of disease) and day 14 (representing the peak of inflammation) after immunization.

Figure 2.

Hierarchical clustering of gene expression levels in LN cells from LEW and F344 rats from induction to resolution of EAU. RNA was isolated from the draining LNs of individual naïve or immunized LEW or F344 rats ($n = 4$ per time point). For analysis, RNA samples were pooled in equal amounts within each group, and the cDNAs generated were used for microarray analysis. Samples were analyzed on three different microarray membranes, each containing a duplicate set of genes. The averages of the three z-normalized data sets per time point per strain were used for hierarchical clustering. Different patterns of gene expression were evident in susceptible LEW and resistant F344 LN cells during the time course of the disease development. *Red*: genes that were upregulated in the LEW strain in comparison to the F344 strain; *green*: downregulated genes. Genes showing major differences at days 0 and 3 are magnified. *Blue outlines* encompass major changes occurring at days 3 and 14 in the F344 compared with the LEW strain.

Figure 3.

Expression levels of genes that are validated by real-time RT-PCR from the time course experiment. Pooled RNA samples ($n = 4$ rats per group) used for the microarray analysis in Figure 2 were used to determine the expression level of genes by RT-PCR. The RT-PCR analysis was repeated three times for each gene. Genes that were validated by real-time PCR analysis are grouped into functional categories. Histograms show relative gene expression rates measured at days 0 and 3 pi and calculated as ratios between gene and internal *Gapdh* values. Error bars, SD of mean results of three independent experiments. * $P = 0.04$, comparing LEW day 3 with F344 day 3; ** $P = 0.01$, comparing LEW day 3 vs. F344 day 3; • $P = 0.05$, comparing LEW day 0 vs. LEW day 3; •• $P = 0.05$, comparing LEW day 0 vs. F344 day 0.

Figure 4.

Expression levels of validated genes: quantitative RT-PCR from the time course experiment. The pooled RNA samples used for the microarray analysis in Figure 2 were used to validate the expression level of various genes. Genes that underwent validation by quantitative RT-PCR analysis are grouped into functional categories. Histograms show gene expression rates at day 3 normalized to that of naïve animals (day 0).

Figure 5.

Flow cytometric analysis of subpopulations of LN cells at days 0 and 14 pi. LN cells from each naïve LEW or F344 rats ($n = 5$ for each strain) or draining LN cells from each immunized LEW or F344 rats ($n = 5$ for each strain) were isolated and fluorescently labeled for CD3, CD8 α , and CD53 surface molecules. The mean frequency of CD53⁺ cells is shown as a percentage of (A) CD3⁺CD8 α ⁺ and (B) CD3⁻CD8 α ⁺ gated cells. Also shown is the frequency of CD3⁺ cells expressing CD8 α (C). Statistically significant results of validation of microarray data obtained by flow cytometry are shown. * $P = 0.001$, comparing day 0 vs. day 14 of both strains; • $P < 0.04$, comparing LEW day 0 vs. F344 day 0; ** $P = 0.0001$, comparing LEW day 14 vs. F344 day 14.

Figure 6.

Hierarchical clustering of gene expression levels of lymphocytes from LEW and F344 rats on in vitro stimulation with the immunizing antigen. Draining LN cells from LEW ($n = 3$) and F344 ($n = 3$) rats were isolated 14 days pi and were individually cultured in the absence (LC and FC) or presence (L14 and F14) of immunizing antigen for 24 hours. RNA was isolated from the cultured cells of individual rats and equal amounts of RNA were pooled within each group to prepare cDNA. cDNA from each group was hybridized to three different microarrays each containing a duplicate set of genes within a membrane. The means of the three z -normalized data sets per group per strain were used for hierarchical clustering. Different patterns of gene expression were evident in susceptible LEW and resistant F344 lymphocytes after stimulation with the immunizing antigen. *Red*: genes upregulated in the LEW strain compared to the F344 strain; *green*: those that were downregulated. Genes showing major differences in gene expression levels are magnified.

Figure 7.

Expression levels of genes that are induced by antigenic stimulation of lymphocytes and validated by real-time RT-PCR. The pooled RNA samples used for the microarray analysis in Figure 6 were used for determining the expression levels of genes induced by antigen stimulation. Genes validated by real-time RT-PCR analysis are grouped into functional categories. Histograms show relative gene expression rates measured in unstimulated or antigen-stimulated lymphocytes shown as ratios between gene and internal *Gapdh* values. Bars, SD of mean results generated in three independent experiments. * $P < 0.05$, comparing LEW stimulated with F344 stimulated; * $P < 0.05$, comparing LEW un-stimulated versus F344 unstimulated.

Figure 8.

Profile of lymphocyte expression of cytokines on stimulation with the immunizing antigen. Shown are the significant variations in the concentrations of cytokines secreted by lymphocytes of susceptible LEW and resistant F344 rats on in vitro stimulation with the immunizing antigen. Bars, SD of mean results generated from three independent experiments, each with five to seven rats per strain using pooled LN cells within a group. * $P < 0.05$, comparing unstimulated with stimulated cells; * $P < 0.05$, comparing unstimulated LEW versus unstimulated F344 cells; ** $P < 0.02$, comparing stimulated LEW cells with stimulated F344 cells.

Table 1

Primers and Probes designed from Rat mRNA Sequences of Genes

	Forward Primer	Probe	Reverse Primer
<i>Bax</i>	5'-GACTCCCCCGAGAGGTCT	6FAM-CGGGTGGCAGCTGACATGTTGC	5'-AGTGCACAGGGCCTTGAGC
<i>Birc5</i>	5'-TGGCCCAGTGTCTTTCTGC	6FAM-AGAGGAGCATAGGAAGCACTCCCCTGG	5'-CGGTCAAGTCTTCCACCTGC
<i>Ccr2</i>	5'-CCACCTTCCAGGAATTCCTGG	6FAM-TGAGTAACTGTGTGGTTGACATGCACCTAGACC	5'-CTCTGTCACCTGCATGGCC
<i>Cd53</i>	5'-CCGTAATCTCCCCTTCCTGAC	6FAM-TGGCAATGTTCTGGTCATTGTGGGC	5'-CCCAAGAAGGCAACTACCATG
<i>Crhr1</i>	5'-CGTGGTTTGTGGTCCAGCT	6FAM-CCGTGAGCCCCGAGGTGCAC	5'-AACCTACACCAGGCCACATTG
<i>Dedd</i>	5'-TGGACGTGACTTCTTGTGGC	6FAM-AGCGCCAGGGCCGCTGTGT	5'-CAGCACCTGGCGAAAGTTACT
<i>Ifnar1</i>	5'-TTTCTTGCCCGGTTTCTGG	6FAM-TTCGGGTGTGCTTTCAAGGCTCTGA	5'-TGCAACATTTAGCCAAAAGGC
<i>Il1b</i>	5'-GTGGCTGTGGAGAAGCTGTG	6FAM-CAGCTACCTATGTCTTGCCCGTGGAGC	5'-CGTCATCATCCCACGAGTCAC
<i>Il6</i>	5'-CGAAAGTCAACTCCATCTGCC	6FAM-TCAGGAACAGCTATGAAGTTTCTCTCCGCA	5'-GGCAACTGGCTGGAAGTCTC
<i>Il8</i>	5'-GGGTGTCCCAAGTAATGGAG	6FAM-AAGAAGATAGATTGCACCGATGGCGTCTG	5'-CAGAAGCCAGCGTTCACCA
<i>Il10r1</i>	5'-ATCCTGGACCTGGAGGCCT	6FAM-CCCAAAGGTGTACCCGAGCTGA	5'-GCTGCCATGCAGGTCTGAGT
<i>Il12rb2</i>	5'-TCGCGTCTCTGGGAAGCTT	6FAM-CCAGCGTCTCTCTCGTGGGC	5'-ACTTGTGCCCACCTCTGCC
<i>Lck</i>	5'-TGGAGAAGTGTGGATGGCC	6FAM-CTACAACAAGCACACCAAAGTGGCGG	5'-TCCCTGGCTTCATTGTCTTCA
<i>Lep</i>	5'-CATTTACACACGCAGTCGG	6FAM-CCGCCAGGCAGAGGGTCAAC	5'-GTGAAGCCCGGGAATGAAGT
<i>Npy</i>	5'-CCTGTGAAACCACTCTGCCTG	6FAM-ATGCATGCCACCACCAGGCTGGT	5'-CAAGGGAAATGGGTGGAA
<i>Pdgfa</i>	5'-CCCACATCGGCAACTTCT	6FAM-TCTGGCCCCATGTGTGGAGGT	5'-GTTACAGCAGCCAGTGCAGC
<i>Ryk</i>	5'-TGACCTCAGCGGTCTAAATCC	6FAM-TCCAAGCGGTGCAGCACGTAGTGT	5'-TGCACAATCAGGCTACTGGG
<i>Scya2</i>	5'-TCCTCCACCACTATGCAGGTC	6FAM-CCTGTTGTTACAGTTGCTGCCTGTAGC	5'-GAGTAGCAGCAGGTGAGTGGG
<i>Sele</i>	5'-GGTCTGCGATGCTGCCTAC	6FAM-CCCCAGCCAACCTCCCGT	5'-GTGAGGTTGCTGCCACAGAG
<i>Sell</i>	5'-GGAATCAGGAAAATCGGGAAA	6FAM-CGTGGACATGGGTGGGAACCAAC	5'-TCTCTGCTTCTTTGGTGAGGG
<i>Sod1</i>	5'-GTCCAGCGGATGAAGAGAGG	6FAM-ATGTTGGAGACCTGGGCAATGTGGCT	5'-ACATTGGCCACACCGTCCT
<i>Stat5b</i>	5'-CAGCTGACGGATACGTGAAGC	6FAM-TGCAGGGAGCGGCCAC	5'-GGGAAGGAGCCTGGTCCAT
<i>Tshr</i>	5'-CACACTGACGGTCATCACCT	6FAM-TCACCTTCGCCATGCGCCTG	5'-GTGCCTGAGGCGGATCTTC
<i>Vtn</i>	5'-AGGCCCTTTTTCATACTAGCC	6FAM-CCAAGAGTCATGCAAGGGCCGC	5'-GCTGGCCATGAAACCTG

Table 2

Primer Sequences of Rat-Specific Genes Analyzed Using the Quantitative RT-PCR Method

Gene	RefSeq ID	Forward Primer (5'–3')	Reverse Primer (5'–3')
<i>Alcam</i>	NM_031753	AGGTGACTGCATTTCAAGAG	GGAGGAAGTCATGGTATACAAC
<i>Casp3</i>	NM_012922	GTTTCTTCAGAGGCGACTACT	CATCGTCAGTTCCTACTGTCT
<i>Cd69</i>	XM_232418	GCTACCCCTTGCTGTTATTGA	AGACCCTGTCACGTTGAAC
<i>Cd9</i>	XM_001063955	ACTCTCAGACCAAGAGCATCT	GAAGAACAATCCCAGCATG
<i>ErbB2</i>	NM_017003	CCTAAAAGAGACGGAGCTAAG	GTTGGCTTTAGGAGATGTGT
<i>Frk</i>	NM_024368	ATAGACCGCAACTCCATACA	TTATCTGTGCCTCCCTGAG
<i>Il12a</i>	NM_053390	TGTGCCTTGGTAGCATCTAT	GTGATTCAGAGACCGCATT
<i>Il1r1</i>	NM_013123	CCCGTCACACGAGTAATAAC	AGACAAGGTCGGTGAAGCTG
<i>Il7</i>	NM_013110	AAATGCAACTGACTCCTGC	CAGTGGCGACTCTGAATCT
<i>Itgb1</i>	NM_017022	GGATGCTTACTGCAGAAAAG	GGACCTATCGCAGTTGAAG
<i>Itgb5</i>	NM_147139	TAGCTAGCGTGAGCAAATG	AGCCCATCTCCACACATAC
<i>Nos2</i>	NM_012611	CGCTGGTTTGAACTTCTC	GTCTGTGACTTTGTGCTTCTG
<i>Npy</i>	NM_012614	GCCATGATGCTAGGTAACAA	GGAGTAGTATCTGCCATGTC
<i>Npylr</i>	XM_344502	AGACTCTCACAGGCTGTCTTAC	CCTGTACTTACTGTCCCTGATT
<i>Runx1</i>	NM_017325	TCAACGACCTCAGGTTTGT	TAGTTTCTGCCGATGTCTTC
<i>Ryk</i>	XM_343458	TCCCAACAAGATCTGGTACA	GGAAACAGTCTCTGGAAAG
<i>Sele</i>	NM_138879	CACCTCTGACATTGTCTCTCA	CTGATTGCTCGCATCTTAG
<i>St13</i>	NM_031122	AGACGCCATCAAGCTAAAC	GCTTTCCTCTCCATTG
<i>Stat3</i>	NM_012747	GCGAAAGGAAACACCTATG	GTGGACATTAACCAAGGTTGA
<i>Stat5a</i>	NM_017064	ACTCCATGCTTCTCTTGGA	CCTTGAGGTCTGAGTGAAAA
<i>Stat5b</i>	NM_022380	GGACTCCATGCTTCTCTTG	CCTTGAGGTCTGAGTGAAAA
<i>Tcf8</i>	XM_341539	AGTGCGGAATCTGTAGAAAAG	TAGGAGTAGCGGTGATTCAT
<i>Tgfb2</i>	NM_031131	CTGTCCAGAAGGCTGTGA	CCACTTCACGGTCAAAAAGT
<i>Tnfaip1</i>	NM_182950	CCTGCAGGACAAGAAAGAC	CAGAGTTGCTGGGTACGA
<i>Txn</i>	NM_053800	CCTTCTTTCATTCCCTCTGT	GAGAACTCCCAACCTTTT
<i>Txnl</i>	NM_080887	GGAGCAATGAGGACACAGA	TCGCAGTCAGACTCCAAGA

Table 3

Genes Selected for Validation from Microarray Expression Profiles: Innate Immune Response

Gene	Accession No. (Human mRNA)	z-Ratio	Presence in Any EAU QTL Region	Validation (Methods Used)
Adhesion and Costimulatory Molecules				
ALCAM	NM_001627	-1.72		Yes (cDNA)
CD8A	NM_001768	1.49	Yes	No (cDNA, FACS)
CD9	NM_001769	-1.62		Yes (cDNA)
CD53	NM_000560	0.11	Yes	No (cDNA, FACS)
CD69	NM_001781	0.66		No (cDNA)
ITGB1	XM_005799	1.60		No (cDNA)
ITGB5	NM_002213	-2.93		No (cDNA)
SELE	NM_000450	0.16		No (cDNA)
SELL	NM_000655	1.50		Yes (cDNA)
VTN	NM_00638	-1.83	Yes	No (cDNA)
Apoptosis				
BAX	NM_004324	0.83		Yes (cDNA)
BIRC	NM_001165	0.57		Yes (cDNA)
CASP3	NM_004346	1.29		No (cDNA)
Cytokines, Chemokines, and Their Receptors				
CCR2	NM_000647	0.97		Yes (cDNA)
IFNAR	NM_000629	-0.89		Yes (cDNA)
IL1B	NM_000576	-1.54		No (cDNA)
ILLR1	NM_000877	-2.22		Yes (cDNA)
IL6	NM_000600	-1.31		Yes (cDNA)
IL7	NM_000880	-0.71	Yes	Yes (cDNA)
IL8	NM_000584	-1.93		Yes (cDNA)
IL10RA	NM_001558	-0.36		Yes (cDNA)
IL12A	NM_000882	-0.61		Yes (cDNA)
IL12RB2	NM_001559	0.56		Yes (cDNA)
PDGFA	NM_002607	1.23		Yes (cDNA)
SCYA2	NM_002982	1.07		Yes (cDNA)
TGFB2	NM_003238	0.62		No (cDNA)
Metabolism and Hormones				
LEP	NM_000230	-0.47	Yes	No (cDNA)
NOS2A	NM_000625	-0.75		Yes (cDNA)
NPY	(Not in the array)		Yes	Yes (cDNA)
NPYLR	NM_000909	-1.77		Yes (cDNA)
SODL	NM_000454	0.06	Yes	Yes (cDNA)
TSHR	NM_000369	-2.45		No (cDNA)
TXN	NM_003329	1.46		No (cDNA)
TXNL	NM_004786	-0.07		Yes (cDNA)
Signal Transduction and Transcription Factors				
ERBB2	NM_004448	-1.51	Yes	Yes (cDNA)
FRK	NM_002031	-0.02		Yes (cDNA)
LCK	NM_005356	-2.19		No (cDNA)
RUNXL	NM_001754	0.86		Yes (cDNA)
RYK	NM_002958	0.22	Yes	No (cDNA)
STL3	NM_003932	-2.30		Yes (cDNA)
STAT3	NM_003150	0.65	Yes	Yes (cDNA)
STAT5A	NM_003152	0.01	Yes	No (cDNA)

A positive z-ratio indicates increased expression in the LEW strain compared to the F344 strain on day 3 after immunization. A yes in validation means both the microarray data and the validation method followed the same direction of differential expression.

Table 4

Selected Genes for Validation from Microarray Expression Profiles: Antigen-Specific Responses

Gene	Accession No. (Human mRNA)	z-Ratio	Presence in any EAU QTL Region	Validation (Methods Used)
<i>Adhesion and Costimulatory Molecules</i>				
CD53	NM_000560	1.00	Yes	No (cDNA)
SELE	NM_000450	-3.05		Yes (cDNA)
SELL	NM_000655	-1.21		Yes (cDNA)
VTN	NM_00638	0.35	Yes	Yes (cDNA)
<i>Apoptosis</i>				
BAX	NM_004324	-0.88		No (cDNA)
BIRC5	NM_001168	0.47		Yes (cDNA)
<i>Cytokines, Chemokines, and Their Receptors</i>				
CCR2	NM_000647	-1.75		Yes (cDNA)
IFNG	NM_000619	-0.42		No (cDNA)
IL1B	NM_000576	2.14		No (cDNA, ELISA)
IL2	NM_000586	0.11	Yes	Yes (ELISA)
IL6	NM_000600	0.76		Yes (ELISA)
IL8	NM_000584	-0.01		No (cDNA)
IL10	NM_000572	1.08		Yes (ELISA)
IL10RA	NM_001558	1.54		No (cDNA)
IL12RB2	NM_001559	2.51		Yes (cDNA)
PDGFA	NM_002607	0.96		Yes (cDNA)
SCYA2	NM_002982	1.00		No (cDNA, ELISA)
SCYA20	NM_004591	0.24		Yes (ELISA)
SCYA5	NM_002985	0.36	Yes	No (ELISA)
<i>Metabolism and Hormones</i>				
LEP	NM_000230	1.34	Yes	Yes (cDNA, KO mice)
NOS2	NM_000625	0.70		
NPY			Yes	Yes (cDNA)
SODL	NM_000454	0.13	Yes	No (cDNA)
TSHR	NM_000369	-2.09		No (cDNA)
<i>Signal Transduction and Transcription Factors</i>				
DEDD	NM_004216	2.74		Yes (cDNA)
LCK	NM_005356	-0.45		Yes (cDNA)
RYK	NM_002958	1.88	Yes	No (cDNA)
STAT5B	NM_012448		Yes	Yes (cDNA)

A positive z-ratio indicates increased expression in the LEW strain compared to the F344 strain after in vitro antigenic stimulation of LN cells collected 14 days after immunization. A yes in validation means both the microarray data and the validation method followed the same direction of differential expression.