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Combining genetic mapping with genome-wide expression in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and candidate genes regulating autoimmunity

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The experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system commonly used to study multiple sclerosis (MS). We combined clinical EAE phenotypes with genome-wide expression profiling in spleens from 150 backcross rats between susceptible DA and resistant PVG rat strains during the chronic EAE phase. This enabled correlation of transcripts with genotypes, other transcripts and clinical EAE phenotypes and implicated potential genetic causes and pathways in EAE. We detected 2285 expression quantitative trait loci (eQTLs). Sixty out of 599 *cis*-eQTLs overlapped well-known EAE QTLs and constitute positional candidate genes, including *Ifit1* (*Eae7*), *Atg7* (*Eae20-22*), *Klrc3* (*eEae22*) and *Mfsd4* (*Eae17*). A *trans*-eQTL that overlaps *Eae23a* regulated a large number of small RNAs and implicates a master regulator of transcription. We defined several disease-correlated networks enriched for pathways involved in cell-mediated immunity. They include C-type lectins, G protein coupled receptors, mitogen-activated protein kinases, transmembrane proteins, suppressors of transcription (*Jundp2* and *Nr1d1*) and STAT transcription factors (*Stat4*) involved in interferon signaling. The most significant network was enriched for T cell functions, similar to genetic findings in MS, and revealed both established and novel gene interactions. Transcripts in the network have been associated with T cell proliferation and differentiation, the TCR signaling and regulation of regulatory T cells. A number of network genes and their family members have been associated with MS and/or other autoimmune diseases. Combining disease and genome-wide expression phenotypes provides a link between disease risk genes and distinct molecular pathways that are dysregulated during chronic autoimmune inflammation.

INTRODUCTION

A predisposition to develop a complex disease such as multiple sclerosis (MS) is regulated by numerous genetic variants that each contribute small effects (1). Clinically, MS is characterized by immune-mediated destruction of myelin sheaths and axons in the central nervous system, leading to progressive disability. Despite recent substantial progress in deciphering genetic variants that contribute to susceptibility (2), little is known about

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the functional outcomes of these risk-associated variants, in part due to limitations in access of relevant human samples. In addition, identified risk alleles together explain only a fraction of disease heritability and variance (2). Additional risk variants conferring small effects may contribute to heritability of complex diseases, and the clustering of genes (below thresholds for significant association with MS) into functional networks supports this hypothesis (3). Unraveling the functions of susceptibility genes through identification of pathways enriched with risk genes can reveal mechanisms central in disease regulation.

An animal model widely utilized to characterize the genetic basis and disease mechanisms of relevance for MS is experimental autoimmune encephalomyelitis (EAE). Myelin oligodendrocyte glycoprotein (MOG)-induced EAE in rats mimics many features of MS (4), including inflammation and demyelination, relapses and remissions and immune cell infiltration. Linkage analysis in experimental animal crosses can readily detect quantitative trait loci (QTLs) related to clinical traits of complex diseases, and over 50 QTLs have been identified in EAE (5). Several genes underlying QTL effects in rats were positionally cloned and a number of them have been subsequently confirmed to regulate human counterpart (6). However, it has been challenging to define single quantitative trait genes (7).

Given the high heritability of variation in gene expression (8), identifying determinants of gene expression may give insights into pathogenic mechanisms of complex traits. The approach of mapping quantitative variation in gene expression was introduced in 2001 (9,10). This approach yields expression QTLs (eQTLs) (11), which influence expression of transcripts either in *cis* or in *trans*, where *cis*-acting eQTLs are located in close proximity of the target gene itself, while *trans*-acting eQTLs are located in a region distant from the gene it regulates. Technical artifacts excluded (e.g. hybridization differences) (12); *cis*-regulatory effects can usually be mapped with high statistical significance and could be explained in most cases by a variation in DNA sequence in the regulatory regions of the target gene (13). *Cis*-eQTLs that overlap trait QTLs constitute plausible candidate genes underlying the trait QTL effect. During the last decade, the use of genome-wide expression profiling combined with linkage analysis in segregating populations has identified genomic variations that regulate complex traits in experimental models (6,14,15). In addition, the approach has been utilized to characterize genetically driven networks of genes giving insights into pathways and functions critical for the trait of interest (16).

In this study, we combined genome-wide expression analysis in spleen from an experimental backcross (BC) between EAE-susceptible Dark Agouti (DA) and EAE-resistant Piebald Viral Glaxo (PVG) rat strains during the chronic phase of EAE with clinical EAE phenotypes and classical EAE QTLs. These two inbred rat strains have been extensively used in our laboratory to characterize EAE QTLs (5,17–27). We characterized several potential positional candidate genes for known EAE QTLs that provide a good base for further functional studies. Genome-wide expression analysis in the chronic stage EAE enabled correlation of transcripts not only with genotypes and to each-other but also with the clinical EAE phenotypes. We defined several disease-correlated gene networks partially genetically regulated by loci that predispose for EAE. Some were enriched for pathways involved in cell-mediated immune mechanisms of relevance for EAE and MS, and also included genes or family members of genes associated with MS.

RESULTS

Overview of eQTLs in the chronic stage of EAE

We used the eQTL approach to identify candidate genes and pathways that regulate EAE. This was achieved by combining clinical EAE phenotypes in a BC with whole-genome transcript expression analysis in splenic tissue from 150 BC male rats. Spleens were collected at day 35 after induction of EAE and expression was measured using Affymetrix Rat Gene 1.0 ST Arrays. The evidence for linkage was tested between genotype and gene expression (27342 transcripts) to identify hereditary components and revealed a total of 2285 eQTLs with genome-wide significance of $P \leq 0.05$ (Table 1, Supplementary Material, Tables S1 and S2). By introducing clinical traits as covariates that had been recorded during the clinical EAE experiments, we evaluated if the detection of eQTLs depends on disease incidence and severity. A majority of detected eQTLs did not depend on disease incidence or severity, as depicted in the Venn diagram (Fig. 1A). Thus, we report only EAE status as a disease covariate in Table 1. An overview of *cis*- and *trans*-acting eQTLs across the genome is presented in Figure 1B.

Co-localized *trans* eQTLs

Trans-acting eQTLs (Fig. 1B and Supplementary Material, Table S2) do not involve DNA variation in the expressed

Table 1. *Cis*- and *trans*-eQTLs detected in EAE spleen day 35 post-immunization

Chr	Transcript		EAE(a)		EAE(i)	
	cis	*trans*	*cis*	*trans*	*cis*	*trans*
1	123 (20)	264 (12)	106 (32)	216 (69)	80 (26)	71 (15)
2	69	196	51	142	35	46
3	23	20	19	15	17	18
4	64 (47)	8 (6)	62 (50)	8 (6)	59 (50)	13 (7)
5	7	17	6	13	9	20
6	45	71	39 (8)	57 (4)	37 (9)	105 (14)
7	14 (2)	5 (1)	13 (1)	3 (1)	8	6 (2)
8	25 (9)	12 (4)	27 (2)	24	26 (3)	89 (3)
9	11	4	11	6	8	8
10	21 (17)	24 (20)	20 (17)	32 (29)	14 (11)	22 (21)
11	14	35	13	45	24	509
12	10	11 (1)	10	10 (1)	10	9
13	14 (3)	15 (5)	13 (2)	12 (2)	11 (3)	10 (1)
14	13 (3)	17 (2)	10 (3)	9 (3)	9 (3)	6 (2)
15	38	49	32	31	24	8
16	31	172	25	74	25	258
17	28 (5)	643 (141)	24 (7)	408 (102)	10 (2)	158 (31)
18	14	20	13	17	5	7
19	15	56	13	44	11	34
20	4	34	5	38	3	14
X	16	13	17	9	15	16
Total	599 (106)	1686 (192)	529 (122)	1213 (217)	440 (107)	1427 (96)

Number of *cis*- and *trans*-regulated transcripts for each chromosome and total (rows) for a selection of clinical phenotypes as covariates (column pairs). eQTLs were selected to have a logarithm of odds score ≥ 2 (generated with the Haley–Knott regression model in R/qtl) and a genome-wide corrected *P*-value of ≤ 0.05 (generated with 1000 permutations). Numbers in parentheses refer to the subset of transcripts controlled by loci in EAE QTLs (see Supplementary Material, Table S5). Abbreviations: eQTL, expression quantitative trait locus; Chr, chromosome; Transcript, no covariate; EAE(a), incidence of EAE as covariate, additive model; EAE(i), incidence of EAE as covariate, interactive model.

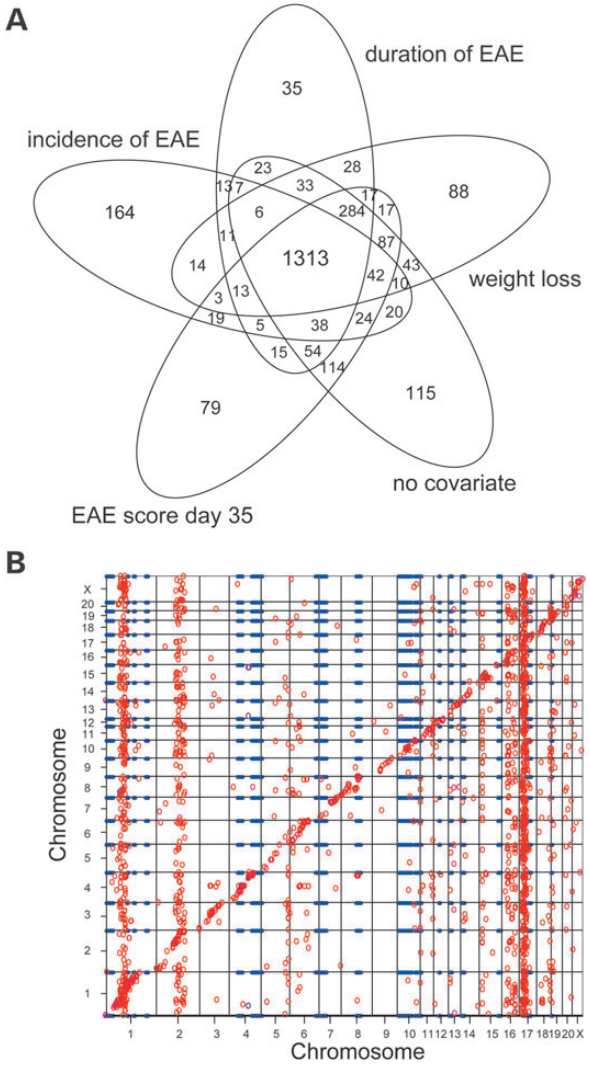

Figure 1. Expression QTLs in chronic stage EAE. (**A**) Venn diagram indicating the number of overlapping transcripts when performing a whole genome scan for eQTLs using different clinical disease variables as covariates. (**B**) Scatter plot with the *x*-axis depicts the genomic position of the eQTL and the *y*-axis depicts the genomic location of the target gene. Each circle represents a significant *cis*- or *trans*-eQTL (no covariates used in the model). Blue lines represent EAE QTLs identified in the DA and PVG strain combination. The diagonal band indicates *cis*-eQTLs and the off-diagonal circles represent *trans*-eQTLs. Vertical *trans*-eQTL bands reflect genomic regions that control many transcripts. eQTLs were generated with the Haley–Knott regression model in R/qtl; LOD $\geq$ 2; genome-wide corrected *P*-value $\leq$0.05.

transcript in question; instead, the transcript is regulated by other more distant genetic variations. Therefore, *trans*-regulation can denote a genomic location of a master regulator of transcription (16). Functional analysis using ingenuity pathways analysis (IPA) revealed a significant association of *trans*-regulated genes with liver × receptor/retinoid × receptor activation, nuclear receptors involved in transcriptional regulation (28) and inactivation of platelet activating factor (Supplementary Material, Tables S3 and S4). Vertical *trans*-bands of co-localized eQTLs reflect the genomic position of a regulator of multiple eQTL transcripts (29, 30). Such bands were observed on rat chromosomes (RNO) 1, 2 and 17, with the *trans*-band on RNO17 overlapping an EAE regulating QTL, *Eae23a,* related to several clinical traits of complex disease (26). Although the *trans*-band on RNO17 was not enriched for a functional pathway, we observed regulation of many small nucleolar RNA genes and spliceosomal small nuclear RNAs from this genomic region (Supplementary Material, Table S2). These have been reported to be involved in epigenetic modifications (31) and the formation of spliceosomes (32), respectively. Additionally, we observed that genes in these families negatively correlate with disease phenotypes.

Candidate genes denoted by *cis* eQTLs

A cis-acting eQTL directly identifies expression variations in a gene that in turn can regulate a physiological trait or molecular pathway. One of the most significant *cis*-eQTLs was the regulator of G-protein signaling 4 (*Rgs4*) (Table 2) on RNO13. We performed IPA analysis and recorded that *Rgs4* and its most correlated transcripts associated with cell proliferation and migration, integrin and endocytosis signaling (Supplementary Material, Tables S3 and S4). Although *Rgs4* does not reach a genome-wide significance in human GWAS, it is of interest that its family member *Rgs1* is associated with MS (33) and that the *Rgs14* gene region is associated with Crohn's disease, another disease with chronic inflammatory features (34).

In the next stage, we combined eQTL analysis with classical EAE QTLs. Analysis of EAE QTLs that segregate between DA and PVG strains was performed in a larger set of 421 BC rats, encompassing 150 rats used for eQTL analysis, and all EAE QTLs are reported in Supplementary Material, Table S5 (Stridh *et al*., unpublished data). A large proportion of the QTLs also showed evidence of linkage in a subset of 150 animals used for eQTL analysis, albeit with less significance due to decreased power in a smaller sample. Therefore, we focused on all well-established EAE QTLs that have been identified in more than one well-powered study (Table 3, Supplementary Material, Table S5), while emphasizing the QTLs identified in this BC (Table 3).

We then examined the eQTL data for candidate genes underlying EAE phenotypes. Of all eQTLs with a *P*-value $\leq$0.05, a total of 60 *cis*-eQTLs (Table 3) with LOD $\geq$ 3.9 resided in previously known EAE QTLs present in the same DA and PVG strain combination (Supplementary Material, Table S5), such as *Ifit1 (Eae7)*, *Atg7 (Eae20-22), Klrc3 (eEae22)* and *Mfsd4* (*Eae17*), among others. These 60 *cis*-eQTLs were mainly associated with functional pathways of natural killer (NK) cell signaling and molecular functions of cellular growth and proliferation (Supplementary Material, Tables S3 and S4). A *cis*-eQTL that maps to a disease QTL can be considered a likely causal gene underlying the disease QTL.

Gene networks of inter-dependent genes that correlate with clinical phenotypes

Complex phenotypes are often the result of a response of multiple functionally interacting genes. Combining genome-wide expression traits with clinical information enables the study of gene networks of inter-dependent genes that can be correlated with clinical phenotypes. With weighted correlation network

Table 2. Strong *cis*-eQTLs mapping outside of known EAE QTLs in DA/PVG.1av1 crosses

Chr	LOD[a] *cis*-eQTL	Mean[b] Expression	Rat eQTL[c] Probe set	Genomic[d] Location	Gene symbol	Gene name
1	27.2	117.7	10703445	56730912	Fpr3	Formyl peptide receptor 3
1	12.0	137.6	10703706	63694428	Lilrb3	Leukocyte immunoglobulin-like receptor, subfamily
1	13.2	217.4	10719648	79504886	Zfp61	Zinc finger protein 61
1	10.3	519.0	10706297	93734935	Siglec5	Sialic acid-binding Ig-like lectin 5
1	12.9	348.9	10724073	159055461	**Art2**	t-Cell ecto-ADP-ribosyltransferase 1
1	16.6	78.5	10724164	159590904	Chrna10	Cholinergic receptor, nicotinic, alpha polypeptide
1	13.3	59.8	10711944	198987150	Lrrc27	Leucine rich repeat containing 27
1	13.7	251.6	10728904	213828858	Ms4a7	Membrane-spanning 4-domains, subfamily A, member 7
1	10.1	355.8	10728918	214162552	Ms4a6a	Membrane-spanning 4-domains, subfamily A, member 6
1	18.5	97.9	10714106	215590616	Fam111a	Family with sequence similarity 111, member A (NP_001102633.1)
2	14.6	413.1	10823903	173683154	Gucy1b3	Guanylate cyclase 1, soluble, beta 3
2	28.6	145.2	10816144	175479320	Sfrp2	Secreted frizzled-related protein 2
2	12.0	263.3	10824357	180974111	Msto1	Misato homolog 1
2	12.7	114.7	10824611	182644098	Slc27a3	Long-chain fatty acid transport protein 3
2	10.7	221.3	10819690	244323411	**Mcoln2**	Mucolipin 2
3	10.2	78.6	10844331	11511403	Lcn2	Lipocalin 2
3	12.3	168.9	10847957	90174952	Prrg4	Transmembrane gamma-carboxyglutamic acid protein 4
3	24.9	121.2	10848652	106548815	Sptbn5	Spectrin, beta, non-erythrocytic 5
4	11.4	400.7	10866507	173884317	Art4	Ecto-ADP-ribosyltransferase 4
4	10.0	334.6	10859337	174009860	Pde6h	Phosphodiesterase 6H, cGMP-specific, cone, gamma
5	14.9	136.4	10875425	24893573	RGD1309085	Similar to F23N19.9
5	10.2	114.7	10867761	32352311	Mmp16	Matrix metalloproteinase-16
5	10.9	62.8	10877130	77010784	Ptgr1	Prostaglandin reductase 1
5	10.8	586.7	10869288	77924255	Snx30	Sorting nexin family member 30
6	10.7	1359.5	10892653	142735827	Igh-6	Immunoglobulin heavy chain 6
6	25.7	267.3	10892662	142977680	IgG-2a	Gamma-2a immunoglobulin heavy chain
7	11.6	260.5	10904539	112913724	Ly6k	Lymphocyte antigen 6 complex, locus K
7	16.1	536.7	10904597	113434499	Ly6a	Lymphocyte antigen 6 complex, locus A (predicted)
7	15.0	509.1	10898196	121816012	Mpped1	Metallophosphoesterase domain containing 1
9	16.3	127.8	10929288	79091425	Serpine2	Serine (or cysteine) proteinase inhibitor, clade E
11	10.5	34.5	10751434	66357714	Csta	Cystatin A (stefin A)
11	12.6	76.6	10754876	70653993	LOC684506	Similar to SMP3 mannosyltransferase
12	15.8	109.3	10762254	36896819	Oas1k	2′–5′ oligoadenylate synthetase 1K
13	29.0	158.8	10769672	85533882	Rgs4	Regulator of G-protein signaling 4
13	10.2	301.6	10765469	86096465	Sh2d1b1	Similar to EWS/FLI1 activated transcript 2
14	24.2	135.0	10776325	33403956	**LOC498350**	Similar to testicular haploid expressed gene product isoform 2
14	10.3	45.5	10778820	109197351	Ccdc85a	Coiled-coil domain containing 85A
16	17.5	229.3	10787757	22852487	Csgalnact1	Chondroitin sulfate *N*-acetylgalactosaminyltransferase 1
16	26.5	409.1	10789670	84885522	**Lig4**	DNA ligase 4
18	15.4	140.2	10804396	40914028	**Cdo1**	Cysteine dioxygenase 1, cytosolic
X	14.4	236.7	10936742	21117225	GPR34	G-protein-coupled receptor GPR34 (predicted)
X	16.9	73.6	10937013	26697034	Kcnd1	Potassium voltage-gated channel, Shal-related subfamily, member 1
X	26.8	106.0	10932773	35143324	Chrdl1	Kohjirin
X	11.8	68.2	10939319	122209367	Armcx6	Armadillo repeat containing, X-linked 6
X	12.8	209.6	10939498	127688736	LOC678934	Similar to CG30327-PA

eQTLs were selected to have a logarithm of odds (LOD) score ≥ 10 and a genome-wide corrected *P*-value ≤ 0.05 (generated with 1000 permutations). *Cis*-eQTLs were considered those having no genetic marker between the peak of the linkage score and the chromosomal region coding the transcript. Genes in bold are differentially expressed between DA and PVG in day 7 post-immunization *ex vivo* LN cells and/or in MOG re-stimulated conditions (49).
Abbreviations: QTL, quantitative trait locus; LOD, logarithm of the odds ratio; eQTL, expression quantitative trait locus; Chr, chromosome.
[a]LOD score of *cis*-eQTL for probe sets generated with the Haley–Knott regression model, no covariates, in R/qtl.
[b]Average probe set expression level.
[c]Probe ID annotation from Affymetrix Rat Gene 1.0 ST array.
[d]Genomic location of the transcript, start, in mega base pairs.

analysis (WGCNA) (35), we identified eight gene networks that significantly correlated with EAE phenotypes (including susceptibility and severity phenotypes, weight loss and anti-MOG IgG titers). We performed IPA of transcripts for each separate gene network to identify their functional properties. Significant associations with canonical pathways were discerned for six of the eight identified gene networks (Supplementary Material, Table S6) and four of these are described below.

A gene network enriched for T cell functions shows strong correlation with EAE

The most striking gene network A (Fig. 2, Supplementary Material, Tables S6 and S7) associated with molecular functions including T cell-mediated immune mechanisms (Tables 4 and 5), which have also been implicated in MS (2). This network gave the strongest positive correlation with EAE

Table 3. *Cis*-eQTLs mapping to EAE QTLs in DA/PVG.1av1 crosses

EAE QTL	Chr	EAE[a] QTL Position	LOD[b] *cis*-eQTL	Mean[c] Expression	Rat[d] eQTL Probe Set	Genomic[c] Location	Gene symbol	Gene name
Eae29	1	0–25	20.8	34.5	10701620	5473	RGD1564110_predicted	Similar to putative pheromone receptor (predicted)
Eae29	1		8.5	33.5	10716526	245161	LOC365029	Similar to vomeronasal 2, receptor, 1
Eae29	1		24.8	80.6	10701668	1498164	LOC678740	Similar to vomeronasal 2, receptor, 1
Eae29	1		42.4	34.5	10701671	1503580	LOC687363	Similar to vomeronasal 2, receptor, 1
Eae29	1		24.5	41.4	10716562	1524789	LOC286986	Putative pheromone receptor Go-VN13C
Eae29	1		15.8	64.7	10716568	1524798	LOC286986	Putative pheromone receptor Go-VN13C
Eae29	1		38.9	63.6	10701674	1636457	LOC308240	Hypothetical LOC308240
Eae29	1		20.5	68.0	10701684	1694318	RGD1565235_predicted	Similar to Retinoic acid early inducible protein 1
Eae29	1		14.0	60.7	10716603	2121666	RGD1565235_predicted	Similar to Retinoic acid early inducible protein 1
Eae30	1		7.5	429.4	10711299	187396184	Itgax	Integrin, alpha X
Eae30	1		6.2	388.2	10711664	190987657	**Acadsb**	Short/branched chain specific acyl-CoA dehydrogenase
Eae30	1		5.7	67.0	10726371	193384041	Adam12_predicted	A disintegrin and metallopeptidase domain 12
Eae7	1	238–258	4.8	623.1	10714907	238609169	**Ifit1**	Interferon-induced protein with tetratricopeptide repeats 1
Eae24	4	59–75	11.9	68.2	10854406	61714290	Akr1b8	Aldo-keto reductase family 1, member B8
Eae24	4		7.5	200.2	10861946	65674808	D630045J12Rik	'ENSRNOT00000040391'
Eae24	4		5.6	221.2	10854637	66232348	Clec2l	c-Type lectin domain family 2, member L
Eae24	4		8.5	77.6	10854942	69011941	RGD1560283_predicted	Similar to trypsinogen 8 (predicted)
Eae24	4		13.4	91.4	10862184	69022614	RGD1560283_predicted	Similar to trypsinogen 8 (predicted)
Eae25	4	75–83	3.9	437.9	10855512	78343646	Mpp6	Membrane protein, palmitoylated 6
Eae20-22	4	133–159	6.7	523.6	10857950	150814706	**Atg7**	Autophagy-related 7 (yeast)
Eae20-22	4		21.1	89.4	10865186	156705371	RGD1310710_predicted	Similar to RIKEN cDNA 2700091N06 (predicted)
Eae20-22	4		8.5	50.5	10865321	158901835	RGD1560851_predicted	Similar to mKIAA1238 protein (predicted)
Eae20-22	4		10.9	141.6	10858581	159745231	Clec4b2	c-Type lectin domain family 4, member B2
eEae22	4	162–172	6.9	167.4	10859090	166090800	LOC689800	Similar to osteoclast inhibitory lectin
eEae22	4		9.3	45.9	10859149	166857707	Klre1	Killer cell lectin-like receptor, family E, member
eEae22	4		5.5	86.2	10859162	166857707	Klre1	Killer cell lectin-like receptor, family E, member
eEae22	4		5.3	234.8	10859164	166857707	Klrd1	Killer cell lectin-like receptor, subfamily D, member 1
eEae22	4		5.6	10.6	10859160	166857707	Klre1	Killer cell lectin-like receptor, family E, member
eEae22	4		4.9	804.2	10866041	166914788	Klrk1	Killer cell lectin-like receptor subfamily K, member 1
eEae22	4		13.2	161.6	10866056	166956618	Klrc2	Killer cell lectin-like receptor subfamily C
eEae22	4		17.6	320.3	10866052	166956618	**Klrc3**	Killer cell lectin-like receptor subfamily C, member 3
eEae22	4		4.2	82.9	10866061	166981332	Klrc1	Killer cell lectin-like receptor subfamily C
eEae22	4		7.4	97.0	10866076	167091333	Klri2	Killer cell lectin-like receptor family I member 2
eEae22	4		11.5	560.2	10866101	167325313	RGD1563110_predicted	Similar to immunoreceptor Ly49si3
eEae22	4		4.1	223.9	10866116	167367359	LOC684059	Similar to immunoreceptor Ly49si1
eEae22	4		4.7	229.4	10866120	167449814	Ly49si1	Immunoreceptor Ly49si1
eEae22	4		10.4	402.8	10866123	167483018	Klra22	Killer cell lectin-like receptor subfamily A, member 22
eEae22	4		11.4	820.8	10866142	167500280	LOC100364751	Immunoreceptor Ly49si3-like
eEae22	4		12.1	472.3	10866144	167504502	LOC100364751	Immunoreceptor Ly49si3-like
eEae22	4		10.7	612.3	10866140	167553043	LOC100364751	Immunoreceptor Ly49si3-like
eEae22	4		10.7	25.4	10866163	167975702	Ly49s4	Ly49 stimulatory receptor 4
eEae22	4		19.6	25.5	10866186	168230133	Ly49i3	Immunoreceptor Ly49i3
eEae22	4		4.3	172.4	10866167	168230133	Ly49s4	Ly49 stimulatory receptor 4
eEae22	4		6.5	94.7	10866215	168575551	NP_001009494.1	Ly49 stimulatory receptor 7
eEae22	4		14.8	32.2	10866236	168747110	Klra5	Killer cell lectin-like receptor, subfamily A
eEae22	4		7.2	58.7	10866243	168794389	Ly49i7	Immunoreceptor Ly49i7
eEae22	4		20.6	59.8	10866359	170271493	LOC689869	Similar to Taste receptor type 2 member 140
Eae_rno4	4	175–187	9.1	55.5	10867020	186999579	RGD1560851_predicted	Similar to mKIAA1238 protein (predicted)
Eae3	10	15–45	10.8	423.5	10742431	35933369	Rufy1	RUN and FYVE domain containing 1
Eae18a	10	55–67	4.7	119.1	10734740	55270065	Pik3r6	Phosphoinositide-3-kinase, regulatory subunit 6
Eae18a	10		4.3	75.7	10744254	56692319	Tnk1	Non-receptor tyrosine-protein kinase TNK1
Eae12	10	85–108	8.2	189.0	10738451	90602484	**Tmem106a**	Transmembrane protein 106A
Eae12	10		4.9	5793.3	10774359	104545644	Rpl38	Ribosomal protein L38
Eae12	10		15.9	238.8	10748857	105084291	RGD1561778	Similar to dendritic cell-derived immunoglobulin(Ig)-like receptor 1
Eae17	13	38–55	6.2	138.9	10763751	43752437	Fcamr	Fc receptor, IgA, IgM, high affinity

Continued

Table 3. *Continued*

EAE QTL	Chr	EAE[a] QTL Position	LOD[b] *cis*-eQTL	Mean[c] Expression	Rat[d] eQTL Probe Set	Genomic[e] Location	Gene symbol	Gene name
Eae17	13		4.3	270.9	10767565	44910207	**Mfsd4**	Major facilitator superfamily domain-containing protein 4
Eae10	14	0–20	22.7	236.9	10771171	5807837	LOC683128	Similar to guanylate binding protein family
Eae10	14		7.4	142.3	10771190	5965433	Abcg3l3	ATP-binding cassette, sub-family G, member 3 family member
Eae23b	17	57–66	12.7	69.2	10795634	63355581	4921524L21Rik	
Eae15	18	69–87	6.0	116.7	10805571	81524266	Timm21	Translocase of inner mitochondrial membrane 21 homolog

The first column gives the name of the previously mapped EAE QTL (See Supplementary Material, Table S5 for related publications). The QTLs given in bold displayed linkage in 421 BC rats, encompassing 150 rats used for eQTL mapping. eQTLs were selected to have a logarithm of odds (LOD) score ≥ 3.9 and a genome-wide corrected *P*-value ≤ 0.05 (generated with 1000 permutations). *Cis*-eQTLs were considered those having no genetic marker between the peak of the linkage score and the chromosomal region coding the transcript. Genes in bold are differentially expressed between DA and PVG in day 7 post-immunization *ex vivo* LN cells and/or in MOG re-stimulated conditions (49).

Abbreviations: eQTL, expression quantitative trait locus; QTL, quantitative trait locus; Chr, chromosome; LOD, logarithm of the odds ratio.

[a]Position of EAE QTL in mega base pairs.

[b]LOD score of *cis*-eQTL for probe sets generated with the Haley–Knott regression model, no covariates, in R/qtl.

[c]Average probe set expression level.

[d]Probe ID annotation from Affymetrix Rat Gene 1.0 ST array.

[e]Genomic location of the transcript, start, in mega base pairs.

susceptibility and severity (Pearson correlations: EAE $\rho = 0.23$, $P = 0.004$; MAX $\rho = 0.23$, $P = 0.006$; SUM $\rho = 0.32$, $P = 0.00006$; s35 $\rho = 0.26$, $P = 0.001$; ONS $\rho = -0.21$, $P = 0.01$; DUR $\rho = 0.31$, $P = 0.0001$; WL $\rho = 0.32$, $P = 0.00007$). Transcripts in gene network A (Supplementary Material, Table S7) included multiple genes associated with autoimmune diseases (*Cd6, Ets1, Tcf7* and *Themis*) (2, 36–38). The *Cd6* MS susceptibility allele is associated with alterations in T cell proliferation (39) and the transcription factor *Ets1* participates in important aspects of early thymocyte development (40, 41). Several genes, including *Lef1, Lck*, *Crtam* and *Itk*, have previously been linked to functions of the adaptive immune system. In the network, the *cis*-regulated major facilitator superfamily (MFS) domain containing protein 4 (*Mfsd4*), overlapping an EAE-regulating QTL on RNO13 that was initially identified in a (LEW × PVG) F2 intercross (42) had a strong (-0.05) albeit peripheral membership. *Lef1*, co-regulated by two loci on RNO1 and RNO18, was the strongest member of this gene network (-0.21). Several members of the network are regulated from EAE QTLs, *Art2* (*Eae31*), *Crtam* (*Eae17* and *Eae23a*) and *Resp18* (*Eae23a*), showing a genetic regulation of the network by loci that predispose for EAE.

Gene network associated with G protein coupled receptor signaling

Gene network B (Fig. 3, Supplementary Material, Tables S6 and S8) correlated negatively with severity phenotypes (MAX $\rho = -0.19$, $P = 0.02$; SUM $\rho = -0.24$, $P = 0.003$; s35 $\rho = -0.19$, $P = 0.02$; DUR $\rho = -0.16$, $P = 0.05$; WL $\rho = -0.27$, $P = 0.0005$). Canonical pathways associated to the gene network included LPS/IL-1-mediated inhibition of RXR function and G protein coupled receptor signaling (Supplementary Material, Table S6). Several transcripts in the network are family members of genes associated with MS (Supplementary Material, Table S8). These include C-type lectins, G protein coupled receptors, mitogen-activated protein kinases, transmembrane proteins and solute carriers (SLCs) which are proteins involved in both inflammation and immunity, as well as in signal transduction that leads to cellular responses. *Jundp2* and *Nr1d1* are in regions associated with Crohn's disease (34) and are both involved in transcriptional regulation (43, 44). The gene network is partially genetically regulated by *Eae17*, *Eae23a*, *Eae29* and *Eae30*.

Genetic regulation of gene network associated with IFN signaling pathways

Gene network C (Fig. 4, Supplementary Material, Tables S6 and S9) correlated with duration of EAE ($\rho = 0.18$, $P = 0.02$) and indicated a genetic regulation by loci that predispose for EAE (*Eae3*, *Eae7*, *Eae18a, Eae18b* and *Eae20-22*). IPA revealed a high significance for IFN signaling pathways (Supplementary Material, Table S6) that are important in neuroinflammation. Network C also included genes and family members of genes associated with MS or other autoimmune diseases; zinc fingers, STAT transcription factors (*Stat2* and *Stat4)* (45), genes involved in the innate immune response (*Irgm*) (46) and interferon regulatory factors (*Irf1, Irf7* and *Irf9)* (45, 47). IFN-induced downstream signaling molecules, several of which associated with MS (48), are evident in the network such as proteins induced by interferons (*Oas*), interferon-induced guanylate-binding proteins (*Gbp*) and a number of interferon-induced proteins.

Furthermore, network D (Supplementary Material, Table S6) correlated with serum anti-MOG total IgG levels day 35 p.i. ($\rho = 0.24$, $P = 0.003$) and was functionally enriched for the regulation of p70S6K, a serine/threonine kinase in the PI3 kinase pathway.

In conclusion, we identified several gene networks involved in specific immune-related pathways that are presumably of importance or crucial for both EAE and MS pathogenesis and

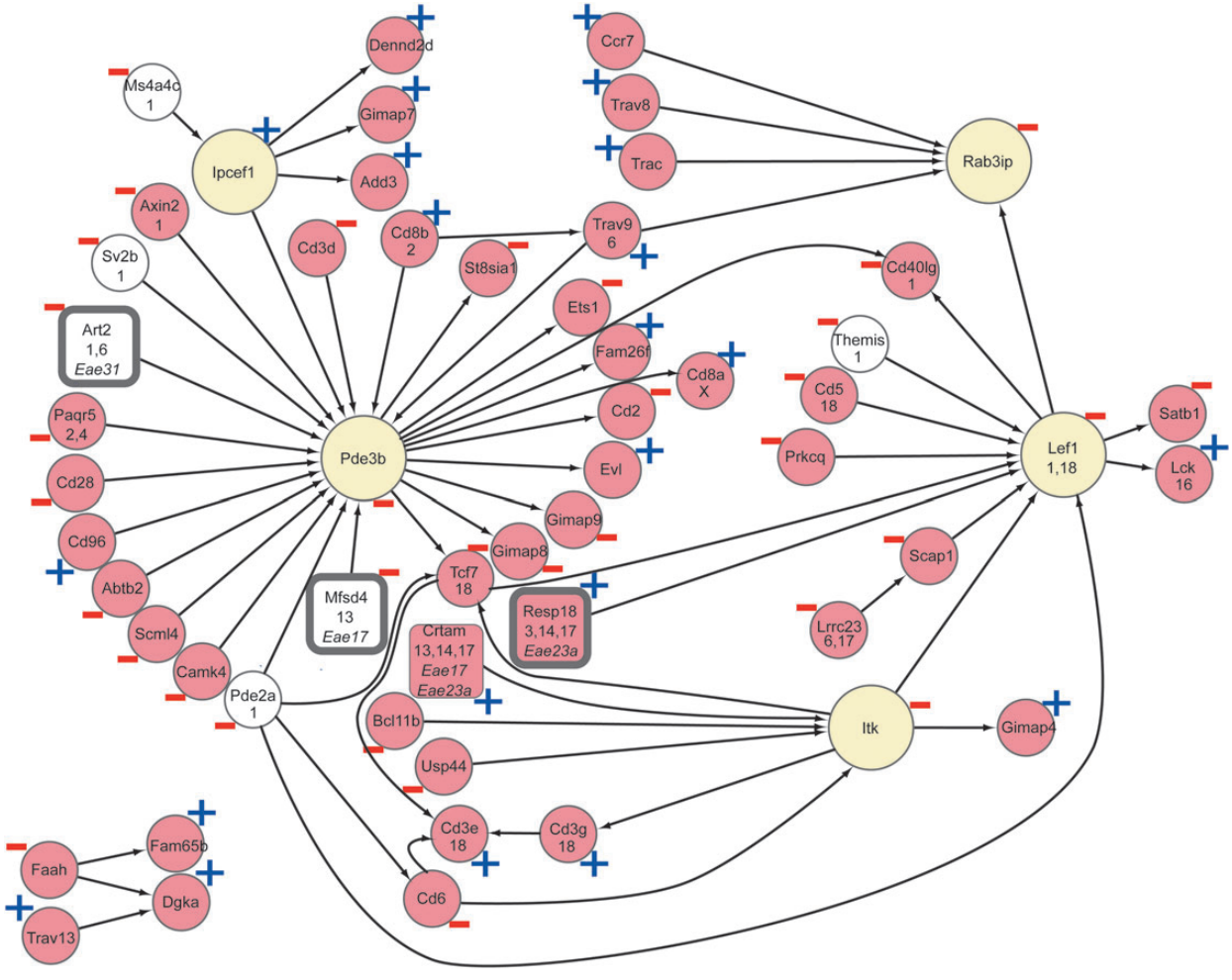

Figure 2. Gene network enriched for T cell functions shows strong correlation with EAE. Expression traits with neighboring locations are drawn next to each other. eQTLs are indicated by their rat chromosome location on a second line and *cis*-eQTLs are indicated in white. eQTLs within EAE QTLs are indicated by a grey box with the QTL name listed in parentheses. Predicted hub genes are presented in yellow and the black arrow heads indicate a directed interaction. Plus and minus indicate association to EAE phenotype. Genes with a higher disease association are placed to the right, whereas causative genes are placed toward the left.

which indicate partial genetic regulation by loci that predispose for EAE. Additionally, the co-expression analyses provided novel insights in the cause–effect interactions of several genes that have not previously been linked to immune related pathways. Furthermore, the approach of integrating clinical data in the analyses distinguished molecular responses important at different stages of disease. For instance, we defined networks of interacting genes that correlated with duration and IgG levels, respectively. Any genetic difference in individuals that could modulate the defined molecular pathways could potentially influence susceptibility to EAE and MS.

DISCUSSION

The eQTL approach can facilitate identification of candidate genes (6,14,15) and in the current study our whole-genome approach identified genetic differences between two inbred rat strains that contribute to the expression of individual genes. We detected a number of *cis*-eQTLs that overlap established EAE QTLs that warrant further biological characterization.

The co-localization of a *cis*-eQTL and an EAE QTL makes it an attractive novel positional candidate for the causal relationship between the disease phenotype and the regulation of the transcript. We identified several *cis*-regulated positional candidate genes in EAE QTLs including *Ifit1 (Eae7)*, *Atg7 (Eae20-22), Klrc3 (eEae22)* and *Mfsd4* (*Eae17*). We have previously described these genes as being differentially expressed between DA and PVG in *ex vivo* LN cells and/or in MOG re-stimulated lymphocytes at day 7 p.i. of EAE (49). *Ifit1* is induced in response to stimuli such as LPS, IL-1 and TNF (50,51) and has been described among genes that could best predict the response to IFN-β treatment in MS patients (52). *Atg7* is involved in macroautophagy, an intracellular pathway that regulates quantity and quality of organelles and proteins. Autophagy has recently been linked to both innate and adaptive immunity (53). *Klrc3*, encodes an activating NK cell receptor, and NK cells are suggested to confer a disease-protective role in MS and EAE (54–56). *Mfsd4* is a transmembrane transporter of the MFS. MFS proteins are the largest group of secondary carriers in the cell and transport small solutes by using chemiosmotic ion gradients (57,58). The availability of whole-genome

Table 4. Top canonical pathways associated with *Network A*

Ingenuity top canonical pathway	Ratio[a]	Genes[b]	*P*-value[c]	Corrected *P*-value[d]
T Cell receptor signaling	11/109	CD247,CD3G,CD28,LCK,CAMK4,PRKCQ,CD3E,CD8A,CD3D,CD8B,ITK	2.1E−15	3.1E−13
iCOS−iCOSL signaling in T helper cells	10/122	CD247,CD3G,CD28,LCK,CD40LG,CAMK4,PRKCQ,CD3E,CD3D,ITK	1.3E−13	9.6E−12
CD28 signaling in T helper cells	9/132	CD247,CD3G,CD28,LCK,CAMK4,PRKCQ,CD3E,CD3D,ITK	1.6E−11	7.9E−10
Calcium-induced T lymphocyte apoptosis	7/70	CD247,CD3G,LCK,CAMK4,PRKCQ,CD3E,CD3D	1.3E−10	4.8E−09
Role of NFAT in regulation of the immune response	9/200	CD247,CD3G,CD28,LCK,CAMK4,PRKCQ,CD3E,CD3D,ITK	4.8E−10	1.4E−08

Transcripts in Network A were uploaded into the ingenuity pathways application (Ingenuity Systems, www.ingenuity.com). The software was used to identify the most significant pathways.
[a]Ratio refers to the number of molecules in a given pathway that meet cutoff criteria, divided by the total number of genes that map to the canonical pathway.
[b]Genes in a given pathway that meet cutoff criteria.
[c]Significance was determined from a single test *P*-value calculated using the right-tailed Fisher's exact test.
[d]Adjusted significance was determined from multiple test-corrected *P*-values using the Benjamini–Hochberg correction (top five categories reaching a corrected statistical significance of ≤ 0.05 are shown).

Table 5. Molecular functions associated with *Network A*

Cellular and molecular functions	Molecules[a]	Representative subgroup	Molecules[b]	*P*-value[c]	*P*-value subgroup[d]
Cellular function and maintenance	25	Lymphocyte homeostasis	23	1.3E−23–1.2E−02	1.3E−23
Cellular development	27	T cell development	22	3.0E−23–1.2E−02	5.9E−23
Cell-to-cell signaling and interaction	23	Activation of T lymphocytes	15	3.6E−16–1.3E−02	3.6E−16
Cellular growth and proliferation	26	Proliferation of lymphocytes	18	3.8E−15–1.2E−02	9.4E−15
Cellular movement	18	Lymphocyte migration	13	5.0E−13–1.2E−02	5.0E−13

Transcripts in Network A were uploaded into the ingenuity pathways application (Ingenuity Systems, www.ingenuity.com). The software was used to identify the most significant molecular functions.
[a]Number of functional analysis molecules related to the pathway.
[b]Number of functional analysis molecules related to the representative pathway subgroup.
[c]Range of significance for pathway subgroups. Significance was determined from a single test *P*-value calculated using the right-tailed Fisher's exact test.
[d]Significance for representative pathway subgroup.

expression profiles provides the opportunity to explore the role of genes with as yet unknown molecular function. This can be achieved by investigating interacting molecules or pathways the gene of interest correlates with. Our analysis identified several candidate genes that can serve to generate novel hypotheses and study of the function of these proteins in EAE and MS pathogenesis.

We additionally identified highly significant *cis*-regulated eQTLs that do not overlap with known EAE QTLs but could still denote functions important for autoimmunity. Particularly interesting are family members of genes that associate with MS including *Rgs4*, *Cyp2r1*, *Tmem184a*, *Zfp61* and *Slc27a3*. *Rgs4* is a member of the protein family of negative regulators of G-protein signaling (RGS) which deactivates G protein coupled receptor signaling (59,60). This signaling was associated with gene network B that correlated negatively to EAE severity phenotypes. The RGS protein family is highly regulated at the transcriptional level and up-regulation of certain RGS proteins decreases immune cell migration and reduced chemokine-dependent calcium flux. Interestingly, these are emerging as potentially important drug targets. Our functional analysis associated *Rgs4* with cell proliferation, cell migration, integrin and endocytosis signaling. Another family member of RGS, *Rgs1,* is associated with MS and is suggested to have a role in immune cell regulation. *Cyp2r1* associated with B cell functions and signaling and *Tmem184a* with migration of cells (Supplementary Material, Table S3 and S4). *Zfp61* is associated with the organization of the ER and autophagy of cells. The SLC superfamily of transporters is responsible for the uptake of amino acids, peptides, ions, hormones and drugs (61) and SLC mRNA levels are dysregulated in inflammatory bowel disease patients (62). Further studies on the functions and pathways affected by MS family member genes identified in this study could add to the understanding of the genetic contribution of susceptibility and/or pathogenesis of autoimmune diseases.

Several *cis*-eQTLs are genes with a recognized role in the immune system. *Ccr6* has previously been demonstrated to play an important role in the mechanism of autoreactive lymphocyte priming and migration to the efferent lymphatics (63), implicated in the migration of lymphocytes to the CNS (64) and has been associated with Crohn's disease and rheumatoid arthritis (47,65). *Tgfb2* is a potent immunosuppressive cytokine that ameliorates EAE and has been associated with remissions in MS. However, cases of nephrotoxicity in clinical trials (66) ruled it out as a treatment alternative for MS patients. *Ifitm6* is part of a family of genes that are induced by IFNs (67) and encode cell surface proteins that modulate cell–cell adhesion and cell differentiation.

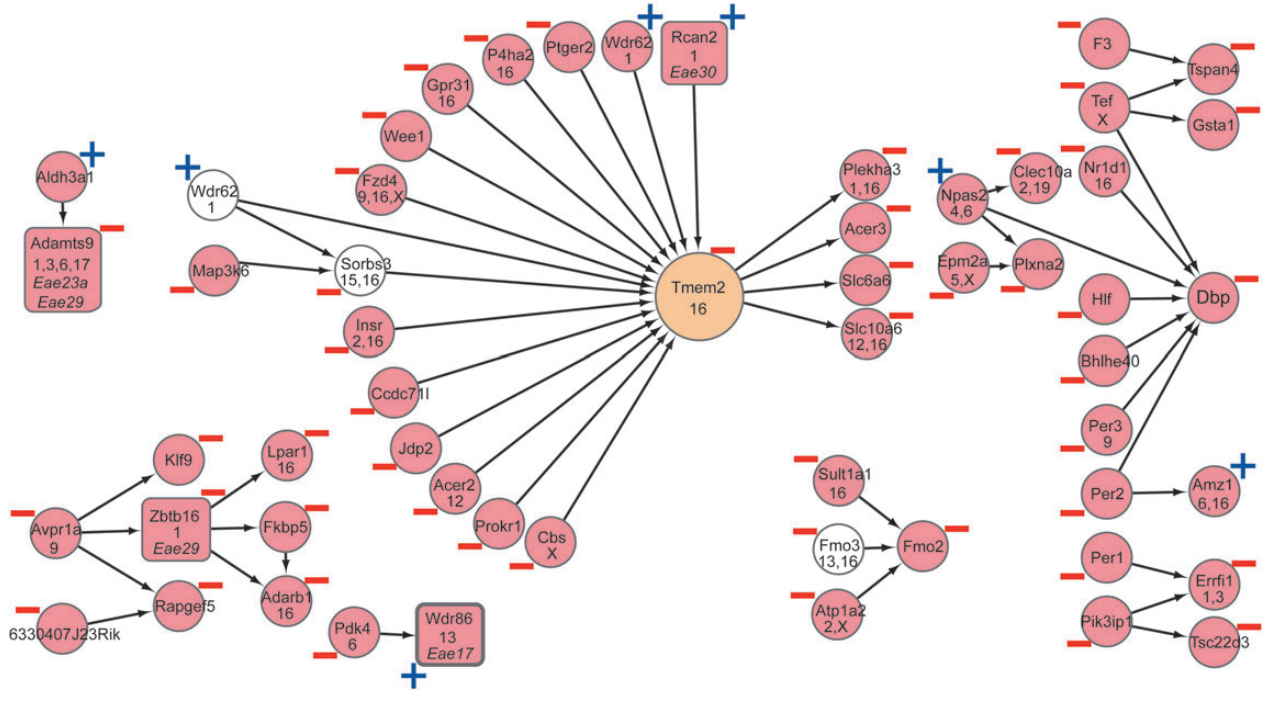

Figure 3. Gene network associated with GPCR signaling correlates negatively with EAE. Expression traits with neighboring locations are drawn next to each other. eQTLs are indicated by their rat chromosome location on a second line and *cis*-eQTLs are indicated in white. eQTLs within EAE QTLs are indicated by a grey box with the QTL name listed in parentheses. Predicted hub genes are presented in orange and the black arrow heads indicate a directed interaction. Plus and minus indicate association to EAE phenotype. Genes with a higher disease association are placed to the right, whereas causative genes are placed toward the left.

Genes can exert a control over the expression of another gene or a set of genes, which are referred to as *trans*-eQTLs. In our study, the sensitivity to detect *trans*-eQTLs has greatly been increased by the use of a BC population originating from two inbred strains and Affymetrix Gene arrays that provide comprehensive genome coverage with multiple probes per each transcript. More than 70% of eQTLs displayed regulation in *trans* at a genome-wide corrected *P*-value of ≤ 0.05, which conforms to previous studies in inbred rodent strains (14, 68). The *trans*-band on RNO17 overlaps *Eae23a*, which we previously demonstrated to control EAE severity in a congenic strain (26). Although this *trans*-band was not significantly enriched for functional pathways, we observed regulation of many small RNA genes such as nucleolar and spliceosomal RNAs, previously implicated in epigenetic regulation (31) and alternative splicing (32), respectively. *Trans*-regulation can denote a genomic location of a master regulator of transcription (16). A transcription factor, *Zeb1* (69), known to regulate the IL-2 pathway (70, 71) that is crucial in MS and EAE, is encoded in *Eae23a*. In addition, we have previously demonstrated that the balance between splice variants of *Zeb1* driven by *Eae23a* could influence the regulation of EAE (26).

With the recorded clinical and molecular parameters during the full EAE experiment (susceptibility, severity and molecular phenotypes), we defined clusters of gene networks that had a direct relation to clinical characteristics of disease. Thus, although our study in chronic stage of EAE does not necessarily address early molecular events that control susceptibility, a possibility to correlate gene expression with clinical phenotypes provides a major benefit in defining genes and pathways that are related to the disease (and not just a consequence of genetically driven expression differences between the strains). Moreover, defining pathways that regulate chronic stage of disease might have better translational prospects considering that all interventions in MS occur long after the onset of disease. The identified genetically driven pathways that regulate susceptibility to autoimmune disease primarily involved T cell activation pathways, G protein coupled receptor and IFN signaling. The most positively correlated network to EAE susceptibility and severity, gene network A (Fig. 2), associated with molecular functions including T cell-mediated immune mechanisms. EAE and MS are described as being T cell-mediated since adoptive transfer of T cells can induce disease, T cell infiltrates are evident in EAE and MS lesions (72–75) and that the major genetic determinant of both MS and EAE is the *HLA/MHC*. Thus our unbiased way of defining functions central in EAE conform to previous knowledge about central mechanisms in autoimmune inflammation, indicating that these mechanisms are partially genetically regulated, as implicated in the MS GWAS.

Themis, Lef1, Lck, *Satb1*, *Crtam* and *Itk* were identified in network A, which strongly correlated with EAE susceptibility and severity and associated with molecular functions including T cell-mediated immune mechanisms. *Cis*-regulated *Themis* act through TCR signaling and has recently been reported to control Treg functions and susceptibility to intestinal inflammation (76). In network A, *Themis* was determined to directionally interact with the lymphoid enhancer-binding factor 1 (*Lef1*) that binds to a functionally important site in the TCR-α enhancer. In turn, *Lef1* was in our co-expression analysis predicted to interact with *Lck*, known to play a key role in the TCR signal transduction pathway. *Lef1* also interacted with *Satb1*, a genome organizer

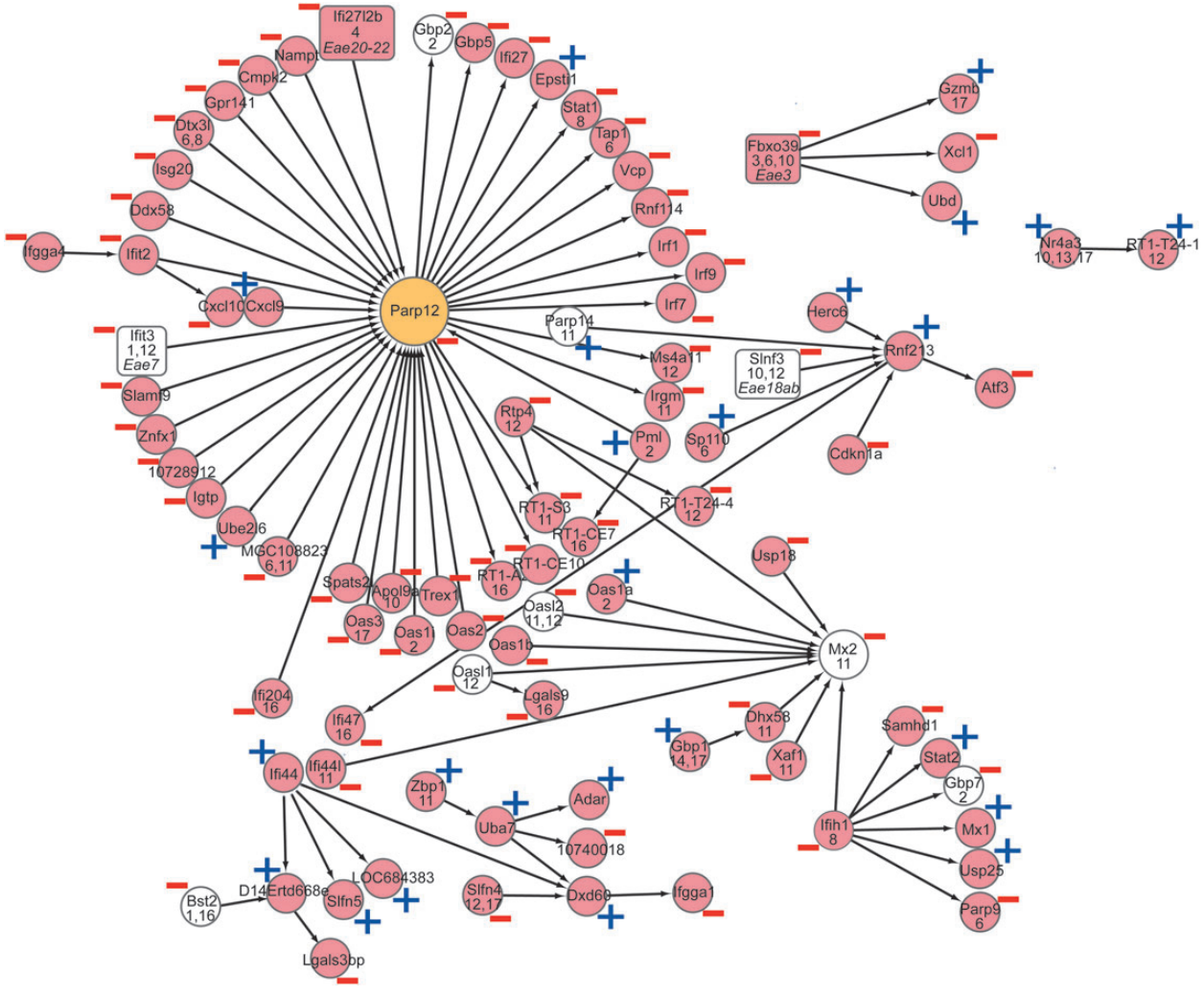

Figure 4. Gene network associated with IFN pathway signaling. Expression traits with neighboring locations are drawn next to each other. eQTLs are indicated by their rat chromosome location on a second line and *cis*-eQTLs are indicated in white. eQTLs within EAE QTLs are indicated by a grey box with the QTL name listed in parentheses. Predicted hub genes are presented in orange and the black arrow heads indicate a directed interaction. Plus and minus indicate association to EAE phenotype. Genes with a higher disease association are placed to the right, whereas causative genes are placed toward the left.

that regulates chromatin structure and gene expression, known to be crucial for the phenotype and function of Tregs (77).

The T-cell-specific HMG box transcription factor family member *Tcf7*, also part of network A, is a transcriptional activator for genes involved in immune regulation (78). The encoded protein can bind an enhancer element and activate the CD3E gene that is part of the T cell receptor–CD3 complex. We also found a directed interaction between cytotoxic and a regulatory T cell molecule (*Crtam*), found to influence the adaptive immune response (79), and the IL-2-inducible T cell kinase (*Itk*), which is thought to play a role in T cell proliferation and differentiation (80). *Lef1* and the hub gene phosphodiesterase 3B (*Pde3b)* interacted with *Cd40l* expressed on activated T cells. *Cd40l* is a costimulatory molecule and induces activation in APC by binding to *Cd40*.

Utilizing information about clinical EAE development (disease status, severity and onset) enabled predictions regarding the direction of interactions demonstrating a causal relationship associated with the development of a clinical phenotype. Additionally, our analyses can serve to reveal novel players that influence specific molecular pathways. For instance, in the gene network associated with T cell functions, the co-expression analysis predicted that *Mfsd4* would interact, in a directed causal–effect relationship *Pde3b. Pde3b* is an enzyme that hydrolyzes cAMP for cell metabolism and has been shown to be regulated downstream of Foxp3, by direct binding, to support Treg homeostasis and lineage stability (81,82).

We identified a gene network highly associated with IFN signaling pathways which included STAT transcription factors, interferon regulatory factors and IFN-induced downstream signaling molecules. A first-line therapy for MS is IFN-β, a type I IFN, although the exact molecular mechanism of action of the drug is still poorly understood. *Mx1* and *Mx2*, part of the network, can be used as biomarkers for monitoring response to interferon therapy in patients (83). *Mx2* expression is regulated in *cis* on RNO11, and in IPA we found *Mx2* to associate with IFN signaling and activation of IRF by cytosolic pattern recognition receptors, attraction of NK cells, homing of T lymphocytes

and cell death of phagocytes (Supplementary Material, Tables S3 and S4). In gene network C, we also recorded the nuclear body proteins (*Pml* and *Sp110*) that regulate genes involved in the immune response (84). *Pml* acts as a transcriptional co-activator with p53 (85). p53 has previously been reported to interact with *Wwox* (86), a *cis*-eQTL on RNO19. WW domain-containing proteins are involved in protein degradation, transcription and RNA splicing. The network associated with IFN signaling included several T cell chemoattractants (*Xcl1*, *Cxcl9* and *Cxcl10*). *Xcl1* is a cytokine that attracts T cells and is expressed in activated thymic and peripheral blood $CD8^+$ T cells (87, 88). *Cxcl9* and *Cxcl10* are induced by IFN-γ and execute their chemotactic functions by interacting with the chemokine receptor *Cxcr3*.

In conclusion, our well powered and unique study of the non-MHC influences on gene expression generates hypothesis of molecular pathways that are genetically regulated and predispose for EAE. We detected *cis*-regulated transcripts, both in EAE QTLs and outside, which may play important roles in regulating key mechanisms in both EAE and human disease owing to a large overlap of genes and functions. These can serve to generate novel hypotheses useful in further dissecting pathogenic molecular mechanisms that are dysregulated during chronic autoimmune inflammation. In addition, the gene network analyses denoted genes linked to functional pathways and described their importance in regulating clinical traits of disease. We demonstrate how MS risk genes translate well to EAE and efforts should now be put into combining clinical and experimental research to evaluate candidate genes and to functionally study their mechanisms of action. Ultimately, these integrated findings can provide insight to possible diagnostic and prognostic biomarkers or potential therapeutic interventions for autoimmunity.

MATERIALS AND METHODS

Experimental subjects

DA rats were originally obtained from the Zentralinstitut für Versuchstierzucht (Hannover, Germany) and MHC-identical PVG.1av1 from Harlan UK Limited (Blackthorn, UK). Colonies have thereafter been established at Karolinska Institutet (DA/Kini and PVG.1av1/Kini). A (DA × PVG.1av1) × DA BC population was established. In short, to create the F1 generation, four breeding pairs with DA female founders were established. The N2 generation was bred from eight breeding pairs, with DA females or males crossed to F1 males and females, respectively. Four N2 litters, 421 BC rats (213 females and 208 males), were subjected to MOG-EAE in four separate experiments, referred to as experimental sets. Out of 421 immunized BC rats, splenic RNA was extracted from 347 rats (182 females and 165 males) that survived until day 35 p.i. All 421 BC rats were used to map EAE pQTLs, which are indicated in Supplementary Material, Table S5 and the details of mapping will be reported in a separate study (Stridh *et al*., unpublished data). To minimize variability introduced by experimental sets and increase mapping power, we decided to perform genome-wide expression analysis in one sex; male rats were selected due to less variability between experimental sets. Out of 165 males, 150 selected rats represent similar susceptibility and severity, and all breeding pairs and experimental sets as the full set of 165 rats. All animals were bred and housed at the Karolinska University Hospital (Stockholm, Sweden) in 12 h light/dark- and temperature-regulated rooms in polystyrene cages containing aspen wood shavings where they had free access to standard rodent chow and water. Rats were tested according to a health-monitoring program at the National Veterinary Institute (Statens Veterinärmedicinska Anstalt, SVA) in Uppsala, Sweden.

Induction and clinical evaluation of EAE

Recombinant MOG, amino acids 1–125 from the N-terminus, was expressed in *Escherichia coli* and purified to homogeneity by chelate chromatography as previously described (89). 421 BC rats, between 10–14 weeks of age, were anesthetized with isofluorane (Forene, Abbott Laboratories, Abbot Park, IL, USA) and immunized with a single subcutaneous injection at the dorsal tail base with 200 μl of inoculum containing rMOG (12.5–15 μg/rat in females and 25–30 μg/rat in males titrated in order to achieve similar disease severity/induction) in saline emulsified in a 1:1 ratio with incomplete Freund's adjuvant (Sigma Aldrich, St Louis, MO, USA). Rats were monitored daily for weight and clinical signs from day 8 until 35 p.i. The clinical score was graded as follows: 0, no clinical signs of EAE; 1, tail weakness or tail paralysis; 2, hind leg paraparesis or hemiparesis; 3, hind leg paralysis or hemiparalysis; 4, tetraplegia or moribund; 5, death. Clinical parameters were assessed and used in the analysis: EAE, incidence of EAE; ONS, onset of EAE (day p.i. of first clinical sign); DUR, duration of EAE (number of days subjects showed clinical signs of EAE); MAX, maximum EAE score during the experiment; SUM, sum of all scores during the experiment; s35, EAE score on day 35 post immunization (p.i.) (last day of experiment when spleen tissue was collected for expression analysis); WL, weight loss (calculating the percentage of weight loss between lowest weight throughout the experiment and day 8 p.i.). At day 35 p.i., 347 rats were sacrificed and spleens were collected, snap-frozen and stored in −70°C until use. Spleens from the remaining 74 animals were not collected due to decease prior to the end of experiment. In the BC experiment, 60.4% of all rats were affected with EAE (average maximum clinical score = 2.46). Of individuals still included in the experiment at day 35 p.i., 61.8% were still affected at the end of experiment (average clinical score at day 35 p.i. score = 2.05). Anti-MOG antibodies were measured at the onset (day 12 p.i.) and chronic phase of EAE (day 35 p.i.) as previously described (90).

DNA isolation and genotyping

Genomic DNA was prepared from tail tips of the BC population as described previously. Information about polymorphic microsatellite markers for the BC (118 markers evenly spaced throughout the genome with an average inter-marker distance of 20 cM) was retrieved from Ensembl Genome Database (http://www.ensembl.org v.50-62) (91). DNA amplification was performed with PCR using forward primers end-labeled with a fluorescent dye (VIC, NED, FAM or PET) with products run on ABI 3730 capillary sequencer and analyzed with GeneMapper v3.7 (Applied Biosystems, Foster City, CA, USA). Primers were obtained from Eurofins MWG Operon (Ebersberg, Germany), Applied Biosystems or Proligo (Paris, France). All

genotypes were evaluated manually and quality assessed by two independent observers.

Expression analysis

One hundred milligrams of splenic tissue from each of 150 BC rats was disrupted using Lysing Matrix D tubes (MP Biomedicals, Irvine, CA, USA) in a FastPrep homogenizer (MP Biomedicals). mRNA was extracted using an RNeasy mini kit according to the manufacturers protocol (Qiagen, Hilden, Germany), including on-column DNA digestion. RNA concentration and purity was determined through measurement of A260/A280 ratios with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For 150 BC male rats selected to represent all breeding pairs and sets, whole-genome expression profiles were determined with Affymetrix Rat Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA). Confirmation of RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Target labeling, array hybridization and washing and staining were performed as described in the GeneChip Whole Transcript (WT) Sense Target Labeling manual (http://www.affymetrix.com). Arrays were scanned in the GeneChip Scanner 3000 7G (Affymetrix). Affymetrix cell intensity (CEL) files from scanning were analyzed with Affymetrix Expression Console (EC, version 1.1). The following settings were used: Summarization: probe logarithmic intensity error (PLIER) described in the Guide to probe logarithmic intensity error (PLIER) estimation (http://www.affymetrix.com); Background correction: PM-GCBG and Normalization: Global Median. The microarray data are available in MIAME-compliant (minimal information about a microarray experiments) format at the ArrayExpress Database (http://www.ebi.ac.uk/arrayexpress) (92) under accession code E-MTAB-784.

Linkage and statistical analysis

For the BC, genome-wide linkage analysis was performed for 27342 expression traits with a set of 118 genetic markers using the Haley–Knott regression model in R/qtl version 1.14 (93), to generate maximum base 10 logarithm of the likelihood ratio of odds (LOD) scores. Logarithmic values of all molecular quantitative traits (transcript expression) were used as an input for R/qtl and analyses were performed independently for several clinical phenotypes to act as covariates as well as single QTL scans for all covariates and pairs of covariates. Genome-wide significance (P-value ≤ 0.05) was generated with 1000 permutation tests (94). Physical locations of probe sets were obtained from Ensembl version 55 or from Affymetrix (http://www.affymetrix.com) and the transformation of cM to Mb was determined with R/qtl. The physical positions (Mb) were retrieved from Ensembl, either directly or by sequence similarity searches with the oligo sequences. For arbitrary cM, the density of the markers was considered sufficient (95) to allow a linear interpolation between the physical positions of flanking markers. Computations and their interpretation were orchestrated with the interactive QTL system (TIQS. http://tiqs.it), where data are available. eQTLs were defined as *cis* when there was no genetic marker between the peak of the linkage score and the chromosomal region coding the transcript. Other eQTLs were considered being regulated in *trans*. Genomic locations were obtained from genomic assembly Rnor3.4. For pathway analysis in IPA, significance was determined with the right-tailed Fisher's exact test and adjusted significance using the Benjamini–Hochberg correction. Venn diagrams are based on the gplots R package version 2.11.0.1 (http://CRAN.R-project.org/package=gplots).

Due to the polygenic nature of EAE, we used a multiple-QTL model, i.e. forward selection followed by backward elimination in R/qtl version 1.14 (93), to identify EAE QTLs in 421 BC rats, encompassing 150 rats used for eQTL analysis (Supplementary Material, Table S5). Similar results were obtained using Hailey–Knott regression (data not shown). EAE QTL analysis was also performed in 150 rats used for eQTL analysis using the Haley–Knott regression method in R/qtl version 2.12 (93). Nominal P-values were used to report EAE QTLs in 150 rats due to prior evidence from 421 BC rats and additional independent studies (Supplementary Material, Table S5).

Functional association analysis and network analysis

Molecular functions and canonical pathways were evaluated with the ingenuity pathways application (Ingenuity Systems, www.ingenuity.com). The functional and canonical pathways (disease-specific pathways were not included) analysis identified the molecular functions and pathways, respectively, from the Ingenuity Knowledge Base that was most significant to the uploaded data set. For analysis of transcripts correlated to *cis*-eQTLs (Pearson's correlation coefficient $r > 0.40$ or $r < -0.40$), the experimental set 1 was excluded due to significant deviation from other sets (the induction dose of 30 μg/rat was used for set 1 males, whereas other sets were induced with 25 μg/rat).

Gene networks were provided by TIQS, created on the basis of WGCNA (35). The Pearson correlation coefficient was used to determine the association of a gene network with clinical EAE phenotypes. Gene network analysis in combination with the clinical parameters measured for each individual in the EAE experiment was used to predict a causal relationship between genes. Directions indicate the gene that is observable to differ in its expression level from healthy individuals after longer disease duration. Directed associations were determined with the use of clinical parameters for all predicted gene interactions and hub genes in the eQTL data as inferred from an association of the gene expression levels with the onset of the disease phenotypes (Gupta *et al.*, unpublished data). The arrows point to the gene that changes its expression at a later stage of the disease. To determine the direction, the expression levels were compared with the onset and the severity. From the onset, the duration of the disease is derived. Every gene is assigned an average score across all individuals to reflect if the expression levels are changing with the duration of the disease, with the strength of the phenotype contributing to the scoring. The directions follow the gradient of that score.

Ethics statement

All experiments were approved and performed in accordance with the guidelines from the Swedish National Board for Laboratory Animals and the European Community Council Directive

(86/609/EEC) under the ethical permits N284/07 (N332/06) that were approved by the North Stockholm Animal Ethics Committee (Stockholms Norra djurförsöksetiska nämnd).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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