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Genetics of EAE Supports the Role of T Helper Cells in MS Pathogenesis Running head: Critical genetic role for T cells in MS and EAE

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Abstract

Objective—The major histocompatibility complex (MHC) is the primary genetic contributor to multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE), but multiple additional interacting loci are required for genetic susceptibility. The identity of most of these non-MHC genes is unknown. In this report, we identify genes within evolutionarily conserved genetic pathways leading to multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE).

Methods—To identify non-MHC binary and quantitative trait loci (BTL/QTL) important in the pathogenesis of EAE, we generated phenotype-selected congenic mice using EAE-resistant B10.S and EAE-susceptible SJL mice. We hypothesized that genes linked to EAE BTL/QTL and MS-GWAS can be identified if they belong to common evolutionarily conserved pathways, which can be identified with a bioinformatic approach using Ingenuity software.

Results—Many known BTL/QTL were retained and linked to susceptibility during phenotype selection, the most significant being a region on chromosome 17 distal to *H2* (*Eae5*). We show in pathway analysis that T helper (T_H)-cell differentiation genes are critical for both diseases. Bioinformatic analyses predicted that *Eae5* is important in CD4 T-effector and/or Foxp3⁺ T-regulatory cells (Tregs), and we found that B10.S-*Eae5*^{SJL} congenic mice have significantly greater numbers of lymph node CD4 and Tregs than B10.S mice.

Interpretation—These results support the polygenic model of MS/EAE, whereby MHC and multiple minor loci are required for full susceptibility, and confirm a critical genetic dependence on CD4 T_H-cell differentiation and function in the pathogenesis of both diseases.

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) with axonal damage leading to progressive paralysis in affected individuals.¹ The heritability of MS is estimated to be ~35%, largely associated with the inheritance of susceptible *HLA* haplotypes that can be present in up to 70% of these MS cases.² However, polymorphisms in many other genes have also been associated with MS in genome-wide association studies (GWAS). In GWAS analyses, the contribution of each susceptibility gene is small, and thus both the importance of different genes in pathogenesis and the mechanisms by which they act are difficult to test individually.

A suitable inbred rodent model is useful for discerning the effect of each of these human disease-modifying genes. Experimental allergic encephalomyelitis (EAE), the autoimmune model for MS, results in a progressive paralysis due to the activation of myelin-specific CD4⁺ T cells that initiate disease pathogenesis.^{3, 4} Like MS, EAE is genetically complex, and susceptibility in *H2^S*-matched progeny of EAE-resistant B10.S and EAE-susceptible SJL mice is modified by at least 30 different binary and quantitative trait loci (BTL/QTL).⁵⁻¹²

Identifying the gene underlying each BTL or QTL can be accomplished by positional cloning using traditional isolation of the QTL interval in congenic mice, facilitated by marker-assisted selection (MAS).¹³ However, complications arise for congenic production based solely on MAS because EAE is highly influenced by intrinsic genetic and environmental factors such as age, sex, and season of induction.^{11, 14} We attempted to circumvent these issues by creating *phenotype*-selected congenic mice, in which the trait is stable by definition. Such analyses have been used in the identification of genetic elements underlying simple and complex traits such as autoimmune and infectious diseases.¹⁵

In the present study, we selected congenic lines based on their EAE susceptibility as the first step toward the identification of positional candidate genes. Our results demonstrate, however, that MAS of advanced generations of originally phenotype-selected congenic lines did not identify regions amenable to positional candidate gene cloning. This finding is likely due to the polygenic nature of the disease and the dilution of multiple epistatic loci in advanced generations.

We nevertheless were able to use results from congenic production to identify the combinations of genes linked to EAE and MS that belong to common evolutionary conserved pathways. We undertook a pathway analysis to find non-MHC genes that underlie full expressivity of EAE. Our results underscore the importance of genetic epistasis in EAE and MS and identify potentially interacting evolutionarily conserved gene pathways that influence susceptibility to CNS autoimmune disease. Furthermore, our results provide evidence that the genetic dysregulation of T_H-cell development, differentiation, and/or activation underlies the disease mechanism of both MS and EAE.

Materials and Methods

Animals

SJL/J (SJL) and B10.S/SgMcdJ (B10.S) were purchased from The Jackson Laboratory (Bar Harbor, ME). For phenotype selection, we selected for EAE resistance or susceptibility among (B10.S $\times$ SJL)F1 $\times$ SJL and (B10.S $\times$ SJL)F1 $\times$ B10.S progeny, respectively. Resistant families from SJL backcrosses were kept for breeding the next generation. Susceptible families from B10.S backcrosses were bred before testing the mothers for susceptibility due to the difficulty in breeding affected animals. Progeny derived from susceptible mothers were used to breed the next generation. We obtained phenotype and genome-wide genotype data from the backcross mice and followed the segregation of donor marker loci for 4 subsequent generations. After this phenotype selection stage, multiply heterozygous progeny of B10.S-SJL-*Eae*^S and SJL-B10.S-*Eae*^R N5 founder pairs were (1) crossed to their respective congenic partners to continue the lines as multi-BTL lines and (2) intercrossed to generate homozygous and heterozygous F2 progeny to test linkage of the retained segments to EAE incidence and severity. Two B10.S-SJL-*Eae*^S lines were selected for derivation into traditional congenic lines by MAS. The experimental procedures performed in this study were approved by the animal care and use committees of the University of Vermont and Drexel University College of Medicine.

EAE induction protocol

For the phenotype selection phase of the study, EAE was induced using SJL mouse spinal cord homogenate (MSCH) in complete Freund's adjuvant without pertussis toxin as previously published.^{7, 16} For the cross-intercross stage of the phenotype-selection study, PLP₁₃₉₋₁₅₁ was used according to established protocols.¹⁷

Genotyping and linkage analysis

Samples of tissue were harvested for DNA, and genotyping was performed as described in our previous publications.⁸ During the phenotype selection stage, the multigenerational groups were analyzed for linkage as follows: a χ^2 goodness of fit test was performed for each marker locus to look for deviations from the expected segregation pattern of heterozygous and homozygous individuals for both backcross lines. Significant deviation in favor of the heterozygous genotypes for a particular marker indicates an effect of this locus on susceptibility. For the cross-intercross stage, traditional F2 linkage analysis was performed with χ^2 and logistic regression testing of the linkage to EAE incidence, and ANOVA was used to determine significant EAE-BTL.^{8, 16} Fully congenic lines were assessed for the strength of their retained QTL by the same statistical methods. A *p* value of 0.01 was considered significant, and *p* = 0.05 was considered suggestively significant.

In silico analysis

Mouse homologues of 40 genes listed as MS-GWAS gene candidates (msgene.org) were identified using Mouse Genome Informatics (<http://www.informatics.jax.org/>) and Ingenuity programs, and their interacting genes were determined using the network tool in Ingenuity Pathway Analysis Software (IPA; Ingenuity® Systems, www.ingenuity.com). This list

(~1600 genes, Supplemental Table 5) was used to create either “known EAE-BTL” (Supplemental Table 6, from previous linkage testing) or significantly retained “phenotype-selected EAE-BTL” (Supplemental Table 7) from the logistic regression described above. The two lists were filtered to contain only genes that were likely to be polymorphic between B10.S (using C57BL/10J as a reference because it is mostly sequenced and is the background strain for B10.S) and SJL using the Mouse Phenome Database (<http://phenome.jax.org/>) and selecting as polymorphic any allelic pair for which there was more than one nucleotide difference between SJL and C57BL/10J. The genes for which SNP information was missing for SJL were evaluated and included in the lists by employing the imputation data available in the Mouse Phenome Database.

We then subjected the three lists to pathway analysis using the core analysis tool in IPA, and used the comparison analysis function to determine which pathways were enriched in all three lists. The P-values associated with each pathway were adjusted for multiple hypotheses testing within IPA.¹⁸ After enriched pathways were identified, epistasis modeling was conducted by first identifying the *Eae5* candidates that were still present in both lists by stage three (Figure 2). Then, connect tool in IPA was used to test for interactions between those *Eae5* candidates and the genes involved in each of the significantly enriched pathways.

Antibodies and flow cytometric analysis

Lymph nodes (inguinal, braquial, axillary) were excised and dissociated into single cell suspensions. For the identification and phenotypic analysis of Treg cells (CD4⁺ CD8⁻ TCRβ⁺ Foxp3⁺), the following surface anti-mouse mAb were used: anti-CD4 (MCD0417, Caltag); anti-CD8, (53-6.7, BD Pharmingen); anti-TCRβ and anti-Foxp3 (H57-5987, FJK-16s; eBioscience). Intracellular Foxp3 was stained with the mouse/rat Foxp3 staining set (eBioscience), according to the manufacturer’s instructions. Viable cells were selected for flow cytometric analysis (LSR II, BD) based on forward and side scatter light properties and analysis was performed using FlowJo software (TreeStar Software, Inc).

Results

EAE phenotype-selected lines

We generated bi-directional, phenotype-selected congenic lines using EAE-resistant B10.S and EAE-susceptible SJL parental strains (creating the reciprocal lines B10.S-SJL-*Eae*^S and SJL-B10.S-*Eae*^R). By the 5th backcross generation (N5), we observed 30.6% susceptibility in the B10.S-SJL-*Eae*^S lines and 64.7% susceptibility in the SJL-B10.S-*Eae*^R lines (Supplemental Table 1). We used microsatellite marker genotypes at each generation to track donor alleles that were retained during trait selection (Supplemental Tables 2, 3). A χ^2 goodness of fit test was performed for each marker (Table 1), where significant deviations in favor of heterozygous genotypes indirectly indicate an effect of this locus on EAE susceptibility. Selection for phenotype for five backcross generations resulted in the retention of donor intervals on chromosomes (chr) 2, 3, 7, 12, 13, 16, and 17 (Table 1, Supplemental Table 4). These seven retained intervals harbor known EAE QTLs; thus, these results confirm previous linkages.

Markers were also analyzed for significant linkage to either resistance or susceptibility to EAE, and an odds ratio (OR) was calculated for each linkage (Table 1). *Eae4* was significantly retained in the SJL-B10.S-*Eae*^R congenics and significantly determined EAE incidence in both crosses, as did intervals located on chr 2 (comprising *Eae8*, *Eae21*, and *Eae33*). *Eae5* was significantly retained in the B10.S-SJL-*Eae*^S lines and nearly significantly retained in the SJL-B10.S-*Eae*^R congenics ($p = 0.08$); this locus showed ORs consistently >1 across the narrow *Eae5* interval on chr 17 in both crosses.

Derivation of single-interval EAE BTL congenic lines from phenotype-selected lines

The generation of SJL-B10.S-*Eae*^R single-interval lines on the SJL background was unsuccessful due to the poor breeding performance of SJL mice. *Eae5* was the only locus in B10.S-SJL-*Eae*^S mice that showed strong significance in the N5 generation by both retention and linkage (Table 1). Therefore, we generated single-interval congenic lines for *Eae5* by MAS (B10.S-SJL-*Eae5*^S). Two separate congenic B10.S-SJL-*Eae5*^S lines were created. In addition to *Eae5*, early generations of these congenic lines carried intervals bearing *Eae3*, *Eae8*, *Eae16*, *Eae20*, *Eae21*, and a region on chr 13, all of which were serially eliminated by MAS.

We observed that EAE susceptibility no longer segregated with *Eae5* in later MAS backcross generations (Fig 1). There was no CNS inflammation in B10.S-SJL-*Eae5*^S congenic mice in the absence of clinical signs in any generation (data not shown). Attempts were made to selectively promote EAE susceptibility in B10.S-SJL-*Eae5*^S congenic mice, using MSCH and pertussis toxin in the induction, which resulted in a modest increase in susceptibility, but at the expense of increased susceptibility in B10.S control animals¹⁹ making positional candidate gene identification in B10.S-SJL-*Eae5*^{SJL} nearly impossible. Thus, although the phenotype selection confirmed the requirement for known EAE BTL, we could not use this approach for physical positional candidate gene identification.

In silico candidate gene identification

Because gene identification by traditional positional cloning alone was not feasible for *Eae5*, we used a bioinformatic approach to identify genes that are important in both EAE and MS. Such genes have been identified using traditional genetic linkage studies and by genetic association studies including GWAS. For this analysis, we selected murine orthologues for the top 44 MS-GWAS genes (<http://www.ms-gene.org>). We used the network tool in IPA to identify interacting genes whose functions directly impact the action of each MS-GWAS homolog (Stage 1 of Fig 2). For example, *IL2* was included in the model, because of its interaction with *IL2RA*, a gene identified in a previous MS-GWAS study.²⁰ We did not include *H2* because B10.S and SJL have identical *H2* class I and class II alleles.⁸ From this list of ~1600 mouse genes (Supplemental Table 5), consisting of GWAS murine orthologues and interacting genes, we generated two additional lists: “known EAE-BTL,” containing BTL known to distinguish B10.S from SJL mice,^{6, 8, 11} and “phenotype-selected EAE-loci,” containing mouse genes that reside in intervals that were significantly retained and/or linked to EAE during the generation of the B10.S-SJL-*Eae5*^S congenic lines (Stage 2 of Fig 2; Table 1). These two lists were filtered to include only polymorphic loci, using genomic SNP data from the Mouse Phenome Database (see Methods). The result was a list of 190 “known

EAE-BTL” and a list of 132 “phenotype-selected EAE-loci” (Stage 3 of Fig 2) (Supplemental Tables 6-9). To determine physiological and functional networks of importance to autoimmune demyelination, we then subjected the three lists to pathway analysis in IPA.

The results of pathway analysis showed a significant enrichment of 10 canonical pathways within the MS-GWAS list, 21 in the known EAE-BTL list, and 22 in the phenotype-selected EAE-loci list ($p < 0.01$). The top 10 MS-GWAS-associated pathways were shared between mouse and human; they were conserved with high significance in all three models (Table 2; all pathways with $p < 0.05$ are in Supplemental Table 10). More than half of the shared pathways function in CD4 T helper (T_H)-cell differentiation or activation.

Genes were chosen from the *Eae5* interval (Chr 17, between 37 and 57Mb) that likely play a functional role in these shared, conserved pathways. Ten polymorphic genes within the *Eae5* interval were found, each of which function in at least one of the “shared MS/EAE pathways”: *Ccnd3*, *C6orf130* homolog AI314976, *Hsp90ab1*, *Mog*, *Runx2*, *Srf*, *Tubb4*, *Trip10*, *Vav1*, and *Vegfa* (Table 2, and Supplemental Table 8). Seven of these *Eae5* candidate genes are expressed by CD4 T cells, and all ten are expressed in Foxp3⁺ T regulatory cells (Tregs)^{21, 22} as determined using the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/sites/geo>).

We then set out to identify epistatic genes (on chr 2 [*Eae21*], chr 3 [*Eae3*, *Eae20*], chr 12 [*Eae16*], or chr 13) whose interactions with *Eae5* are critical for EAE but that were lost in the B10.S-SJL-*Eae5*^S congenic lines during MAS (Supplemental Table 9). We therefore located genes from these intervals that (1) are known to interact with an *Eae5* candidate and (2) do so in the context of one of the top “shared MS/EAE pathways.” For each of the CD4 T-cell functional pathways, there was a “core” of molecules present, with distinct interactions (Fig 3). The core consists of four *Eae5* candidates on chr 17 (*Runx2*, *Srf*, *Vegfa*, and *Vav1*) and *IL2* (on chr 3 [*Eae3/20*]), all of which biologically unify five of the top canonical pathways involving CD4 T cells (Fig 3, Table 2). Again, the identification of evolutionarily conserved pathways that lead to demyelination in human and mouse species underscores the importance of T_H cells in both MS and EAE. Epistasis modeling also shows there is clearly the potential for interaction of the *Eae5* candidates with other genes in each of the significant pathways and that these interactions may play a critical role in susceptibility to disease.

A test of this hypothesis is to show that *Eae5* functions in T cells. The expression of *Eae5* candidates in T_H -cell pathways suggested that either CD4 T_H cells or Tregs might be affected as a result of the inheritance of SJL alleles at *Eae5*. The frequency of natural Tregs (nTregs) in the lymph node is under the control of a gene within or tightly linked to *H2*.²³ The *Eae5* locus lies just distal to *H2* (Fig 4). We hypothesized that the *Eae5* candidate genes identified by *in silico* analysis would regulate the frequency or number of nTregs and susceptibility to EAE. We therefore quantified the total number of CD4⁺TCRβ⁺ T cells and the number of CD4⁺TCRβ⁺FoxP3⁺ nTregs in the lymph nodes of B10.S and B10.S-*Eae5*^{SJL} congenic mice. Surprisingly, the absolute numbers of both T_H cells and nTregs were significantly greater in B10.S-*Eae5*^{SJL} mice compared to B10.S mice (Fig 5). These data

establish that *Eae5* plays a significant role in controlling the number of T_H and nTregs and confirm the *in silico* approaches used.

Discussion

The overpowering impact of *HLA* on MS susceptibility impinges on the identification of other genetic contributors necessary for disease. Therefore, we used the EAE model to identify non-MHC loci essential for disease by generating *H2^s*-matched phenotype-selected congenic lines. Over the period of this selection, the incidence of EAE did not vary substantially in either of the crosses or as a consequence of trait selection (Supplemental Table 1). The phenotype selection approach was useful, however, for accumulating progeny mice that were significantly more likely to carry selected alleles of known EAE BTL and to transmit the selected EAE phenotype to their progeny.

We were able to selectively retain donor intervals that significantly conveyed resistance or susceptibility in the host strain. However, selection of mice at each generation that had the right phenotype and, presumably, the right EAE BTL alleles, did not lead to a high proportion of mice bearing the selected trait after five generations. This result indicates that numerous BTL must be retained in individual mice to govern EAE incidence. Therefore, in this strain combination, the generation of phenotype-selected and MAS congenic lines cannot be used for positional cloning of EAE candidate genes due to the polygenic nature of the disease. However, this finding does not apply to all animal models of complex genetic diseases, as witnessed in the non-obese diabetic (NOD) model.²⁴ It is widely accepted that the selection of phenotypes for gene discovery is more successful when a susceptible genetic background is converted to resistance, as seen in NOD.B6 congenic mice. Unfortunately, we were unable to maintain SJL-B10.S-*Eae^R* lines because of the poor breeding performance and aggressive nature inherent in the SJL strain.

To circumvent the difficulties pertaining to positional cloning and subsequent gene identification in the B10.S-SJL-*Eae5^S* congenic lines, we developed an *in silico* method to ascertain polymorphic positional candidate genes within *Eae5*. This approach identified a core of *Eae5* candidates—*Runx2*, *Srf*, *Vegfa*, and *Vav1*—that interacted with genes within the top ten canonical pathways. Of these genes, *Vav1* has been shown to influence both EAE and MS susceptibility by controlling T-cell expansion and proinflammatory cytokine production,^{25, 26} and *Vav1* has itself been suggested as a candidate gene.²⁵ Additionally, *TUBB4* (6.49 Mb), *TRIP10* (6.74 Mb), and *VAV1* (6.77 Mb), syntenic with human chr 19, are in linkage disequilibrium with *TNFSF14* (*LIGHT*) (6.61 Mb), which has been confirmed as an MS-GWAS candidate in a forthcoming study from The International Multiple Sclerosis Genetics Consortium and the Wellcome Trust Case Control Consortium.²⁷ Dysregulation of *TNFSF14/LIGHT* activity results in the disturbance of T-cell homeostasis, including an enlarged T-cell compartment, and the breakdown of peripheral tolerance.²⁸

The most highly significant set of pathways emerging from our analysis governs T_H-cell differentiation. Cellular and molecular techniques have defined a clear role for CD4 T cells in the pathogenesis of MS and EAE. Our results genetically support these data and suggest that the dysregulation of T_H-cell differentiation may be the primary mechanism leading to

MS and EAE susceptibility. Furthermore, pathways involving the communication between innate and adaptive immune systems; regulation of IL-2 expression; vitamin D receptor and retinoic acid signaling; and ICOS signaling are all unified by the core of *Eae5* candidates through their function within CD4 T cells (Figure 2). Therefore, the altered regulation of one or more *Eae5* candidate genes may impact CD4 T-cell differentiation and function and contribute to MS and EAE susceptibility, perhaps due to their role in the differentiation and/or function of Tregs. In the B10.S $\times$ SJL intercrosses and in the phenotype selection cohort, homozygous or heterozygous expression of the SJL-derived allele of *Eae5* was associated with higher incidence of EAE, indicating that either the increased number of lymph node CD4 T cells or nTregs seen in the B10.S-*Eae5*^S congenic mice leads to an increased susceptibility to disease. Studies to determine which of these alternative explanations is true are underway.

Our *in silico* approach also identified genes in other retained loci that epistatically interact with the *Eae5* candidates within the context of CD4 T cells. Interestingly, *Il2*, which resides within *Eae3/20* on chr 3, is central to all five top canonical pathways that signal in CD4 T cells. In human subjects, functional polymorphisms in the *IL2* and *IL2RA* genes have been identified in genetic studies^{29, 30} and in GWAS for other autoimmune disorders (<http://t1dbase.org/page/Regions>). IL-2 signaling plays a key role in immunomodulation through control of regulatory T-cell homeostasis and function. Indeed, small perturbations in IL-2 levels or in Treg frequency have been associated with differential susceptibility to EAE.^{23, 31} In addition, the environmentally derived factor VitD is physiologically immunoregulatory due, in part, to positive effects on Tregs.^{32, 33} In humans, low VitD levels are an environmental factor that is strongly associated with increased MS³⁴ and relapse severity³⁵ and exogenous VitD confers protection from EAE in mice.^{23, 36-38}

Importantly, the EAE results accurately reflect the polygenic and multifactorial nature of MS in humans. In line with the transient nature of interval retention during phenotype selection, each GWAS-determined SNP associated with MS is relatively weak and contributes only a small fraction to the variance seen in patients. Our data are not incompatible with the hypothesis that stronger MS risk alleles are rare and would probably not be included in any GWAS that limited the minor allele frequency to > 0.05 .³⁹ Searching for the rare alleles of MS risk genes has already yielded some interesting results.⁴⁰ A rare allele of *TYK2* (present in the homozygous state in only 0.164% of individuals studied [<http://www.msgene.org>]) is associated with MS resistance. In this regard, the autoimmune-resistant *Tyk2*^{E775K} allele is also rare (it is present only in B10.D1-*H2^g*/SgJ mice and not found to be segregating among >100 different classical laboratory and wild-derived inbred strains [unpublished data]), and it controls full susceptibility and resistance to EAE.⁴¹

In summary, by using an evolutionarily conserved network-based pathway analysis, we have established a critical role for CD4 T-cell differentiation and function in the pathogenesis of EAE and MS. Importantly, our results provide a functional framework in which animal models can provide insight into understanding the evolutionary overlap between the non-MHC genetic basis for susceptibility to different autoimmune diseases, i.e., the shared autoimmune disease susceptibility gene hypothesis, that first arose from animal studies looking at the genetic control of susceptibility to EAE and experimental autoimmune

orchitis.^{42, 43} Moreover, such studies may identify pathways that may be optimally suited to therapeutic intervention for the prevention or treatment of autoimmune disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The risk of EAE susceptibility in B10.S-SJL-*Eae5*^S cross-intercross progeny bearing *Eae5*^{S/S} or *Eae5*^{S/b} is compared to progeny bearing *Eae5*^{b/b} after immunization with 2×PLP +CFA. The relative odds ratio in B10.S-SJL-*Eae5*^S mice that carried susceptible alleles at *Eae5* over those that carried resistant *Eae5*^{b/b} alleles, classified by the year of the study. The year 2003 corresponds primarily to N6 mice, 2004 to N7 and N8 mice, etc. The *Eae5*^{S/S}-dependent susceptibility was lost after several generations of backcrossing to B10.S. One attempt to rescue the phenotype of these *Eae5*⁻ bearing mice is shown for 2008, when they were inoculated with 1×MSCH+PTX. s/s, homozygous SJL at *Eae5*; s/b, heterozygous SJL/B10.S at *Eae5*; b/b, homozygous B10.S at *Eae5*; *, $P < 0.05$; **, $P < 0.01$. Percentage of affected mice (*Eae5*^{s/-} vs. *Eae5*^{b/b}): 2008 with PTX 97% vs.77%; 2008, 10% vs.15%; 2007, 32% vs.42%; 2006, 38% vs.55%; 2005, 49% vs.67%; 2004, 51% vs.34%; 2003, 39% vs.10%

Figure 2.

In silico analysis to determine epistatic interactions in EAE and MS. Starting with known 44 MS-GWAS genes and their interactors (defined as protein-protein interactions or gene regulation relationships), a total of 1612 murine orthologues formed our initial analysis point in Stage 1. For Stage 2, we filtered these genes against positions of known EAE-BTL or against regions retained during phenotype selection. In Stage 3 we queried these genes against the mouse phenome database to determine polymorphisms (≥ 1 bp) in the coding and noncoding regions of these genes as well as 20MB up- and downstream of the boundaries. These refined lists were then combined and analyzed by Core pathway analysis in Ingenuity. A highly significant ($p < 0.001$ based on permutation test) interaction map for Conserved EAE/MS Pathways was then generated to elucidate epistatic interactions important in EAE and MS.

Figure 3.

IPA analysis of polymorphic genes retained in B10.S-SJL-*Eae5*^S phenotype selected congenic mice. *Eae5* candidate genes on chr 17 are indicated in green, and the polymorphic genes retained in the B10.S-SJL-*Eae5*^S congenic mice that interact with the *eae5* candidates are in blue (chr 2), purple (chr 3), yellow (chr 12), and brown (chr 13). The *Eae5* candidate genes are present in pathways that function within CD4 T cells, and these 5 pathways are unified by IL2, VEGFA, VAV1, SRF, and RUNX2. The loss of any one or more of the connected non-chr 17 genes could lead to the loss of the EAE-phenotype associated with *Eae5*.

Markers and Genes	Mb	B10	A.SW	B10.S	SJL	Eae5
SNP	3.13	B	B	B	S	B
SNP	4.18	B	B	B	S	B
D17Mit97	9.88	B	B	B	S	B
SNP	11.03	B	B	B	S	B
SNP	16.10	B	B	B	S	B
rs33756396	17.50	B	S	S	S	S
rs47291045	19.70	B	S	S	S	S
rs45810365	20.47	B	S	S	S	S
rs3155825	25.01	B	S	S	S	S
SNP	30.33	B	S	S	S	S
H2-K1	34.13	B	S	S	S	S
SNP	35.10	B	S	S	S	S
H2-D1	35.40	B	S	S	S	S
Mog	37.15	B	B	B	S	S
SNP	37.25	B	B	B	S	S
D17Mit105	41.43	B	B	B	S	S
D17Mit176	42.87	B	B	B	S	S
D17Mit51	43.64	B	B	B	S	S
SNP	43.70	B	B	B	S	S
Runx2	44.77	B	B	B	S	S
SNP	45.35	B	B	B	S	S
Hsp90ab1	45.70	B	S	B	S	S
Ccnd3	45.71	B	S	B	S	S
Vegfa	46.16	B	S	B	S	S
Srf	46.68	B	S	B	S	S
rs52092199	48.50	B	S	B	S	S
Al314976	48.56	B	S	B	S	S
D17Mit70	52.83	B	S	B	S	S
Tubb4	57.21	B	S	B	S	S
Trip10	57.39	B	S	B	S	S
Vav1	57.44	B	S	B	S	S
D17Mit89	63.90	B	S	B	S	B

Figure 4.

The *Eae5* haplotype and location of candidate genes. Traditional microsatellite mapping and SNP genotyping by phototyping⁴⁴ were used to identify breakpoints in chr 17 for key strains. SNPs used are labeled by their position in megabases (Mb) and were developed from genomic databases at MPD. C57Bl/10J and A.SW were the parental strains used to create the B10.S *H2* congenic and B10.S and SJL were the parental strains used to create the B10.S-*Eae5*^{SJL} congenic strain. The distal border of *Eae5* in the B10.S-*Eae5*^{SJL} congenics is indeterminate.

Figure 5.

B10.S-*Eae5^{SL}* mice have an increased number of lymph node CD4⁺ T cells and Foxp3⁺ Treg cells. Total number of cells (A), CD4⁺ T cells (B), and CD4⁺Foxp3⁺ Treg cells (C) in the lymph nodes of naïve B10.S (■, n = 10) and B10.S-*Eae5^{SL}* (□, n = 6) mice. Statistical significance was determined using the Mann-Whitney test (**p<0.01; ***p<0.001). Data are representative of two independent experiments.

Table 1

Significantly retained and linked BTL in phenotype-selected generations (N2-N5).

Retained intervals Known EAE BTL	Retention in B10.S-SJL- Eae ^S (p) ^a	Linkage to EAE (p) ^b	OR, range ^c	Retention in SJL-B10.S- Eae ^R (p) ^a	Linkage to EAE (p) ^b	OR, range ^c
Chr 2: 18-122 Mb ^d <i>Eae8,21, 33</i>	0.03	0.001	0.6 - 3.1	6.1×10^{-22}	0.03	1.4 - 4.2
Chr 3: 29-141 Mb ^d <i>Eae3, 10, 20a, b</i>	0.05	0.03	1.2 - 4.2	0.004	NS	0.7 - 1.8
Chr 7: 63-132 Mb <i>Eae4</i>	NS	0.02	2.1 - 4.7	3.0×10^{-15}	0.05	1.6 - 2.4
Chr 12: 12-120 Mb ^d <i>Eae16</i>	1.2×10^{-06}	NS	1.1 - 2.2	2.0×10^{-09}	0.02	0.4 - 0.5
Chr 13: 3-117 Mb ^d	0.02	NS	0.7 - 1.4	4.4×10^{-05}	NS	0.3 - 2.7
Chr 16: 4-57 Mb <i>Eae11</i>	0.0001	NS	1.9 - 2.1	0.001	0.001	3.8 - 15.2
Chr 17: 37-63 Mb ^b <i>Eae5</i>	1.6×10^{-25}	0.005	2.7 - 3.9	NS	NS	1.2 - 4.0

Intervals that were retained during phenotype selection are shown. All but those on chr 7 and chr 17 are bi-directional and were retained in both B10.S-SJL-*Eae*^S congenics as well as SJL-B10.S-*Eae*^R congenics, often with significant direct linkage to EAE, as assessed by logistic regression. The regions in total cover ~18% of the mouse genome.

NS, not significant

^a p value for most significantly retained marker in interval.

^b p value for most significant linkage to EAE in interval.

^c Odds ratios (OR) were calculated by logistic regression, corrected for the sex of the mice, and those greater than 1 indicate a ratio consistent with the expected effect of a retained allele (B10.S alleles that convey greater resistance, or SJL-derived alleles that convey greater susceptibility). The "range" of OR for individual markers in each interval is listed. Bold font indicates intervals where linkage to EAE was consistently seen with OR > 1.

^d Intervals on chr 2, 3, 12, and 13, together with *Eae5* on chr 17, were also retained in the N6 progenitor of the B10.S-SJL-*Eae*^S lines. The chr 2, 3, 12, and 13 regions were selected for the analysis of genes that are candidates for epistatic interactions with *eae5* on chr 17.

Table 2

Shared MS/EAE pathways show the close correspondence of human MS-GWAS pathways and both H2s mouse EAE BTL pathways.

Ingenuity Canonical Pathways	List Name	p value	Molecules
1. T Helper Cell Differentiation	MS-GWAS	1.0E-09	IL12A, CD40, TGFB1, TNFRSF1A, IL21R, HLA-DRB1, IL2RA, STAT3
	Known EAE-QTL	3.0E-09	IL21, IFNG, IL4R, IL5, CD40, IL2, IL12RB1, TNFRSF1A, NGFR, IL21R, RORC, IL4
	Phenotype-selected	1.7E-04	IL21, IL6ST, IL4R, IL2, IL21R, RORC, TRG (TCR gamma family)
2. Role of JAK1 and JAK3 in γ c Cytokine Signaling	MS-GWAS	4.0E-05	IL7R, IL21R, IL2RA, STAT3, IL7
	Known EAE-QTL	7.0E-05	IL21, IL4R, FES, IL2, IL21R, JAK3, IL7, IL4
	Phenotype-selected	5.1E-03	IL21, IL4R, FES, IL2, IL21R
3. Altered T Cell & B Cell Signaling in Rheumatoid Arthritis	MS-GWAS	9.0E-05	IL12A, CD40, TGFB1, HLA-DRB1, CHUK
	Known EAE-QTL	6.0E-03	IL21, IFNG, CD40, IL2, CSF2, IL4
	Phenotype-selected	2.5E-02	TLR2, IL21, IL2, TRG(TCR gamma family)
4. ICOS-ICOSL Signaling in T Helper Cells	MS-GWAS	2.0E-04	CD40, HLA-DRB1, IL2RA, VAV1, CHUK
	Known EAE-QTL	8.0E-04	GAB2, LCK, CD40, IL2, NFATC2, NFATC1, VAV1, ITK
	Phenotype-selected	1.4E-02	GAB2, AKT1, IL2, VAV1, TRG(TCR gamma family)
5. Communication between Innate & Adaptive Immune Cells	MS-GWAS	2.0E-03	CXCL10, IL12A, CD40, HLA-DRB1
	Known EAE-QTL	8.0E-03	IFNG, IL5, CD40, IL2, CSF2, IL4
	Phenotype-selected	2.8E-02	TLR2, B2M, IL2, TRG@
6. IL-12 Signaling & Production in Macrophages	MS-GWAS	3.0E-03	IL12A, CD40, TGFB1, CHUK
	Known EAE-QTL	4.5E-02	IFNG, CD40, IL12RB1, IL4, PRKCB
	Phenotype-selected	4.1E-02	TLR2, TRAF6, AKT1, PRKCB
7. Colorectal Cancer Metastasis Signaling	MS-GWAS	5.0E-03	TGFB1, TNFRSF1A, TYK2, STAT3, PTGER4
	Known EAE-QTL	3.5E-02	VEGFA, IFNG, RHOG, ARRB1, TNFRSF1A, LEF1, JAK3, WNT5B
	Phenotype-selected	9.0E-03	VEGFA, TLR2, IL6ST, AKT1, RHOG, ARRB1, EGF, LEF1
8. Role of Macrophages, Fibroblasts & Endothelial Cells in Rheumatoid Arthritis	MS-GWAS	9.3E-03	TGFB1, TNFRSF1A, STAT3, CHUK, IL7
	Known EAE-QTL	3.0E-04	TNFRSF1A, NFATC1, FCGR1A, IL7, IL16, VEGFA, NLK, CCL2, NGFR, NFATC2, LEF1, CSF2, WNT5B, PRKCB
	Phenotype-selected	2.0E-04	IL6ST, VCAM1, PDIA3, DAAM1, FCGR1A, VEGFA, TLR2, TRAF6, IL16, AKT1, RIPK1, LEF1, PRKCB
9. VDR/RXR Activation	MS-GWAS	9.0E-03	CXCL10, IL12A, CYP27B1
	Known EAE-QTL	4.0E-03	DEFB4A/DEFB4B, IFNG, IL2, RUNX2, CSF2, PRKCB
	Phenotype-selected	7.0E-03	WT1, YY1, IL2, RUNX2, PRKCB
10. Regulation of IL-2 Expression in Activated & Anergic T Lymphocytes	MS-GWAS	9.5E-03	TGFB1, VAV1, CHUK
	Known EAE-QTL	1.5E-02	IL2, VAV3, NFATC2, NFATC1, VAV1

Ingenuity Canonical Pathways	List Name	p value	Molecules
	Phenotype-selected	2.1E-02	IL2, VAV3, MAP3K1, VAV1

Results are listed in order of significance for human GWAS pathways. P values for each pathway's significance in the three lists, as determined by Ingenuity, are listed in center column. Significance of each was calculated after correction for multiple testing in IPA.