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http://dx.doi.org/10.1073/pnas.0813310106

National Academy of Sciences

Final published version

Thu Apr 04 22:36:44 EDT 2019

http://hdl.handle.net/1721.1/50257

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The role of the CD58 locus in multiple sclerosis

Philip L. De Jager\textsuperscript{a,b,c,1}, Clare Baecher-Allan\textsuperscript{a}, Lisa M. Maier\textsuperscript{a,c}, Ariel T. Arthur\textsuperscript{d}, Linda Ottoboni\textsuperscript{a,c}, Lisa Barcellos\textsuperscript{a}, Jacob L. McCauley\textsuperscript{a}, Stephen Sawyer\textsuperscript{a}, An Goris\textsuperscript{a}, Janna Saarela\textsuperscript{e}, Roman Yelenosky\textsuperscript{c,d}, Alkes Price\textsuperscript{c,k}, Virpi Leppä\textsuperscript{a}, Nick Patterson\textsuperscript{a}, Paul I. W. de Bakker\textsuperscript{a,b}, Dong Tran\textsuperscript{a,b}, Cristin Aubin\textsuperscript{a,b}, Susan Pobywajlo\textsuperscript{a}, Elizabeth Rossin\textsuperscript{a,c}, Xinli Hu\textsuperscript{a}, Charles W. Ashley\textsuperscript{a}, Edwin Choy\textsuperscript{a}, John D. Rioux\textsuperscript{c,k}, Margaret A. Pericak-Vance\textsuperscript{a}, Adrian Ivinston\textsuperscript{a}, David R. Booth\textsuperscript{a}, Graeme J. Stewart\textsuperscript{a}, Aarno Palotie\textsuperscript{b}, Leena Peltonen\textsuperscript{a,b}, Bénédicte Dubois\textsuperscript{a}, Jonathan L. Haines\textsuperscript{a}, Howard L. Weiner\textsuperscript{a}, Alastair Compton\textsuperscript{a}, Stephen L. Hauser\textsuperscript{a,c}, Mark J. Daly\textsuperscript{a}, David Reich\textsuperscript{a}, Jorge R. Oksenberg\textsuperscript{a,c}, and David A. Hafler\textsuperscript{a,c}

\textsuperscript{a}Division of Molecular Immunology, Center for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115; \textsuperscript{b}Partners Center for Personalized Genetic Medicine, Boston, MA 02115; \textsuperscript{c}Program in Medical and Population Genetics, Broad Institute of Harvard University and Massachusetts Institute of Technology, Cambridge, MA 02139; \textsuperscript{d}Department of Medicine and the Nerve Research Foundation, University of Sydney, Sydney NSW 2145, Australia; \textsuperscript{e}Division of Epidemiology, School of Public Health, University of California, Berkeley, CA 94720-7360; \textsuperscript{f}Miami Institute for Human Genomics, Miller School of Medicine, University of Miami, Miami, FL 33136; \textsuperscript{g}Department of Clinical Neurosciences, Addenbrooke’s Hospital, University of Cambridge, Box 165, Hills Road, Cambridge CB2 0QQ, United Kingdom; \textsuperscript{h}Section of Experimental Neurology, University of Leuven, B-3000 Leuven, Belgium; \textsuperscript{i}Department of Molecular Medicine, National Public Health Institute and the Program of Molecular Medicine of the University of Finland, Biomedicum, Haartmaninkatu 8, 00290, Helsinki, Finland; \textsuperscript{j}Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA 02114; \textsuperscript{k}Department of Genetics, Harvard Medical School, Boston, MA 02115; \textsuperscript{l}Montreal Heart Institute and Universite de Montreal, Montreal, Quebec H3C 3J7, Canada; \textsuperscript{m}Harvard Neurodiscovery Center, Harvard Medical School, Boston, MA 02115; \textsuperscript{n}Institute for Immunology and Allergy Research, Westmead Millenium Institute, Sydney, Australia; \textsuperscript{o}Center for Human Genetics Research, Vanderbilt University Medical Center, Nashville, TN 37232-0700; \textsuperscript{p}Department of Neurology, School of Medicine, University of California, San Francisco, CA 94143-0435; and \textsuperscript{q}Institute for Human Genetics, School of Medicine, University of California, San Francisco, CA 94143-0435

Communicated by Elliott D. Kieff, Harvard University, Boston, MA, December 29, 2008 (received for review July 23, 2008)

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system associated with demyelination and axonal loss. A whole genome association scan suggested that allelic variants in the CD58 gene region, encoding the costimulatory molecule LFA-3, are associated with risk of developing MS. We now report additional genetic evidence, as well as resequencing and fine mapping of the CD58 locus in patients with MS and control subjects. These efforts identify a CD58 variant that provides further evidence of association with MS (P = 1.1 × 10\textsuperscript{-6}, OR 0.82) and the single protective effect within the CD58 locus is captured by the rs2300747\textsuperscript{a} allele. This protective rs2300747\textsuperscript{a} allele is associated with a dose-dependent increase in CD58 mRNA expression in lymphoblastic cell lines (P = 1.1 × 10\textsuperscript{-19}) and in peripheral blood mononuclear cells from MS subjects (P = 0.0037). This protective effect of enhanced CD58 expression on circulating mononuclear cells in patients with MS is supported by finding that CD58 mRNA expression is higher in MS subjects during clinical remission. Functional investigations suggest a potential mechanism whereby increases in CD58 expression, mediated by the protective allele, up-regulate the expression of transcription factor FoxP3 through engagement of the CD58 receptor, CD2, leading to the enhanced function of CD4\textsuperscript{+}CD25\textsuperscript{high} regulatory T cells that are defective in subjects with MS.

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ultiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system associated with demyelination, axonal loss, and brain atrophy; susceptibility to this disease is affected by both genetic variation and environmental risk factors (1, 2). The initial episode of neurologic dysfunction results in a clinical diagnosis of a clinically isolated demyelinating syndrome (CIS), and a second episode leads to a diagnosis of MS (1). Increasing evidence suggests that activated, autoreactive T cells are affected by both genetic variation and environmental risk factors. The role of the CD58 locus in multiple sclerosis

Results

Fine Mapping, Resequencing, and Validation of the CD58 Susceptibility Locus in MS. We recently performed a whole genome association screen for MS susceptibility genes and identified a suggestive association at SNP rs12044852 both in the screening and replication phase (P = 1.9 \times 10\textsuperscript{-5} in the combined analysis) (Fig. 1; ref. 5). We therefore initiated a fine mapping effort in the chromosomal region that contains rs12044852 to better characterize this association to MS. Using our collection of subjects with MS from the Brigham and Women’s Hospital in Boston, MA [supporting information (SI) Table S1], we initially surveyed two novel MS susceptibility loci that have recently been identified using a genome-wide association scan approach, and these 2 loci, IL2RA and IL7R, have now been validated in independent subject collections (5–9). In the genome scan, several other loci, including the CD58 locus, displayed suggestive evidence of association with MS susceptibility. Since CD58 (LFA-3) co-stimulates and enhances T cell receptor signaling by engaging CD2 (10), the CD58 locus is an attractive target for understanding the role of genetic variation in immune system dysfunction associated with MS. Here, we first refine and then enhance the association between a polymorphism in CD58 and MS susceptibility. Then, using data from Epstein-Barr virus (EBV) transformed B cells (lymphoblastic cell lines), we find the putative CD58 protective allele to be significantly associated with higher CD58 mRNA levels, and we validate this observation by measuring mRNA expression in circulating mononuclear cells isolated ex vivo from subjects with MS. Moreover, we present evidence that a higher level of CD58 mRNA expression is seen during the clinically quiescent phase of MS and, finally, that higher CD58 expression may function in part by enhancing FoxP3 expression in regulatory T cells.
24 SNPs that capture common variation within this region of the genome (Table S2). These SNP data allowed us to define groups of markers that are correlated and define chromosomal segments that tend to be inherited as a block (Fig. 1). Further analysis in the context of this linkage disequilibrium structure shows that the association to MS is located within the central chromosomal segment (Fig. S1). More specifically, one version of this chromosomal segment, a haplotype found at 8% frequency in our subjects, contains the minor “A” allele of rs12044852 (rs12044852A) and is under-represented in subjects with MS (P = 0.0015). None of the haplotypes containing the more common “G” major allele at rs12044852 has significant evidence of association (Fig. S1). These results suggest that an allele protecting subjects from MS exists somewhere within a 76 kb segment of DNA that only contains the CD58 gene (Fig. 1).

We then sequenced 16 selected individuals over this 76,048 bp DNA segment. Seventeen putative new SNPs were identified and underwent validation (Table S3). To identify a better marker for the CD58 association to MS, we then genotyped those SNPs that had been validated and demonstrated some level of correlation with rs12044852. We also genotyped additional SNPs that provided information regarding genetic variation within the CD58 gene region but which had not yet been assessed by the initial panel of 24 SNPs (Table S2). Using these data, we assessed the possibility of other independent associations to MS susceptibility within the CD58 locus (allelic heterogeneity) by performing a conditional analysis in our fine mapping data to account for the effect of the most associated SNP (Table S4). Once the effect of the associated CD58 protective allele is accounted for, as estimated by either of the 2 best markers (rs12044852A or rs2300747G), there is no residual evidence of association to MS susceptibility within the CD58 locus. This result suggests that there is a single allele or a group of alleles that are strongly correlated which explains the association of the CD58 locus to MS susceptibility.

We then extended our mapping effort by genotyping the 15 most associated SNPs from our original fine mapping screen in an additional 1,278 trio families with MS (Table S5a). The 2 best SNPs from this trio analysis [rs2300747 and rs12044852, which are strongly correlated (r2 = 0.929) in HapMap CEU samples; ref. 11] were then genotyped in an additional 3,541 MS cases and their controls. Once all data are pooled, rs2300747 is the most associated marker (P = 1.1 × 10−6, odds ratio 0.82, 95% confidence interval 0.75–0.89) (Fig. S2 and Table S5b). The minor allele rs2300747G is found within the protective haplotype that contains the CD58 gene (Fig. 1), and we therefore consider this allele to be a marker for a protective effect in MS susceptibility.

The CD58 Protective Allele Affects RNA Expression. The rs2300747 polymorphism is found within the first intron of CD58 and does not have a known functional consequence. Thus, we investigated the effect of the MS associated allele on expression of CD58 RNA using data generated by the Sanger Institute from EBV-transformed lymphoblastic cell lines (LCL) used in the HapMap project (11, 12). Using the quantitative trait analysis module implemented in the PLINK toolkit (13), we find that the protective rs2300747G allele is associated with increased expres-
sion of CD58 RNA in 60 unrelated LCLs of European ancestry (Centre d’Etude du Polymorphisme Humain (CEPH), Utah residents with ancestry from northern and western Europe (CEU) LCLs) (P = 0.038) and in 89 unrelated LCLs of East Asian ancestry (Han Chinese in Beijing (CHB) and Japanese in Tokyo (JPT) LCLs) (P = 1.1 × 10^{-10}). The higher frequency of rs2300747G in the larger sample of East Asian LCLs (frequency = 0.66 vs. 0.13 in the CEU LCLs) explains in part the more extreme association of rs2300747G with higher levels of CD58 expression in the East Asian LCLs. This association of rs2300747G with higher expression of CD58 RNA is best illustrated by plotting the RNA expression values of individual LCLs and organizing them by genotype class (Fig. 2A): the rs2300747GG homozygote class has a higher level of CD58 RNA expression than does the rs2300747AA homozygote class, and the rs2300747AG heterozygote class has an intermediate level of expression. This effect on CD58 RNA expression is also observed in an independent set of 400 independent LCLs from subjects of British ancestry for which similar data have been collected (14). In these samples, rs2300747G has not been genotyped, but the minor allele of rs6077309, a SNP which is strongly correlated with rs2300747 (r^2 = 0.87 in CEU HapMap samples; ref. 14), is seen to be associated with higher levels of CD58 RNA expression (P = 2.1 × 10^{-5}). In addition, the correlation of rs2300747G with RNA expression is specific to CD58: it is not seen with the 2 genes flanking CD58. Using the more informative East Asian HapMap LCLs, we repeated the quantitative trait analysis and found no evidence for association of rs2300747G with RNA expression of the flanking ATP1A1 gene (P = 0.96) on the telomeric side or of the IGSF3 gene (P = 0.50) on the centromeric side. The LCLs from individuals of European ancestry showed similar results (data not shown). Thus, this putative “protective” CD58 allele for MS may exert its effect on disease risk by specifically increasing the expression of CD58 RNA in a dose-dependent manner.

We next validated this in vitro observation using ex vivo data: we examined a data set derived by extracting mRNA from circulating mononuclear cells from 239 subjects with remitting-relapsing MS (RR MS) or CIS for evidence of correlation between rs2300747 and the expression of CD58 RNA. Since some of these subjects with MS were treated at the time of sampling, we first established that there was no significant difference in CD58 RNA expression among subjects that are untreated (n = 81), treated with glatiramer acetate (n = 64), or treated with an IFN beta formulation (n = 94) (Fig. 3). We then pooled all of these subjects into a genotypic analysis. There is only one rs2300747GG homozygote, so we compared the level of CD58 RNA expression in rs2300747AA homozygotes to the expression level observed in subjects bearing at least one rs2300747G allele. This analysis validates our in vitro LCL results by demonstrating that the protective rs2300747G allele is associated with higher expression of CD58 RNA (P = 0.0037) in mononuclear cells of RRMS and CIS subjects (Fig. 2B).

The CD58 Locus and Clinical Manifestations of MS. To assess whether CD58 mRNA levels correlated with clinical disease activity, we analyzed RNA data captured from a different set of subjects with MS who were experiencing either a clinical relapse or remission. These data had been generated as part of an independent project that analyzed changes in RNA expression of 9,381 genes to discover relapse- and remission-specific patterns of gene expression in whole blood of untreated subjects with MS (15). Of the 38 putative MS susceptibility loci with evidence of replication in the recent whole genome association scan for MS (5), CD58 is the only one whose RNA expression is enhanced in subjects in clinical remission (Fig. S4). The expression of CD58 RNA in whole blood during a remission is, on average, 1.7-fold greater than baseline expression in healthy control subjects, and this remission-associated increased expression of CD58 is significantly greater than the levels of CD58 expression seen in subjects with MS that are sampled during a relapse (P = 0.011) (Fig. 3).
These data suggest that an enhanced level of CD58 RNA expression is correlated with a clinically quiescent state and may therefore have a role in limiting inflammation in MS.

**Activation of the CD58/CD2 Pathway Enhances FoxP3 Expression in Regulatory T Cells.** We examined one potential mechanism by which alterations in CD58 expression may influence immune function. CD58 engagement of its receptor, CD2, provides an activation signal for human T cells, including CD4^{+}CD25^{high} regulatory T cells. While the precise mechanism of action of human regulatory T cells has not been established, the transcription factor FoxP3 is associated with regulatory T cell activity. However, FoxP3 expression alone does not necessarily confer suppressor activity to human T cells (16). Nonetheless, the importance of FoxP3 in mice and humans is highlighted by the association of mutations in FOXP3 with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (17) and by the observations that murine null alleles of FOXP3 or even attenuated expression of FoxP3 in murine regulatory T cells are associated with aggressive autoimmune disease (18, 19).

Recent experiments have implicated CD2 in regulatory T cell activity, including the binding of FoxP3 to the CD2 promoter region in chromatin immunoprecipitation experiments (20) and the induction, by CD2 coactivation, of suppressor function in human T cells characterized by CD4^{+}CD25^{high}DR^{+}CD62L^{high} expression (21). Our group demonstrated that while the proportion of regulatory T cells is normal in subjects with MS, these cells are dysfunctional (4). Subsequent studies have both confirmed these observations (22) and demonstrated that the expression of FoxP3 is diminished in regulatory T cells from patients with MS, suggesting a central role for this transcription factor in regulatory T cell dysfunction (23, 24).

Based on these observations, we compared the induction of FoxP3 by the costimulatory signal provided through the CD58 receptor, CD2, to the effect of the strong CD28 costimulatory signal in regulatory T cells; both costimulatory signals were given in the context of T cell receptor (TCR) activation using cross-linking of the TCR by anti-CD3 monoclonal antibody. Of note, T cells express CD58, and thus exogenous CD28 costimulation occurs in the context of endogenous interaction between CD58 on T responder cells and CD2 on regulatory T cells. Both costimulatory signals are capable of triggering substantial suppression of T cell proliferation in vitro by activation of CD4^{+}CD25^{high} regulatory T cells: 39% suppression with anti-CD2 and 30% suppression with anti-CD28 (Fig. 4A). Both stimuli result in enhanced FoxP3 expression in regulatory T cells when these cells are compared to regulatory T cells analyzed ex vivo (Fig. 4B and C) and the effect of anti-CD2 is dose-dependent (Table S6). There is significantly more induction of FoxP3 with CD2 as compared to CD28 engagement in CD4^{+}CD25^{high} regulatory T cells after 4 days of culture (Fig. 4C). On average, FoxP3 expression is 2.1-fold greater (log scale) following CD2 as compared to CD28 engagement (Fig. 4D).

Functionally, we have previously reported that in vitro anti-CD2 costimulation of this regulatory population results in suppression of T cell proliferation within 3 days of stimulation, as compared to 5 days with anti-CD28 (22). Thus, it appears engagement of the CD58 receptor, CD2, has a significant effect on FoxP3 expression and a more rapid impact on regulatory T cell function as compared to engagement of the CD28 costimulatory pathway. Of note, the higher proliferation of the T responder cell population in response to anti-CD2 vs. anti-CD28 stimulation (Fig. 4A) suggests that the costimulatory effect of anti-CD2 is broad and not specific to FoxP3 expression.

**Discussion**

Here, we report the detailed characterization of the CD58 locus that affects susceptibility to MS and propose a mechanism of action for its putative protective effect. After studying 5,326 subjects with MS, we have discovered a better susceptibility marker (rs2300747) within the CD58 locus, and, by adding 1,530 new subjects with MS to the previously published analysis (5), the evidence for this locus affecting disease risk in MS has been enhanced (P = 1.1 × 10^{-6}). Although the magnitude of this allelic variant’s effect on susceptibility to MS is modest, functional characterization of this polymorphism uncovers compelling evidence that the protective allele has an effect on the level of CD58 RNA expression both in vitro and ex vivo. We also show that enhanced CD58 expression, which is associated with protection from MS, is further associated with a clinically quiescent disease state.

While CD58 is widely expressed in immune and non-immune cells, we propose that its role in the pathogenesis of MS is related to alterations in immune function. This is consistent with the pronounced inflammatory lesions associated with CNS demyelination. Moreover, enhanced CD58 expression may both mediate protection from the onset of MS and moderate acute attacks of inflammatory demyelination once the disease has begun.

Our analyses of CD58 RNA expression led us to examine whether the protective effect of the CD58 locus on CNS inflammation may be mediated in part through the function of regulatory T cells. Functional investigations indeed suggest a potential mechanism whereby the CD58 risk allele leads to decreases in CD58 expression, with subsequent down-regulation of FoxP3 leading to the dysfunction of regulatory T cells observed in subjects with MS (4, 22–24). Nonetheless, the ubiquitous ex-
expression of CD58 and its role as both an adhesion and signaling molecule necessitate caution in a single interpretation of the effect of CD58 expression in the pathogenesis of MS.

The association of allelic variants in the CD58 gene with MS susceptibility may open new avenues of investigation into the pathogenesis of MS and offer another pathway and set of targets for the development of novel therapies. In particular, these studies suggest that manipulation of the CD58/CD2 pathway, perhaps with the CD58/IgG1 fusion protein (Alefacept) approved for the treatment of psoriasis, may be of utility (25). The in vivo immune effects associated with infusions of this fusion protein are pleiotropic and may be cell-type specific: its use in patients with psoriasis is associated with reduced numbers of memory T cells (26, 27). However, it may also have important agonistic properties that are evidenced by changes of in vivo and in vitro peripheral blood mononuclear cell gene expression within 6 hours of infusing the CD58 fusion protein (28). Finally, further characterization of immune effects driven by CD58 gene variants in the context of other immune genes associated with susceptibility to develop MS may provide direct insight into the pathogenesis of the disease.

Materials and Methods

Subjects. All subjects (MS and healthy controls) were enrolled under study protocols approved by the Institutional Review Board of each institution. Subjects with MS all meet McDonald criteria for MS (29). Details of the clinical composition of each collection of subjects are presented in Table S1. For the control samples, only complete trio families were used in transmission disequilibrium test (TDT) analyses. Control subjects in the Brigham and Women’s Hospital collection are spouses or friends of subjects with MS. The control subjects for the Belgian, United Kingdom and University of California, San Francisco, CA, collections have been previously described (4), and the Finnish controls are anonymized individuals who underwent a blood count at the University hospital.

Genotyping and Sequencing. Details of the genotyping and sequencing platforms used in these experiments are presented in the SI Methods section as are descriptions of the resequencing strategy and SNP selection approach.

Disease Association Analyses. For the case/control analyses, we used a standard χ2 calculation to establish the level of significance of an observation. For the trio analysis, we used only complete trio families and performed a TDT analysis (30). Both were implemented using the Haploview software (31), which was also used to estimate the number of each category of haplotypes used in our study. A Mantel-Haenszel approach was used for the pooled analysis of the replication data (32). Before performing a combined analysis, we performed a Pearson χ2 goodness-of-fit test to assess the validity of combining our different replication samples; the result of this analysis (P = 0.17) suggests that differences in allele frequencies between the sample sets are not significant. The analyses conditional on the genotype of rs12044852 or rs2300747 were performed using logistic regression as implemented in the PLINK toolkit v0.99r by S. Purcell (13).

Analyses of Cell Line and ex Vivo RNA Data. Details of the source and processing of the cell line and ex vivo RNA data are provided in the SI Methods section.

Cell Isolation, Culture, and Cytometric Analysis. Cell isolation: CD4+ T cells, isolated by negative selection (Miltenyi Biotec) from whole blood mononuclear cells after Ficoll-Hypaque (Amersham Pharmacia) gradient centrifugation of heparinized blood, were FACs-sorted on a FACS ARIA (BD Biosciences) after staining for HLA-DR (PerCP, clone L243), CD62L (APC, clone Dreg 56), CD32 (FITC, clone 3D3), CD14 (FITC, clone M5E2) and CD116 (FITC, clone M5D12) (all from BD Pharmingen) and CD25 (Pacific Blue, clone BC96 from BioLegend) to typically 92% purity. Cells were then plated at 2.5 × 10^3 cells/well with or without CD4+ CD25high regulatory T cells at a 2:1 ratio in the previously described in vitro accessory cell-free micro coculture assay (22). The in vitro assay was modified...
to include the different plate-bound stimuli of αCD3 (UCHT1, BD Biosciences, 0.5 μg/ml) with αCD2 (RPA-2.10, BD Pharmingen, 0.125 μg/ml) or αCD28 (28.2, BD Pharmingen, 0.5 μg/ml). The cells were harvested on day 4, stained for FoxP3 using the eBioscience FoxP3 staining buffers and the αFoxP3-PE (PCH101, eBioscience), acquired on a FACS Calibur using CellQuest Software (BD Biosciences), and analyzed with FlowJo software (TreeStar Industries).

ACKNOWLEDGMENTS. We thank the following clinicians for contributing to sample collection efforts: B.W.H. Dr. Guy Buckle, Dr. Susan Gauthier, Dr. Maria Houtchens, Dr. Samia Khoury, Lynn Stazzone NP. We also thank the following University of Finland clinicians for contributions to sample collection efforts: Dr. Pentti J Tienari, Dr. Marja-Liisa Sumpelmahti, Dr. Irina Elovaaara, Dr. Keijo Koivisto, Dr. Tuula Pirttilä, Dr. Mauri Reunanen. This work was supported by grants from the NIH R01SN24247, NIH P01A039671. P.L.D. has a Harry Weaver Neuroscience Scholar Award from the National Multiple Sclerosis Society; he is also a William C. Fowler Scholar in Multiple Sclerosis Research and is supported by National Institute of Neurological Disorders and Stroke K08 grant, N54341. D.A.H. is a Jacob Javits Scholar of the National Institutes of Health. B.D. is a Clinical Investigator and A.G. is a Postdoctoral Fellow of the Research Foundation Flanders, Belgium (F.W.O.-Vlaanderen). J.L.M. is supported by a postdoctoral fellowship (FG-1718-A1) from the National Multiple Sclerosis Society. The International Multiple Sclerosis Genetics Consortium is supported by R01NS049477. LP is supported by NINDS, ROI NS43559, and the Neuropromise European Union project, LSHM-CT-2005–018637. D.R. is supported by a Burroughs Wellcome Career Development Award in the Biomedical Sciences. The RNA data on MS and CIS subjects from the Comprehensive Longitudinal Investigation of Multiple Sclerosis at Brigham and Women’s Hospital study was generated as part of a collaboration with Affymetrix, Inc.