Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis

CHRISTOPHER LOCK[1], GUY HERMANS[1], ROSETTA PEDOTTI[1], ANDREA BRENDOLAN[2], ERIC SCHADT[4], HIDEKI GARREN[1], ANNETTE LANGER-GOULD[1], SAMUEL STROBER[2], BARBARA CANNELLA[7], JOHN ALLARD[8], PAUL KLONOWSKI[8], ANGELA AUSTIN[8], NAGIN LAD[8], NAFTALI KAMINSKI[6], STEPHEN J. GALLI[3], JORGE R. OKSENBERG[5], CEDRIC S.RAINE[7], RENU HELLER[8] & LAWRENCE STEINMAN[1]

Departments of [1]Neurology and Neurological Sciences, Beckman Center, [2]Medicine and [3]Pathology, Stanford University, Stanford, California, USA
[4]Informatics, Rosetta Inpharmatics, Kirkland, Washington, USA
[5]Department of Neurology and [6]Lung Biology Center and Cardiovascular Research Institute, University of California at San Francisco School of Medicine, San Francisco, California, USA
[7]Department of Pathology (Neuropathology) and Neurology, Albert Einstein College of Medicine, Bronx, New York, USA
[8]Roche Bioscience, Palo Alto, California, USA
C.L., G.H., R.P. and A.B. contributed equally to this study.
Correspondence should be addressed to R.H. or L.S.; email: rheller@stanford.edu or steinman@stanford.edu

Microarray analysis of multiple sclerosis (MS) lesions obtained at autopsy revealed increased transcripts of genes encoding inflammatory cytokines, particularly interleukin-6 and -17, interferon-γ and associated downstream pathways. Comparison of two poles of MS pathology—acute lesions with inflammation versus 'silent' lesions without inflammation—revealed differentially transcribed genes. Some products of these genes were chosen as targets for therapy of experimental autoimmune encephalomyelitis (EAE) in mice. Granulocyte colony-stimulating factor is upregulated in acute, but not in chronic, MS lesions, and the effect on ameliorating EAE is more pronounced in the acute phase, in contrast to knocking out the immunoglobulin Fc receptor common γ chain where the effect is greatest on chronic disease. These results in EAE corroborate the microarray studies on MS lesions. Large-scale analysis of transcripts in MS lesions elucidates new aspects of pathology and opens possibilities for therapy.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of central nervous system (CNS) white matter. Environmental factors and a genetically determined susceptibility are both implicated in a misdirected immune response against myelin antigens. Microarray technology provides an image of gene expression in MS lesions on an unprecedented scale.

We analyzed samples of fresh frozen brain lesions obtained at early autopsy (1.5–4.0 h post mortem) from four MS patients, and compared the pattern of gene expression against two controls without nervous system pathology. Comparison of transcripts from acute and 'chronic active' lesions versus 'chronic silent' lesions revealed several genes not previously known to be important in MS, and they were differentially expressed in the acute/active versus chronic silent lesions. By targeting the products of two of these new candidates, we were able to ameliorate experimental autoimmune encephalomyelitis (EAE) in mice, a model with similarities to MS.

Histopathology of MS lesions obtained rapidly at autopsy

The clinical characteristics of the MS subjects and controls are listed in Table 1. The MS lesions examined in this study fell into three categories: 1) acute MS lesions, comprising areas of recent inflammation and edema, ongoing demyelination, abundant astroglial hypertrophy and an ill-defined margin showing myelin vacuolation but little or no fibrous astrogliosis (Fig. 1*a* and *b*); 2) chronic active MS lesions, comprising areas of long-term demyelination and fibrous astrogliosis, the well-demarcated margins of which had superimposed regions of recent inflammation and ongoing demyelination (Fig. 1*c* and *d*; and 3) chronic silent lesions, displaying no inflammatory activity, abundant gliosis and well-demarcated margins[1]. The neurologically normal cases examined displayed no CNS neuropathology, and were devoid of inflammatory lesions.

Microarrays of MS lesions reveal patterns in transcripts

Genes with at least a two-fold upregulation in expression in all four MS samples were clustered and visualized using cluster analysis[2] (Fig. 1). Cluster analysis determines correlation coefficients betweens pairs of genes, and organizes genes by similarities in expression pattern. The numerical output consists of a color-coded table and dendrogram that are displayed by the program TreeView[2]. Genes with increased expression are colored red, whereas genes with decreased expression are colored green. Several regions were chosen from the clustering (Fig. 1), and genes having 2-fold or greater expression changes in 3 of 4 MS samples are provided (see Supplementary Tables A and B online). Genes that were increased (39) or decreased (49) in 4 of 4 MS samples were reclustered in Figs. 2 and 3.

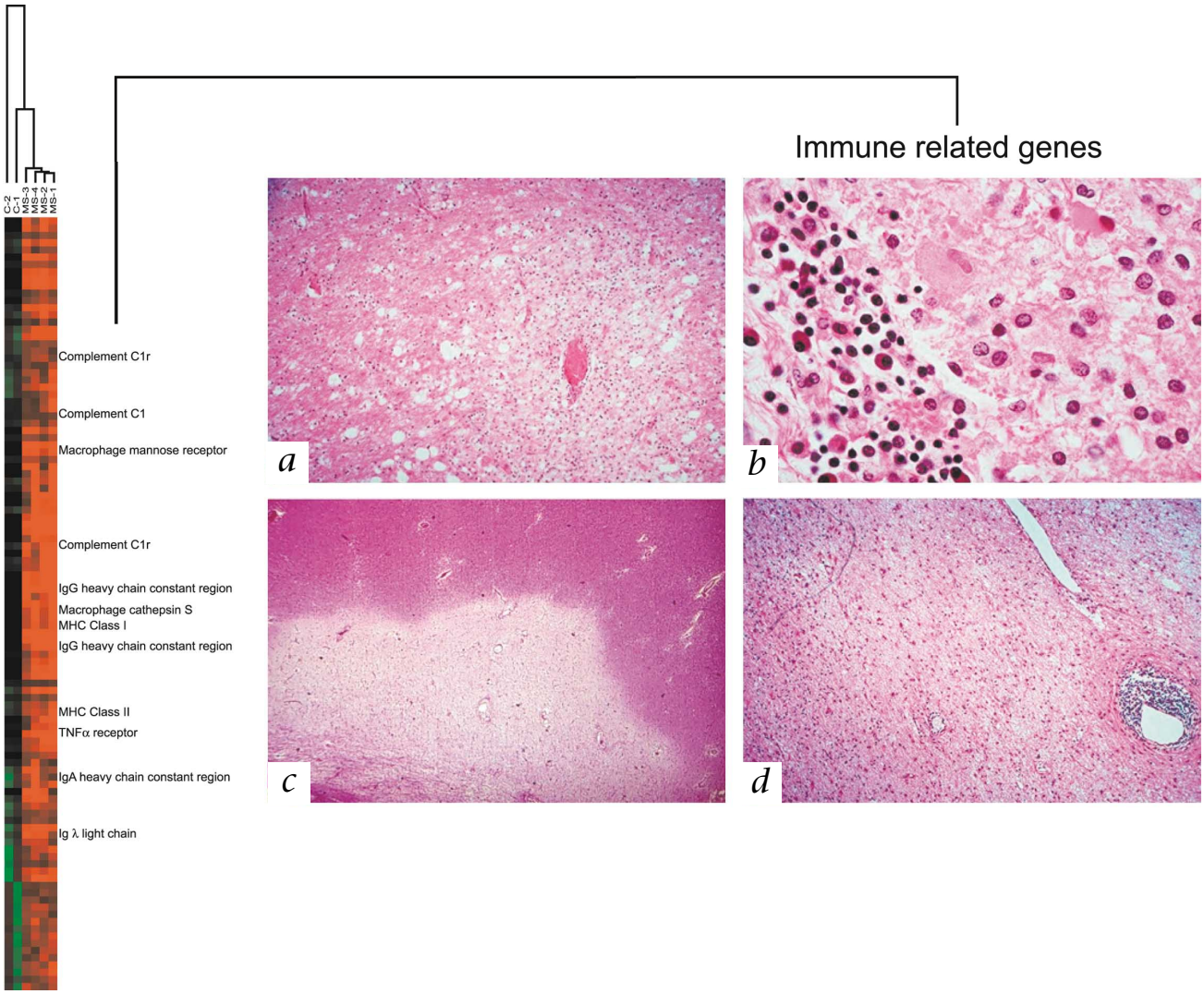

Fig. 1 Histopathology of analyzed MS plaques. ***a***, Low-power image of an acute MS lesion. Note intense inflammation, edema and non-fibrotic nature of the tissue and the indistinct lesion margin showing myelin vacuolation. H&E preparation; magnification, ×175. ***b***, Adjacent to a perivascular cuff containing small lymphocytes and plasma cells, hyptertrophic astrocytes are also apparent in the edematous lesion parenchyma. H&E preparation; magnification, ×750. ***c***, Chronic active MS lesion shows a well-demarcated edge and a fibrotic, demyelinated center. H&E preparation; magnification, ×60. ***d***, Recent inflammation around the edge of the lesion in (*c*). H&E preparation; magnification, ×250. In the left-hand panel is a cluster analysis of MS samples and controls. Genes with at least a 2-fold change in expression in 4 of 4 MS samples (1,080 genes) were clustered. The change in red or green in the control columns indicates how much each control departs from the control mean. Genes colored in red are more highly expressed in the MS sample than in the controls, and genes colored green are more highly expressed in the control samples.

One of the strengths of the Affymetrix GeneChip technology is that there are multiple probes to detect each gene. Here we analyze four samples from the MS population and two samples from the non-MS population. Although these sample sizes are not large enough to obtain entirely reliable estimates of the true variation structures of each of the genes tested in the respective populations, it did allow us to obtain *P* values, which in this setting are useful in rank-ordering genes for further validation[3–5]. The last column in Supplementary Tables A and B summarizes the *P* values for some of the relevant genes.

Genes with increased expression

We observed a picture of active migration of lymphocytic cells. Both $CD4^+$ and $CD8^+$ T cells were present in lesions, but alterations in levels of transcription of T-cell receptor (TCR), CD4 and CD8 genes were less than two-fold in the lesion material studied. $CD4^+$ and $CD8^+$ T-cell counts in the four MS samples revealed some $CD4^+$ and $CD8^+$ T cells in samples MS 2, 3 and 4, with much larger numbers of these T cells in the highly active sample, MS 1. Other T-cell transcripts such as that for interleukin-17 (IL-17) were elevated.

Upregulation of immune response genes included class II major histocompatibility complex (MHC) molecules and immunoglobulin G (IgG). Adhesion molecules integrin β_4 and P-selectin ligand are increased. B-cell presence is reflected by a novel B-cell activation gene, *BL34*, which is characteristic of mitogen-activated B-cells[6]. Several genes provide evidence for the presence of macrophages. Macrophage capping protein is a gelsolin-like molecule expressed primarily by differentiated macrophages[7]. MCL1 is expressed in early differentiation of the monocyte/macrophage pathway and is similar to BCL2 in inhibiting cell death[8]. Gp-39 is a secreted member of the chitinase

Table 1 MS and control subjects

	Age/ethnicity/gender HLA type	Diagnosis & clinical signs	Disease duration	Past medication	Lesion type	Cause of death	Autopsy interval
MS 1	31/W/F DRB1*1501 DRB5*0101	Chronic progressive MS with acute MS lesions, quadriparesis, seizures	11 y	Prednisone Solumedrol	Acute Chronic active	Respiratory failure	1.5 h
MS 2	59/W/F DRB1*1504 DRB5*0101	Chronic MS, secondary progressive quadriparesis	35 y	None	Chronic active Chronic silent	Bronchopneumonia	4 h
MS 3	38/W/F	Secondary progressive MS with chronic active lesions, diplopia, ataxia, dysphagia	11 y	Cytoxan	Chronic active Chronic silent	Bronchopneumonia	4 h
MS 4	46/W/M DRB1*03	Chronic progressive MS, paraplegia	15 y	Lioresal Compazine	Chronic silent	Cardiac arrest	4 h
Control 1	84/B/F	Bowel disease	4 mo			Pulmonary embolism	8 h
Control 2	57/W/M	Sudden death				Trauma	

Characteristics of the 4 MS and 2 control subjects studied are shown. Subject MS 1 had acute, active MS with widespread inflammatory involvement in the white matter. 2 separate samples of brain lesions were obtained from subject MS 1. All brain samples were rapidly frozen after early autopsy. Controls 1 and 2 died of non-neurologic illnesses. Full CNS autopsies were performed on all MS subjects and control 1. Control 2 was from a subject who died from trauma and was purchased as whole human brain polyA$^+$ mRNA.

family and is considered a marker for late stages of macrophage differentiation[9]. Complement activity is suggested by increases in C1r and C3 and increases in several molecules involved in the regulation of the complement cascade. Complement is an important mediator of damage to myelin and expression of a soluble inhibitor of complement activation, sCrry, prevents EAE in the mouse[10]. CD59, or protectin, has a role particularly in inhibition of the formation of complement membrane attack complexes[11] and is involved in T-cell and natural killer–cell function. Pregnancy-associated plasma protein A (PAPPA) is a large zinc glycoprotein whose function is not fully known. The sequence shows conserved motifs resembling the short consensus repeats of complement control proteins.

IL-1 receptor (IL-1R), IL-8 receptor type 2, IL-11 receptor α, IL-17 and p75 tumor necrosis factor–receptor transcripts were elevated reflecting pro-inflammatory cytokine activity. The type 1 IL-1R mediates all biological responses to IL-1α and IL-1β, and there are two or more signaling pathways leading to induction of nuclear factor-κB (NF-κB) and mitogen-activated serine–threonine protein kinase (MAPK) activity. IL-1 is detected in cerebrospinal fluid (CSF) in MS (ref. 12), and IL-1 exacerbates EAE while soluble IL-1R suppresses EAE (ref. 13). There is an increased number of IL-17-expressing mononuclear cells (MNCs) in blood and CSF (ref. 14). Tumor necrosis factor (TNF) is a key cytokine in the pathogenesis of MS and EAE. We have previously demonstrated both TNF and TNF receptor with immunohistochemistry in MS plaques[15].

A number of cytokine related signaling molecules and transcriptional activators are seen in the MS samples. Most intriguing is the upregulation of nuclear factor-interleukin-6 (NF-IL6), also known as CCAAT/enhancing-binding protein (C/EBP). NF-IL6 is increased with a range of 3.6 to 12.9-fold in all four MS samples. NF-IL6 was originally isolated as a DNA binding protein mediating IL-1 induced IL-6 production[16]. NF-IL6 regulates transcription of IL-6 via a 14-bp palindromic binding site. Elevated levels of IL-6 have been noted in autoimmune diseases such as rheumatoid arthritis and MS. A monoclonal antibody against IL-6 can reverse EAE (ref. 17). As well as regulating transcription of the gene encoding IL-6, NF-IL6 also controls downstream

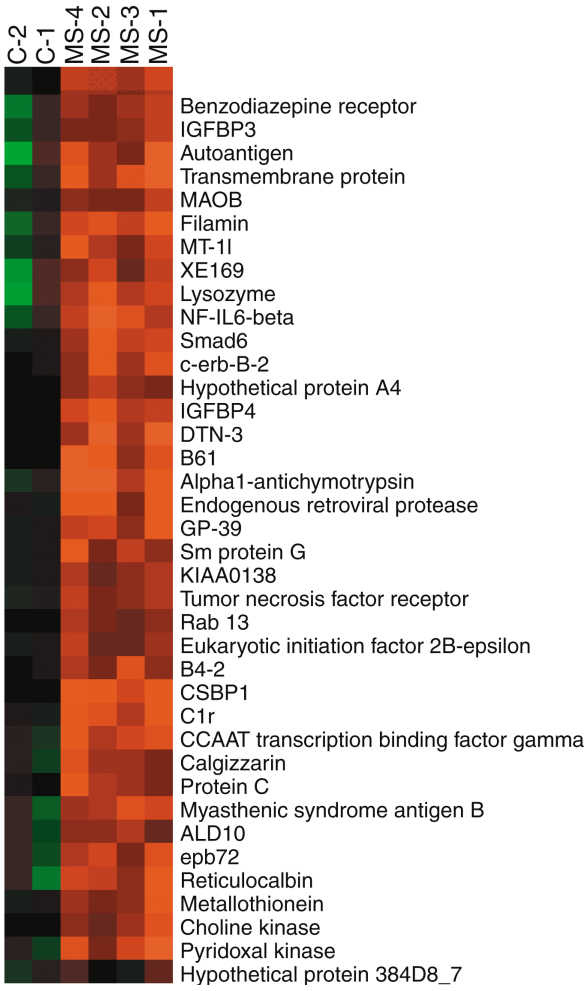

Fig. 2 Genes increased in 4 of 4 MS samples. Samples from Fig. 1 were reclustered using TreeView[2].

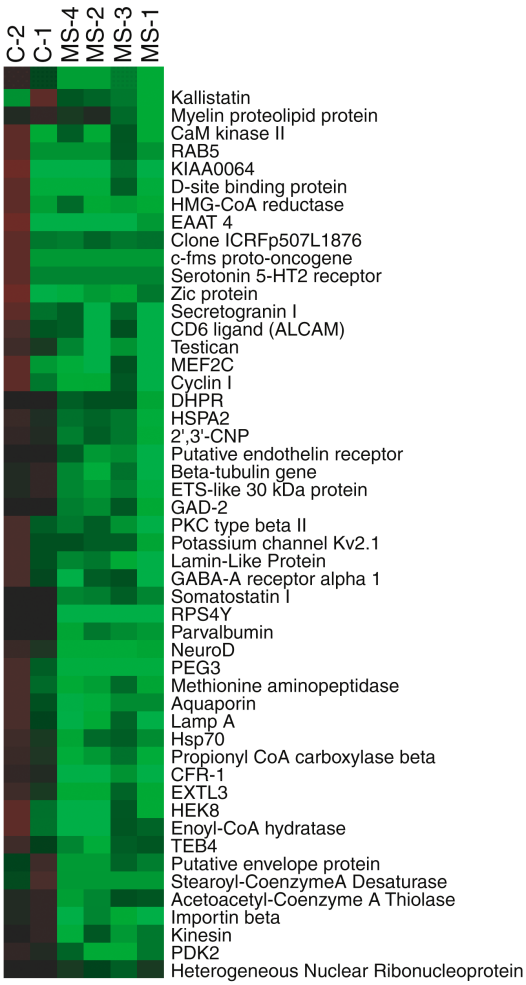

Fig. 3 Genes decreased in 4 of 4 MS samples. Samples from Fig. l were reclustered using TreeView[2].

genes induced by IL-6, and can bind to the regulatory regions of genes encoding IL-1, IL-8, granulocyte colony-stimulating factor (G-CSF) and immunoglobulin, as well as genes such as antichymotrypsin. Another gene connected with IL-6 signaling, that encoding c-erb-B-2, a surface-receptor tyrosine kinase, is elevated in the MS samples.

Other cytokine-signaling molecules seen in the MS material are noted. NF-κB p65 subunit RNA is increased in the MS samples. NF-κB binding sites are present in regulatory regions of TNF, IL-6, IL-8, interferon-γ (IFN-γ), IL-2R and MHC class I and II. TNF-receptor signaling occurs partly via NF-κB. B61 (ephrin A1, LERK1) is part of the TNF signaling pathway. It is induced by TNF and binds to the erythropoietin-producing hepatoma amplified sequence (EPH) group of receptor tyrosine kinases. Increased expression of the p75TNF receptor in the samples may indicate its expression as a mechanism to reduce active TNF by binding to its soluble components, or indicate prolonged inflammatory signaling[18]. The MAPKs are mediators of the TNF signaling pathway, and the upregulation of MAPKs in MS tissue suggests inflammatory signaling. CSAIDS (mitogen-activated protein kinase 14; p38 MAPK) was significantly increased. p38-γ (ERK6, MAPK12) is a member of the p38 family which is activated by IL-1β and TNF. MEF2 can regulate the expression of genes that are critical for survival of newly differentiated neurons[19].

Glial scarring or sclerosis occurs in MS. An increase in glial fibrillary acidic protein (GFAP) may be a reflection of increased astrocyte activity. Several stress related genes are increased, such as heat-shock proteins, cytochrome and metallothionein genes. Metallothionein knockouts have been shown to have more severe EAE than wild-type controls[20]. NMDA (*N*-methyl-D-aspartate) receptor transcripts were observed in some samples. An NMDA antagonist has been shown to reduce severity of EAE (ref. 21). A full listing of these increased genes is shown in supplementary Table A.

Genes with decreased expression

Genes encoding proteins associated with myelin were decreased, including myelin associated glycoprotein, peripheral myelin protein 2, proteolipid protein, oligodendrocyte-myelin glycoprotein and myelin-associated oligodendrocytic basic protein. Transcription of UDP-galactose ceramide galactosyl transferase (cerebroside synthase) were decreased. UDP-galactose ceramide galactosyl transferase is a key enzyme in the biosynthesis of glycosphingolipids, cerebrosides and sulfatides, which are essential lipid constituents of the myelin membrane. Reduced expression of these myelin-associated proteins and enzymes may indicate not only reduced levels of myelin, but also a reduced capacity for repair.

Axonal loss is a key pathological process in MS (ref. 22) and neuron-specific genes were generally decreased in the MS samples. These genes included those encoding pentraxin 1; neuronal growth factors such as neuronal growth protein GAP-43 and neuron-specific growth-associated protein SCG10; structural proteins such as neurofilament light and medium proteins; and synaptic proteins such as synaptogamin, synaptobrevin 1 and 2, axonal transporter of synaptic vesicles and synapsin. M6 is a membrane glycoprotein expressed on central neurons, and was decreased in the MS samples. M6 shows high homology with the CNS myelin protein PLP/DM20, but is neuron-specific[23]. A full listing of these decreased genes is shown in supplementary Table B.

Comparison of acute/active lesions with silent lesions

In most MS brains, there is a spectrum, approximating a continuum, of different types of MS lesions. This is not readily apparent in the limited volumes available on needle biopsy specimens. Sample 1 stands at one extreme with acute inflammation, whereas sample 4 stands at the opposite end with only

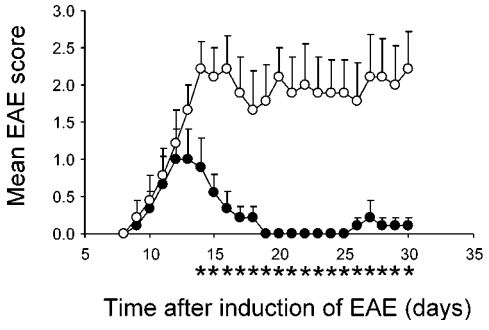

Fig. 4 EAE in immunoglobulin FcRγ-chain-knockouts. EAE was induced with MOG35-55 in 8–12-wk-old female knockout (●) ($n = 9$) and wild-type mice (○) ($n = 9$). Data represent mean EAE score ± s.e.m. *, $P < 0.05$; determined by Mann–Whitney statistics.

Table 2 Genes differentially expressed in acute/active or chronic/silent lesions

Upregulated in acute/active plaques only							
Accession number	Entrez definition	MS-1	MS-3	MS-2	MS-4	Ratio	*P* value
T cells/B cells							
M63438	Ig rearranged γ chain mRNA	3265	1764	< 20	< 20	125.73	0.00000
L05624	MAP kinase kinase mRNA	746	2123	< 20	< 20	71.72	0.11472
X69398	mRNA for OA3 antigenic surface determinant	792	228	47	< 20	15.17	0.00192
X05323	MRC OX-2 gene, signal sequence	385	679	110	< 20	8.18	0.52582
U47686	Stat5B mRNA	590	264	91	< 20	7.72	0.29461
Granulocytes/mast cells							
U52518	Grb2-related adaptor protein (Grap)	1387	283	< 20	< 20	41.74	0.87836
X55990	ECP gene for eosinophil cationic protein	1794	278	93	< 20	18.37	0.00000
Scar tissue formation/remyelination/neurogenesis							
L32961	4-aminobutyrate aminotransferase (GABAT)	907	616	< 20	< 20	38.07	0.99603
U50822	Neurogenic helix-loop-helix protein NEUROD gene	652	221	< 20	< 20	21.83	0.47202
U64573	Connexin 43 gap junction protein (connexin43) gene	559	210	< 20	< 20	19.21	0.80434
M19878	Calbindin 27 gene, exons 1 and 2, and Alu repeat	403	407	25	< 20	17.86	0.01671
M86849	Connexin 26 (GJB2) mRNA	239	207	< 20	< 20	11.15	0.29995
S38953	XA, genomic	481	417	188	< 20	4.33	0.08224
Growth factors/cytokines							
M13755	Interferon-induced 17-kD/15-kD protein mRNA	964	1043	< 20	< 20	50.16	0.37022
X57025	IGF-I mRNA for insulin-like growth factor I	1141	211	< 20	< 20	33.80	0.00001
X03656	G-CSF protein gene	1151	512	< 20	107	13.08	0.02625
Z70276	mRNA for fibroblast growth factor 12	226	200	< 20	< 20	10.65	0.24924
U66198	Fibroblast growth factor homologous factor 2	428	402	< 20	156	4.72	0.05970
Endocrine factors/receptors							
U55764	Estrogen sulfotransferase mRNA	1375	455	44	< 20	28.42	0.22099
D49487	mRNA for obese gene	1200	299	172	59	6.51	0.54287
S77415	Melanocortin-4 receptor	640	375	143	110	4.01	0.18428
Pregnancy-related							
M23575	Pregnancy-specific β1 glycoprotein mRNA	559	244	153	< 20	4.65	0.05666
Upregulated in chronic/silent plaques only							
Accession number	Entrez definition	MS-1	MS-3	MS-2	MS-4	Ratio	*P* value
T-cells/B-cells							
M14159	T-cell receptor β-chain J2.1 gene	< 20	< 20	653	582	30.86	0.84407
K02882	IGHD gene (IgD chain)	< 20	54	402	355	10.29	0.33429
M21934	Rearranged and truncated Ig γ heavy chain disease (RIV) protein gene V-J6 region	< 20	113	571	645	9.17	0.64176
L35253	p38 mitogen activated protein (MAP) kinase	103	151	359	1313	6.57	0.88474
X92521	mRNA for MMP-19 protein	< 20	127	219	710	6.32	0.75678
Macrophages/microglia							
Z48481	mRNA for membrane-type MMP-1	< 20	63	311	1456	21.41	0.08352
M35999	Platelet glycoprotein IIIa (GPIIIa) mRNA	< 20	< 20	335	264	14.98	0.18171
U10492	Mox1 protein (MOX1) mRNA	< 20	126	647	1462	14.43	0.05954
D10202	mRNA for platelet-activating factor receptor	< 20	129	379	920	8.73	0.37058
M63835	IgG Fc receptor I gene	< 20	157	350	1000	7.64	0.59965
M34344	Platelet glycoprotein IIb (GPIIb) gene	< 20	183	527	642	5.76	0.67570
X13334	CD14 mRNA for myelid cell-specific leucine-rich glycoprotein	< 20	195	270	798	4.96	0.54780
Granulocytes/mast cells							
U30998	nmd mRNA, 3'UTR.	< 20	< 20	276	919	29.90	0.60368
M33493	Tryptase-III mRNA, 3' end	< 20	61	499	1170	20.53	0.30590
D25303	mRNA for integrin a subunit	< 20	49	595	794	20.15	0.36080
M89796	High affinity IgE receptor β chain gene	< 20	< 20	254	486	18.51	0.01804
Y10205	mRNA for CD88 protein	< 20	28	261	344	12.60	0.79376
Z34897	mRNA for H1 histamine receptor	< 20	110	266	330	4.59	0.00484
Scar tissue formation/remyelination/neurogenesis							
M94250	Retinoic acid inducible factor (MK) gene exons 1-5	< 20	< 20	1008	1503	62.77	0.33031
X78565	mRNA for tenascin-C	< 20	56	412	1578	26.15	0.49690
L41162	Collagen α 3 type IX (COL9A3) mRNA	< 20	106	286	2755	24.20	0.00002
HG2730-HT2827	Fibrinogen	< 20	24	305	627	21.07	0.87339
M26682	T-cell translocation gene 1 (Ttg-1) mRNA	< 20	< 20	425	348	19.32	0.76570
U26403	Receptor tyrosine kinase ligand LERK-7 precursor	< 20	27	529	386	19.30	0.60340
Y07683	mRNA for P2X3 purinoceptor	< 20	119	580	2023	18.77	0.60841
X14885	Gene for TGF-β 3 (TGFb 3) exon 1	50	< 20	266	986	17.91	0.41797
HG3248-HT3425	Fibroblast growth factor, antisense mRNA	< 20	125	393	425	5.64	0.03060
X06700	mRNA 3' region for pro-α1(III) collagen	181	42	229	519	3.36	0.91389
Growth factors/cytokines							
U32659	IL-17 mRNA	< 20	39	211	841	17.71	0.01032
Endocrine factors/receptors							
X65633	adrenocorticotropic hormone receptor	< 20	166	677	1767	13.12	0.49983
X04707	c-erb-A mRNA for thyroid hormone receptor	< 20	82	434	363	7.81	0.20754
Pregnancy-related							
U25988	Pregnancy-specific glycoprotein 13	< 20	189	460	569	4.93	0.07192

List of genes whose transcripts were elevated 2-fold or more in either the acute/active cases 1 and 3 only, or in the chronic silent cases 2 and 4 only. A more detailed analysis of all of the genes uniquely transcribed in either the acute active or chronic silent cases will be made available online. Genes have been tentatively grouped according to known or suspected functions in MS lesions.

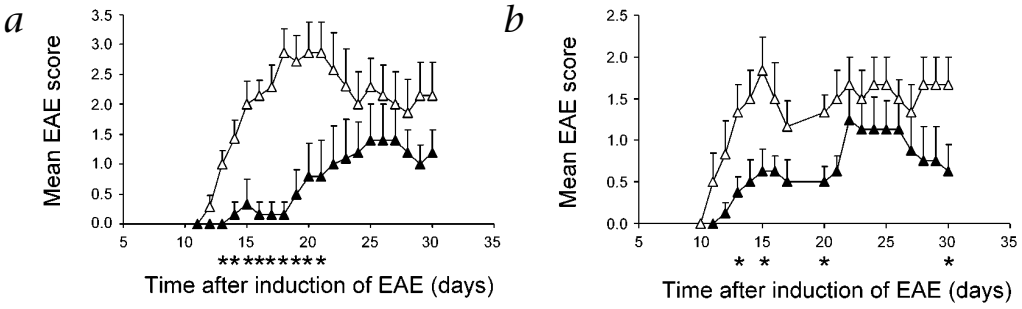

Fig. 5 Effect of G-CSF on EAE, two consecutive experiments. ***a*** and ***b***, EAE was induced with MOG35-55 after G-CSF (▲) or vehicle (△) was injected s.c. for 6 consecutive days from days –7 to –1 (*a*), or from days –14 to –8. (*b*), Data represent mean EAE score ± s.e.m. *, $P < 0.05$; as determined by Mann–Whitney statistics.

chronic silent lesions. Samples 2 and 3 had a mix of both chronic active inflammatory plaques, and chronic silent plaques. Electronic cluster analysis of samples either increased in all four specimens or decreased in all four specimens, placed samples 1 and 3 together, and samples 2 and 4 next to each other and nearer to the controls, which had no inflammatory lesions (Figs. 2 and 3).

Table 2 demonstrates some of the genes whose transcripts were elevated two-fold or more in either the acute/active cases 1 and 3 only, or in the chronic silent cases 2 and 4 only. Some of the highlights of Table 2 include rearranged variable-joining-constant region (VJC) immunoglobulin elevated 125-fold in the 2 active samples. A MAP kinase kinase is elevated 72-fold in the acute active samples. Various growth factors are elevated in the acute active cases, including insulin growth factor-1, which may facilitate remyelination is elevated 34-fold. G-CSF is elevated 13-fold. Fibroblast growth factor-12 (FGF-12) and FGF-2 homolog are elevated 10.7-fold and 4.7-fold, respectively.

Integrin α was elevated in chronic silent MS. We have shown previously that antibodies against α_4 integrin reverses and reduces relapses in relapsing EAE (ref. 24). A humanized antibody to α_4 integrin is now in phase 3 clinical trials.

Various gene transcripts associated with Th2 or allergic responses are elevated in these studies: eosinophil cationic protein transcripts are elevated 18-fold in acute MS, whereas IgE receptor is elevated 18-fold in the chronic silent cases. Histamine receptor H1 is elevated 4.6-fold in the chronic silent cases. IgG Fc receptor 1 is elevated 7.6-fold in the chronic silent cases.

IL-17 was elevated 17.7-fold in the chronic silent cases, while transforming growth factor-β (TGF-β) is increased 17.9-fold in the chronic silent cases, perhaps reflecting gliosis or suppression of the inflammatory response. Matrix metalloprotease (MMP) inhibitors reverse EAE and MMP-2 and MMP-9 are elevated in MS (ref. 25). A distinct MMP, MMP-19, is elevated 19-fold in chronic silent MS lesions .

Various targets of neuroendocrinological interest are elevated in MS brain, including leptin, melanocortin receptor type 4 and adrenocorticotropic hormone receptor (ACTH-R). Leptin is elevated 6.5-fold in acute/active MS versus chronic silent MS, melanocortin receptor type 4 is elevated 4-fold and ACTH-R is elevated 13-fold in chronic MS versus acute. Some of these genes have products that are targets for MS therapeutics, or have been successfully modulated in treating EAE, an animal model of MS (ref. 25).

Validation of new targets in the EAE model

EAE has proven to be a durable model for validation of targets for therapy of MS (ref. 25). Currently the β interferons and Copaxone, all approved for treatment of MS in Europe and the US, have proven effective in EAE (ref. 25). Various drugs in different stages of clinical testing, including anti-α_4 integrin and altered peptide ligands, have also proven effective in EAE. We have chosen two genes whose level of transcription was altered in either acute/active or chronic silent MS lesions (Table 2), and have explored the role of their gene products in EAE (see below). Such validation would be important before pursuing further clinical studies of these targets.

Ig FcR influences the recovery phase of EAE

To assess the importance of FcγR and FcεR in the development of EAE, we used mice with targeted mutations of the common γ chain of the high-affinity receptor for IgE (FcεRI) and the low-affinity receptor for IgG (FcγRIII)[26,27]. In these mice, functional expression of the high affinity FcγRI is also diminished. EAE was ameliorated in immunoglobulin FcR γ-chain knockouts compared with wild type (Fig. 4). We demonstrate highly significant changes in the disease score during the chronic or recovery stage of the disease, particularly from day 20 onwards, where disease is absent in the immunoglobulin Fcγ-receptor knockout mice. This is concordant with microarray data on MS lesions, where Fc receptor transcripts are elevated in chronic lesions, but not in acute lesions (Table 2). For example, the incidence of EAE (4/9 in the FcRγ-chain knockout versus 9/9 in the wild type), the mean disease score at day 15 (0.56 ± 0.24 in the FcRγ-chain knockout versus 2.11 ± 0.39 in the wild type; $P < 0.0037$) and the mean peak of disease severity (1.0 ± 0.41 in the FcRγ-chain knockout versus 2.89 ± 0.35 in the wild type; $P < 0.012$) were reduced in the FcRγ chain-deficient mice compared with the wild type. Intravenous immunoglobulins may act via targeting the inhibitory receptor FcγRIIb (ref. 28). Intravenous immunoglobulin (IVIG) has been shown to be therapeutic in EAE, with some evidence of efficacy in MS (ref. 29).

G-CSF influences the acute phase of EAE

G-CSF is elevated in the acute active cases, 13-fold relative to the chronic silent cases. We have performed two experiments to analyze the influence of G-CSF on the development of EAE. G-CSF was injected subcutaneously (s.c.) for 6 consecutive days at the dose of 250 μg/kg, before EAE was induced with MOG35-55. EAE was prevented in the G-CSF-treated group compared with the control (mean day of disease onset 19.2 ± 1.9 in G-CSF treated group versus 13.1 ± 0.5 in vehicle-treated group, $P < 0.0047$; mean disease score at day 15, 0.4 ± 0.4 in G-CSF-treated group versus 2.0 ± 0.38 in vehicle-treated group; $P < 0.048$) (Fig. 5*a*). In a second experiment, G-CSF treatment still reduced the mean EAE score at day 15, albeit with a milder effect (0.63 ± 0.26 in G-CSF treated group versus 1.83 ± 0.4 in vehicle treated group; $P < 0.0426$) (Fig. 5*b*).

Discussion

In MS, evidence points to an immune response against components of the myelin sheath. A widely accepted view of the process of demyelination suggests that T cells, immunoglobulin and complement components have roles in pathogenesis. Adhesion molecules, cytokines, chemokines, HLA molecules and metalloproteases are critical participants in the development of the inflammatory response in brain. Autoantibodies against myelin antigens, such as myelin basic protein and myelin oligodendroglial glycoprotein also have an important role in demyelination[30–32]. Many of these participants in the pathogenesis of MS have been demonstrated at the sites of lesions by standard methods of immunohistochemistry, *in situ* hybridization and reverse-transcriptase PCR. In each of these approaches, only one or at most a few genes or proteins were assessed. Here we compared the simultaneous transcriptional behavior of thousands of genes, thus providing a comprehensive transcriptional profile of MS.

Most microarray expression studies published to date have used cell culture systems where conditions are more easily controlled and the cell population is homogeneous. Tissue samples from MS have been used less frequently, with one report describing two MS lesions from one brain[33]. In the paper studying two MS lesions from one brain by Whitney *et al.*[33], 29 genes were found to have increased expression in acute MS plaques. These 29 genes were represented on the HuGeneFL chip, except for α-2-chimerin, which was replaced by β chimerin. We found 8 of these 29 genes increased in at least 2 of the 4 MS samples.

Our investigation of MS brain tissue revealed several new targets for potential therapy. Modulating these targets can lead to amelioration of EAE, and we will report soon on other examples from Table 2. For instance, we examined the transcriptional profile of genes activated in encephalitogenic T cells. T cells specific for the mouse encephalitogenic epitope myelin basic protein amino acids 1 through 11, amino terminal acetylated (MBP Ac1-11) were obtained from naive non-immunized MBP Ac1-11 T-cell receptor-transgenic mice. After purification, the T cells were stimulated *in vitro* for various time periods with either the encephalitogenic peptide MBP Ac1–11 or the same peptide altered at the fourth position with a substitution of a tyrosine for a lysine residue (MBP Ac1–11 (4Y)). 4Y is known to prevent the onset of EAE and is generally considered a superagonist for MBP Ac1-11 TCRs (ref. 34). One of the genes that was highly upregulated in MBP Ac1–11 stimulated T cells but not in 4Y stimulated T cells, is MAP kinase kinase 2, which is also known as Mek2. Expression levels were elevated approximately 7-fold over baseline unstimulated T cells after four hours of stimulation with the peptide MBP Ac1–11, but was not significantly elevated after stimulation with 4Y. Analogous findings are demonstrated in the human brain samples. As shown in Table 2, MAPKK is elevated 72-fold in acute active demyelinating lesions versus chronic silent lesions. These findings in mouse T cells and human brain plaques are consistent: MAPKK2, the mouse homolog of the related but not identical MEK2, is elevated in encephalitogenic T cells stimulated with the pathogenic peptide MBP Ac1–11, causing acute EAE, and within acutely diseased MS plaques (Table 2).

Histamine or serotonin is involved in the development of EAE (ref. 35). Cyproheptadine, an anti-histamine, anti-serotoninergic drug, ameliorated clinical disease. Cyproheptadine has a K_i of 2.1 nM for histamine H1 receptor, 4.1 for 5-HT2 receptor, 27 for muscarinic cholinergic receptor and 94 for 5-HT1A receptor[35]. Muscarinic m5 acetylcholine receptor (AchR) that is also targeted by cyproheptadine is also elevated 11-fold in chronic MS (Table 2). Experiments with cleaner drugs more specific for histamine 1 receptor or muscarinic M5 AChR are in progress, with positive results to be reported for a pure histamine H1 antagonist in EAE (unpublished data). One of the potential virtues of medications like cyproheptadine is that they cross the blood–brain barrier.

Gene microarrays have revolutionized the pathological diagnosis of cancer, and may similarly aid in the categorization of MS lesions[33]. Further studies will elucidate how the lesions studied here correspond to other classifications of MS pathology, using more classical techniques on paraffin-embedded biopsy and autopsy specimens[36]. Several whole-genome mapping projects have identified a number of loci associated with susceptibility to MS (refs. 37–39). Genes examined here can be correlated with linkage studies.

Our data implicate several new genes. We exemplified two of these new discoveries in MS lesions, with validating data from EAE. We corroborated the EAE result with the transcriptional analysis of active and silent MS lesions: G-CSF is upregulated in acute, but not in chronic MS lesions, and the effect on EAE is more pronounced in the acute phase of the disease, in contrast to FcRγ-chain where the reverse is true, again in concordance with MS studies. Interestingly when given before the onset of EAE, G-CSF has its major influence on the acute, rather than the chronic stage of the disease. Reversal of ongoing EAE with G-CSF has been reported[40]. G-CSF production in acute EAE and acute MS may be one of the regulatory molecules that naturally suppress acute attacks.

Other genes in the neuroendocrine area are worthy of comment. We have recently found that serum from pregnant females reduces relapses of EAE, without inducing a Th1/Th2 shift (data not shown). Two genes related to pregnancy, Pregnancy-associated plasma protein A (PAPPA), and pregnancy related glycoprotein, were described here. We are currently investigating whether these two proteins are the key factors in sera from pregnancy.

Leptin, melanocortin 4 receptor and ACTH-R are differentially elevated in either chronic or acute EAE (refs. 41–43). Lechler and colleagues showed that leptin ameliorated EAE and induced a Th2 shift[41]. We have shown that corticotropin-releasing factor (CRF) and urocortin, which leads to ACTH secretion, suppresses EAE (ref. 43). ACTH and corticosteroids are somewhat successful in reducing the intensity and duration of relapses of MS (ref. 42).

Finally some proteins associated with stress responses in neurodegeneration, such as IL-6 and metallothioneins, were shown to be elevated in MS lesions. Metallothioneins have a modulating affect in EAE, where studies with knockouts have shown worsening of EAE (ref. 20). IL-6 is elevated in several neurodegenerative diseases, and we have previously shown that anti-IL6 ameliorates EAE (ref. 17).

Microarray studies provide a powerful technological innovation for the simultaneous imaging of large ensembles of genes in MS tissue. A variety of new therapeutic targets could possibly emerge from such studies.

Methods

Human brain samples. MS brain samples and control-1 were obtained at autopsy under an IRB approved protocol. Samples were snap frozen in liquid nitrogen and stored at –80 °C. Control-2 was purchased from Clontech as poly(A$^+$) mRNA.

EAE. EAE was induced in 8–12-wk-old C57Bl/6 female mice (Jackson Labs, Bar Harbor, Maine) as described[35]. Briefly, MOG35-55 peptide was dissolved in PBS to a concentration of 2 mg/ml and emulsified with an equal

volume of IFA supplemented with 4 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories, Detroit, Michigan). Mice were injected s.c. with 0.1 ml of the peptide emulsion and on the same day and 48 h later, were injected intravenously with 0.2 ml of 1 μg/ml *Bordetella pertussis* toxin in PBS. Mice were assessed daily for clinical signs of EAE. The same protocol was used to induce EAE in 8–12-wk-old female FcRγ-chain deficient and wild-type (B6129PF2) mice (Jackson Laboratory, Bar Harbor, Maine). To evaluate the effect of G-CSF on the development of EAE, hrG-CSF (Neupogen; Amgen, Thousand Oaks, California) was given s.c. for 6 d before induction of EAE at a dose of 250 μg/kgs. Mice were maintained in the Research Animal Facility at Stanford, or at Roche, and experiments were conducted in accordance with NIH guidelines.

Sample preparation and chip hybridization. Brain was homogenized in TRIzol reagent (Invitrogen, Carlsbad, California) using a Polytron (Kinematica AG, Luzern, Switzerland) and total RNA prepared according to the recommended protocol. mRNA was purified by 2 rounds of selection using oligo(dT) resin (Oligotex, Qiagen, Hilden, Germany). 2 μg mRNA were used to prepare double-stranded cDNA (Superscript, Invitrogen). The primer for cDNA synthesis contained a T7 RNA polymerase promoter site. 1 μg cDNA was used for an *in vitro* transcription reaction (T7 Megascript, Ambion, Austin, Texas) with biotinylated CTP and UTP (Enzo Diagnostics, New York, New York). The labeling procedure amplified the mRNA population ~60-fold. Microarray chips (GeneChip System, Affymetrix, Santa Clara, California) were hybridized for 16 h in a 45 °C incubator with constant rotation at 60*g*. Chips were washed and stained on a fluidics station, and scanned using a laser confocal microscope.

Data analysis. Affymetrix HuGeneFL7026 microarrays were analyzed with GeneChip v3.1 software, and scaled to a value of 150. Average difference values of less than 20 were arbitrarily rounded up to a value of 20. The mean average difference for each gene was calculated for the combined control samples C-1 and C-2. Then the average differences of C-1 and C-2, and the individual MS sample average differences, were then divided by the control mean, to calculate fold-changes. 1,080 genes had a fold-change of ≥ 2 in at least 2 of the 4 MS samples, and these genes were used for cluster analysis. A mean average difference value for the 4 MS samples was also calculated and divided by the mean of the controls. Fold-changes were converted into a $\log_2$ value, and imported into the clustering program. Cluster and TreeView software was downloaded (http://rana.lbl.gov).

Statistics. To assign *P* values to gene presence and differential expression calls, we employed permutation tests described by Schadt *et al.*[3–5]. Before these tests were conducted to assess the significance of differential expression calls, the arrays were normalized to the intensity differences of one of the control sample arrays using a nonlinear regression technique described by Schadt *et al.*[4]. Each array was subdivided into 16 regions and normalized region by region to help control for spatial variation in intensity across the surface of the array. The permutation test operates on the perfect match (PM) and mismatch (MM) pairs for a given probe set, and tests the simple hypotheses for gene presence and differential expression calls, respectively:

$$PM_{k_i} \overset{D}{=} MM_{k_i} \text{ and/or } \frac{PM_{k_i}}{MM_{k_i}} \overset{D}{=} \frac{MM_{k_i}}{PM_{k_i}}$$

For $i = 1..M$, where M is the number of probe pairs for gene k, and D indicates the PM and MM intensities and ratios are equal in distribution. These hypotheses are tested by empirically estimating the distribution of the PM/MM intensity differences and ratios, and then comparing the observed intensity differences and ratios to the empirically derived distribution, to obtain a quantitative measure of significance (*P* value). This test avoids the distribution assumptions made by parametric tests like the paired *t*-test.

Genes that were detected as significantly present or significantly differentially expressed in at least one of the samples were put through an additional analysis step using the Rosetta Resolver v3.0 Expression Data Analysis System (see www.rosettabio.com for more details). In these cases, log ratios between the control and disease samples were computed by generating all possible control-disease pairs, computing the $\log_{10}$ of the resulting expression ratios for all probe pairs, and then averaging the log ratios of these probe pairs, where the expression ratio for a probe pair was taken to be the ratio between normalized, error-weighted PM/MM difference intensities. An error model for the log ratio was then applied to quantify the significance of expression changes between the control and disease samples. This error model assumed the log ratio statistic followed a standard normal distribution. As discussed by Waring *et al.*[5], the main purpose of such an error model is to generate *P* values for the log ratio statistic so that genes can be rank-ordered according to the significance of this statistic. Using *P* values to rank order genes, as opposed to using *P* values in a classic hypothesis-testing context, is not sensitive to departures in normality. In addition, this model allowed us to optimally combine the control and disease samples to assess the significance of differential expression, and it provided a somewhat independent assessment of the significance of differential expression for each gene called differentially expressed using the permutation test. The *P* values reported in Table 2 and Supplementary Table A and B were obtained using this error model.

Note: Supplementary information is available on the Nature Medicine *website.*

Acknowledgments

We thank J. Woody, R. Booth and H. Van Wart for support during the course of this work; H. Gmuender for help optimizing use of the technology; S. Wilson for developing methods to handle and isolate RNA from human samples; F. Zuo for helpful comments on the manuscript; J. Kumm for advice on bioinformatics; M.C. Jeong for technical help; and Roche Bioscience for support and access to gene-chip technology. This study was supported in part by post-doctoral fellowships from the National Multiple Sclerosis Society to C.L., G.H. and R.P. J.O. is supported by grants from the NIH (NIHAI35761) and the National Multiple Sclerosis Society (RG2901). C.S.R. is supported by NIH grants NS08952 and NS11920. L.S. is supported by NIH grants NIH18235, 30201, 41402 and 28579.

Competing interests statement

The authors declare competing financial interests: see the website (http://medicine.nature.com) for details.

RECEIVED 12 FEBRUARY; ACCEPTED 12 MARCH 2002

1. Raine, C.S. The neuropathology of multiple sclerosis. in *Multiple Sclerosis: Clinical and Pathogenetic Basis* (eds. Raine, C.S., McFarland, H.F. & Tourtelotte, W.W) 151–171 (Chapman and Hall, London, 1997).
2. Eisen, M.B., Spellman, P.T., Brown, P.O. & Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* **95**, 14863–14868 (1998).
3. Waring, J.F. *et al.* Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. *Toxicol. Appl. Pharmacol.* **175**, 28–42 (2001).
4. Schadt, E.E., Li, C., Su, C. & Wong, W.H. Analyzing high-density oligonucleotide gene expression array data. *J. Cell Biochem.* **80**, 192–202 (2000).
5. Schadt, E.E., Li, C., Ellis, B. & Wong, W.H. Feature extraction and normalization algorithms for high-density oligonucleotide gene expression array data. *J. Cell Biochem.* **Suppl.**, 120–125 (2001).
6. Hong, J.X., Wilson, G.L., Fox, C.H. & Kehrl, J.H. Isolation and characterization of a novel B cell activation gene. *J. Immunol.* **150**, 3895–3904 (1993).
7. Dabiri, G.A., Young, C.L., Rosenbloom, J. & Southwick, F.S. Molecular cloning of human macrophage capping protein cDNA. A unique member of the gelsolin/villin family expressed primarily in macrophages. *J. Biol. Chem* **267**, 16545–16552 (1992).
8. Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P. & Craig, R.W. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc. Natl. Acad. Sci. USA* **90**, 3516–3520 (1993).
9. Rehli, M., Krause, S.W. & Andreesen, R. Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. *Genomics* **43**, 221–225 (1997).
10. Davoust, N. *et al.* Central nervous system-targeted expression of the complement inhibitor sCrry prevents experimental allergic encephalomyelitis. *J. Immunol.* **163**, 6551–6556 (1999).
11. Walsh, L.A., Tone, M., Thiru, S. & Waldmann, H. The CD59 antigen—a multifunctional molecule. *Tissue Antigens* **40**, 213–220 (1992).
12. Hauser, S.L., Doolittle, T.H., Lincoln, R., Brown, R.H. & Dinarello, C.A. Cytokine accumulations in CSF of multiple sclerosis patients: Frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6. *Neurology* **40**,

1735–1739 (1990).
13. Jacobs, C.A. *et al.* Experimental autoimmune encephalomyelitis is exacerbated by IL-1 α and suppressed by soluble IL-1 receptor. *J. Immunol.* **146**, 2983–2989 (1991).
14. Matusevicius, D. *et al.* Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult. Scler.* **5**, 101–104 (1999).
15. Raine, C.S., Bonetti, B. & Cannella, B. Multiple sclerosis: Expression of molecules of the tumor necrosis factor ligand and receptor families in relationship to the demyelinated plaque. *Rev. Neurol.* (*Paris*) **154**, 577–585 (1998).
16. Akira, S. & Kishimoto, T. IL-6 and NF-IL6 in acute-phase response and viral infection. *Immunol. Rev.* **127**, 25–50 (1992).
17. Gijbels, K., Brocke, S., Abrams, J.S. & Steinman, L. Administration of neutralizing antibodies to interleukin-6 (IL-6) reduces experimental autoimmune encephalomyelitis and is associated with elevated levels of IL-6 bioactivity in central nervous system and circulation. *Mol. Med.* **1**, 795–805 (1995).
18. Akassoglou, K., Probert, L., Kontogeorgos, G. & Kollias, G. Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *J. Immunol.* **158**, 438–445 (1997).
19. Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M. & Greenberg, M.E. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. *Science* **286**, 785–790 (1999).
20. Penkowa, M. *et al*. Altered inflammatory response and increased neurodegeneration in metallothionein I+II deficient mice during experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **119**, 248–260 (2001).
21. Pitt, D., Werner, P. & Raine, C.S. Glutamate excitotoxicity in a model of multiple sclerosis. *Nature Med.* **6**, 67–70 (2000).
22. Trapp, B.D. *et al*. Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* **338**, 278–285 (1998).
23. Yan, Y., Lagenaur, C. & Narayanan, V. Molecular cloning of M6: Identification of a PLP/DM20 gene family. *Neuron* **11**, 423–431 (1993).
24. Yednock, T.A. *et al*. Prevention of experimental autoimmune encephalomyelitis by antibodies against α4β1 integrin. *Nature* **356**, 63–66 (1992).
25. Steinman, L. Assessment of the utility of animal models for MS and demyelinating disease in the design of rational therapy. *Neuron* **24**, 511–514 (1999).
26. Takai, T., Li, M., Sylvestre, D., Clynes, R. & Ravetch, J.V. FcRγ chain deletion results in pleiotrophic effector cell defects. *Cell* **76**, 519–529 (1994).
27. Miyajima, I. *et al*. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and FcγRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1- dependent passive anaphylaxis. *J. Clin. Invest.* **99**, 901–914 (1997).
28. Samuelsson, A., Towers, T.L. & Ravetch, J.V. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science* **291**, 484–486 (2001).
29. Achiron, A. *et al.* Intravenous immunoglobulin treatment of experimental T cell-mediated autoimmune disease. Upregulation of T-cell proliferation and downregulation of tumor necrosis factor α secretion. *J. Clin. Invest.* **93**, 600–605 (1994).
30. Wucherpfennig, K.W. *et al.* Recognition of the immunodominant myelin basic protein peptide by autoantibodies and HLA-DR2-restricted T-cell clones from multiple sclerosis patients. Identity of key contact residues in the B-cell and T-cell epitopes. *J. Clin. Invest.* **100**, 1114–1122 (1997).
31. Warren, K.G., Catz, I. & Steinman, L. Fine specificity of the antibody response to myelin basic protein in the central nervous system in multiple sclerosis: The minimal B-cell epitope and a model of its features. *Proc. Natl. Acad. Sci. USA* **92**, 11061–11065 (1995).
32. Genain, C.P., Cannella, B., Hauser, S.L. & Raine, C.S. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nature Med.* **5**, 170–175 (1999).
33. Whitney, L.W. *et al.* Analysis of gene expression in mutiple sclerosis lesions using cDNA microarrays. *Ann. Neurol.* **46**, 425–428 (1999).
34. Wraith, D.C., Smilek, D.E., Mitchell, D.J., Steinman, L. & McDevitt, H.O. Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. *Cell* **59**, 247–255 (1989).
35. Pedotti, R. *et al.* An unexpected version of horror autotoxicus: Anaphylactic shock to a self-peptide. *Nature Immunol.* **2**, 216–222 (2001).
36. Lucchinetti, C. *et al.* Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. *Ann. Neurol.* **47**, 707–717 (2000).
37. Haines, J.L. *et al.* A complete genomic screen for multiple sclerosis underscores a role for the major histocompatability complex. The Multiple Sclerosis Genetics Group. *Nature Genet* **13**, 469–471 (1996).
38. Ebers, G.C. *et al.* A full genome search in multiple sclerosis. *Nature Genet.* **13**, 472–476 (1996).
39. Sawcer, S. *et al.* A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. *Nature Genet.* **13**, 464–468 (1996).
40. Zavala, F. *et al.* G-CSF therapy of ongoing experimental allergic encephalomyelitis via chemokine- and cytokine-based immune deviation. *J. Immunol.* **168**, 2011–2019 (2002).
41. Matarese, G. *et al.* Requirement for leptin in the induction and progression of autoimmune encephalomyelitis. *J. Immunol.* **166**, 5909–16 (2001).
42. Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. & Cone, R.D. The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248–1251 (1992).
43. Poliak, S. *et al.* Stress and autoimmunity: The neuropeptides corticotropin-releasing factor and urocortin suppress encephalomyelitis via effects on both the hypothalamic-pituitary-adrenal axis and the immune system. *J. Immunol.* **158**, 5751–5756 (1997).