the amplification, and was processed for antisense RNA (aRNA).[24] The aRNA of MS patients and CN subjects was labeled with a fluorescent dye Cy5, while pooled aRNA of three independent healthy volunteers who were not included in the present study was labeled with Cy3 for a universal reference to standardize the gene expression levels throughout the experiments. The arrays were hybridized at 62℃ for 10 hours in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The average of fluorescence intensities (FI) of duplicate spots was obtained after global normalization between Cy3 and Cy5 signals. The gene expression level (GEL) was calculated according to the formula: GEL = FI (Cy5) of the sample/ FI (Cy3) of the universal reference.

Hierarchical Clustering Analysis, Principal Component Analysis and Statistical Analysis

The genes whose expression was significantly different between MS and CN groups were identified by using *pierre* of the "R" statistical software system (www.cran.r-project.org) based on a Bayesian framework for analysis of microarray expression data.[25] It was considered as significant when the error rate of this test was smaller than 0.25 following the Bonferroni correction. Hierarchical clustering analysis was performed on a set of 286 differentially expressed genes between MS and CN groups as a discriminator by using a standard x standard algorithm on GeneSpring (Silicon Genetics, Redwood City, CA). Principal component analysis (PCA) was performed on GeneSpring to reduce all of the variance in the original dataset to three dimensions that account for a significant fraction of the variance.[26] The differences in clinical parameters among MS subgroups were evaluated by multiple comparison test with the Bonferroni correction.

Results

Microarray Analysis Identified 286 Genes Differentially Expressed in Peripheral Blood T Cells between MS and Control Subjects

Among 1,258 genes on the cDNA microarray, 286 genes were expressed differentially in peripheral blood $CD3^+$ T cells between 72 untreated MS patients and 22 CN subjects. Among these genes, 78 genes were upregulated, while 208 genes downregulated in MS patients as compared to CN (All the data sets are shown in Supplementary Table 1 online.). We also conducted the microarray analysis of $CD3^-$ non-T cells that are composed of B cells, monocytes/macrophages and NK cells, and found that 96 genes were differentially expressed in the non-T cell fraction between MS patients and CN subjects (data not shown).

Hierarchical Clustering Analysis Identified Four Distinct Subgroups of MS and Five Gene Classes

Hierarchical clustering analysis was performed on all $CD3^+$ T-cell samples of untreated MS patients and CN subjects, using the set of 286 differentially expressed genes described above as a discriminator. Genes and samples with a similar expression pattern were arranged as a cluster in the dendrogram (Fig 1). This analysis identified four distinct subgroups of MS separated from the CN group. In contrast, when we studied $CD3^-$ non-T cells, hierarchical clustering analysis could not separate MS subgroups from CN clearly (data not shown). We operationally designated each subgroup of MS as A, B, C and D, following the position in the dendrogram (Fig 1). The subgroup D is located the most distantly from CN, whereas the subgroup A is the closest to CN. The subgroups B and C are equally distant from CN. Among 94 subjects examined, two MS patients and three CN subjects were considered as being unclassifiable (UC). Principal component analysis (PCA) further displayed a clear discrimination of four MS subgroups (Fig 2). Hierarchical clustering analysis categorized 281 of 286 differentially

expressed genes into five distinct classes numbered #1 to #5 (Fig. 1 and Supplementary Table 1 online). The remaining five genes, including TOP1, CHST4, SLC35A1, ST1B2, and TAF2H, were unable to be categorized into any classes. All the class #5 genes were upregulated in MS, whereas the genes of classes #1 to #4 were downregulated in MS when compared with CN (Fig 1). Upregulated expression of some of the class #5 genes in MS versus CN was verified by quantitative real-time RT-PCR analysis (data not shown).

Association of MS Subgroups with Gene Clusters

The subgroup A showed the gene expression pattern that is the most similar to CN. This was supported by PCA (Fig 2) and by the observations that one CN subject was incorporated in the subgroup A, while two subgroup A MS patients were included in the CN population on hierarchical clustering (Fig 1). Expression of the class #5 genes were augmented in all MS subgroups, whereas the classes #1 to #4 genes were downregulated in MS. Notably, subgroup B showed a greatest level of class #5 gene upregulation. The most prominent suppresion of classes #1 to #4 genes was also found in subgroup B (Fig 1), suggesting that this subgroup may represent a most active state of MS. Of note, the class #5 genes, composed of 78 genes, were found to contain nine chemokines (11.5%), including small inducible cytokine subfamily A member 1 (SCYA1 or CCL1), member 3 (SCYA3, CCL3 or MIP1-α), member 13 (SCY13 or CCL13), member 18 (SCYA18 or CCL18), member 24 (SCYA24 or CCL24), subfamily B member 1 (SCYB1, CXCL1 or GRO1), member 2 (SCYB2, CXCL2 or GRO2), member 9 (SCYB9, CXCL9 or MIG), and member 14 (SCYB14, CXCL14) (Supplementary Table 1 online). In contrast, the classes #1 to #4 genes composed of 203 genes contained only two chemokines (1.0%), SCYB5 (CXCL5) and SCYB10 (CXCL10 or IP10). Taken together, it is arguable that the class #5 gene cluster is highly enriched in chemokine genes.

Clinical Characteristics of MS Subgroups Classified by Hierarchical Clustering Analysis

Next, we investigated clinical characteristics of each MS subgroup (Table 2 and Fig 3). No statistically significant differences were observed among MS subgroups in the age, disease duration, EDSS score, and the number of lesions on T2-weighted MRI at enrollment. There was a trend that subgroup D showed a greater EDSS score and had a larger number of MRI lesions suggestive of an advanced stage of the disease. However, the difference did not reach the level of statistical significance. The female patients outnumbered the male patients in all the subgroups, although the male to female ratio was relatively higher in subgroup C, while no male patient was included in subgroup D. The patients with RRMS outnumbered those with SPMS in all the subgroups, although there was a mild bias for SPMS in subgroup B.

Previous studies showed that the patients with opticospinal form of MS (OSMS), characterized by the lesion distribution confined to the optic nerve and spinal cord, were accumulated in Asian countries. However, recent evidence indicated that the proportion of OSMS versus conventional form of MS (CMS) in Japanese MS patients has been markedly reduced in the past 30 years.[6] Although genetic as well as environmental factors may affect the pattern of lesion distribution in MS, it is only speculative at this moment. Here, we determined a possible association between the pattern of the lesion distribution and microarray-determined MS subgroups. In all MS subgroups, CMS greatly outnumbered non-CMS. The latter was composed of OSMS and multifocal recurrent myelitis. Although no obvious association was identified between a particular MS subgroup and the spinal cord involvement, 5 of 6 patients having the lesions restricted to the cerebrum (CBR only) were clustered in subgroup C (Table 2), suggesting that the lesion distribution in the subgroup C patients might be affected by the status of T cell gene expression.

The number of relapse, the day of IVMP treatment, and the day of hospitalization during preceding two years before enrollment were the largest in subgroup B. This

postulate was verified by a statistically significant difference when compared between the subgroups B and C (p = 0.0128, 0.0183, and 0.0329 for each parameter) (Table 2 and Fig 3). These observations indicate that subgroup B included the patients who were clinically most active at enrollment before starting IFNβ.

IFNβ Responders were Clustered in Subgroups A and B

According to the patient's own determination on entry, 72 MS patients were separated into two groups; 46 who started to receive IFNβ treatment for the following two years and 26 who were followed up without IFNβ treatment for the successive two years (Supplementary Table 2 online). The IFNβ-treated patients were evaluated by the IFNβ responder/nonresponder score (Table 1) at the end of the two year-treatment. Forty-six IFNβ-treated MS patients were classified into 19 IFNβ responders, 7 nonresponders, 13 undermined subjects, and 7 dropouts (Table 3). Importantly, 16 of 19 IFNβ responders were included either in subgroup A or B. The subgroup A patients were judged as being the most IFNβ responsive, because a highest proportion of the patients were classified as IFNβ responders [8 of 14 IFNβ-treated patients (57.1%)], and because all the responder patients expressed a satisfaction on IFNβ treatment. The subgroup B patients also showed a good response to IFNβ equivalent to subgroup A (57.1%), although the number of the patients satisfied with the treatment was somewhat smaller. In contrast, only 2 of 11 IFNβ-treated patients in subgroup C (18.2%) and none of the patients in subgroup D were judged as IFNβ responders. Although the difference in the IFNβ responder/nonresponder score among MS subgroups (A: 2.5±2.3; B: 2.1±2.6; C: 1.3±2.1; and D: -0.3±4.3) did not reach the level of statistical significance (Table 3), these observations indicate that the gene expression profiling of MS T cells at the time before starting IFNβ treatment could help us to predict the therapeutic response for successive two years.

The subgroup C patients showed the greatest increase in the number of brain MRI lesions during two years of IFNβ treatment, in accordance with the poor response to

IFNβ, although it was not statistically significant (Table 3). A battery of side effects related to IFNβ treatment, including skin reactions, flu-like symptoms, leukocytopenia, depression, and amenorrhea, were observed in more than 50% of the IFNβ-treated patients. The patients suffering from such side effects were distributed in all the subgroups (Table 3). Seven patients in the IFNβ-treated group discontinued the treatment: five due to IFNβ-related adverse effects, one due to a severe relapse possibly related to IFNβ treatment, and another by a personal reason.

We also performed the microarray and hierarchical clustering analysis of T-cell samples of MS patients obtained at 3 or 6 months after starting IFNβ therapy. Although IFNβ-treated patients could be classified on their gene expression pattern into several clusters, these new clusters did not match with the subgroup A, B, C, or D determined at enrollment before IFNβ treatment (data not shown). Furthermore, no significant association was identified between these new clusters and IFNβ response, suggesting that T cell gene expression profiling before starting IFNβ treatment is the most valuable to predict the clinical outcome, whereas a single sampling of T cells after starting IFNβ treatment might be less informative.

The Temporal Profile of IFN-Responsive Gene Expression in the First Six Months Discriminated Responders and Nonresponders

Finally, we investigated the temporal profile of gene expression levels before and after starting IFNβ treatment at 3 and 6 months. As we have previously shown,[21] IFNβ treatment enhanced the expression of a battery of the genes with IFN-responsive promoter elements named IFN-responsive genes (IRGs) (Fig 4). Strikingly, a remarkable difference was observed between IFNβ responders and nonresponders in the temporal profile of the expression of several known IRGs,[21,27,28] including IFN-stimulated protein 15 (ISG15), small inducible cytokine A2 (SCYA2, alternatively named monocyte chemoattractant protein-1; MCP-1), TNF receptor subfamily member 1B (TNFRSF1B, alternatively named TNFRp75), and IFNα-inducible protein 27

(IFI27) (Fig 5). The IFNβ responders (R) exhibited persistent upregulation of these IRGs during 6 months after initiation of IFNβ treatment. In contrast, the nonresponders (NR) showed higher upregulation at 3 months than the responders (R), but this increase was followed by substantial downregulation at 6 months, presenting with the seesaw pattern. This was verified by a statistically significant difference between R and NR in the change of levels of TNFRSF1B and IFI27 from 3 to 6 months ($p = 0.0092$ and 0.0307, respectively) (Fig 5). These observations suggest that IFNβ nonresponders could also well respond to IFNβ at 3 months but they could not maintain the responsiveness until 6 months.

Discussion

Although MS is a disease manifesting remarkable variability in clinical and pathological manifestations, very little is known about the immunological and molecular basis that may account for the heterogeneity. To explore the molecular mechanism underlying heterogeneity of MS, we designed a comprehensive study combining cDNA microarray analysis of peripheral blood T cells and analysis of clinical information during four years before and after starting IFNβ. Our initial analysis of 72 untreated MS patients and 22 CN subjects revealed 286 genes differentially expressed between MS and CN. By applying hierarchical clustering analysis for these 286 genes, we found that MS patients could be divided into four distinct MS subgroups. Notably, IFNβ responders were clustered in two of the subgroups (subgroups A and B). When clinical profiles in each subgroup were studied, the subgroup B was found to be the most clinically active MS before treatment with IFNβ. Furthermore, by adding kinetic analysis for six months after starting IFNβ, we showed that IFNβ responders are significantly different from responders in the kinetics of two IFN-responsive genes; TNFRSF1B and IFI27. Although these results are not readily utilized for predicting IFNβ responsiveness in

individual patients, they undoubtedly support a potential power of DNA microarary analysis of peripheral blood T cells for evaluating of the clinical profile of patients.

Hierarchical clustering analysis for the untreated MS and CN also identified five different gene classes numbered #1 to #5. Among these, upregulated exclusively in MS were the class #5 genes, including nine chemokines, such as CCL1, CCL3, CCL13, CCL18, CCL24, CXCL1, CXCL2, CXCL9 and CXCL14. Interestingly, the most clinically active subgroup B showed the highest upregulation of the class #5 genes. These observations suggest that the higher disease activity in the subgroup B might be in part attributable to T-cell secretion of the chemokines within the CNS, which promotes lymphocyte and macrophage trafficking into the CNS.[29] It is also possible that these T cell-derived chemokines amplify the inflammation in the CNS by serving as a coactivator of IFNγ-activated macrophages.[30]

It is of particular note that the proportions of IFNβ responders greatly differed among the MS subgroups: the percentage of IFNβ responders was 57.1% in subgroup A, 57.1% in B, 18.2% in C and 0% in D. Unexpectedly, the patients in subgroup B exhibited a good response to IFNβ, despite its highest clinical activity. However, this is consistent with a recent clinical observation that IFNβ responders were characterized by higher relapse rates during the year prior to initiation of IFNβ treatment.[7] In contrast, the patients in subgroup C with the poor response to IFNβ showed the greatest increase in the number of MRI lesions during two years after IFNβ treatment. This is in accord with a recent study showing that the number of on-treatment new T2 MRI lesions correlates with poor response to IFNβ-1a.[31] Although subgroup D did not include any IFNβ responders, the number of MRI lesions did not increase during two years of treatment. This raises a possibility that subgroup D could undergo a neurodegenerative process that is independent of active inflammation. The present study suggests that the microarray-based classification of MS could be useful to predict therapeutic response to IFNβ. Obviously, further studies on the larger cohort of MS patients are required to obtain the conclusive evidence.

IFNα and β (type I IFNs) are produced principally by virus-infected host cells, whereas IFNγ (type II IFN) is secreted by activated T cells and NK cells. Type I IFNs activate JAK protein tyrosine kinases linked with the cell surface receptors for IFNs, leading to formation of the complex composed of STAT molecules and the IFN regulatory factor (IRF) family of transcription factors.[32] Then, the STAT/IRF complex translocates into the nucleus, where it interacts with transcriptional coactivators such as p300 and CREB-binding protein (CBP), and binds to the DNA sequences termed the IFN-stimulated response element or the IRF-recognition element. This activates transcription of a wide range of IFN-responsive genes (IRGs) primarily responsible for biological effects of IFNs. It is of particular interest that the genes differentially expressed between MS and CN included those having IFN-responsive elements in their promoter regions: p300 (EP300) and IFNα receptor 1 (IFNAR1) in the class #1, SCYB10 (CXCL10, IP10), ATP-binding cassette (ABC) subfamily E member 1 (ABCE1, alternatively named RNase L inhibitor),[33] IFNγ-inducible protein 16 (IFI16), and STAT1 in the class #2, myxovirus resistance 2 (MX2) in the class #3, NF-κB-repressing factor (NRF),[34] IRF9 and IRF2 in the class #4, and IFNα-16 (IFNA16) and SCYB9 (CXCL9, MIG) in the class #5 (see all the datasets in Supplementary Table 1 online). These results suggest that the IFN-signaling pathway is aberrantly regulated in peripheral blood T cells of MS patients before treatment with IFNβ. Although the precise reason for this remains unclear, the dysregulation of IRGs might play a role in the pathogenesis of MS, and have some relevance to therapeutic effects of IFNβ in MS. We previously showed that upregulated expression of SCYB9 was suppressed by a long-term treatment with IFNβ.[21] This supports the possibility that IFNβ produces a beneficial effect on MS by correcting the preexisting disturbance in regulation of the IFN-signaling pathway.

We also investigated T cell gene expression profile of MS at 3 or 6 months after starting IFNβ. Again, hierarchical clustering analysis classified IFNβ-treated patients into several subgroups, but none of these new subgroups matched with either IFNβ

responders or nonresponders. Therefore, we conclude that T cell gene profiling at pretreatment (Pre) was the most informative to identify the population of IFNβ responders in MS. A possible explanation could be provided that a defect in the IFN-signaling pathway in untreated MS patients was adjusted by IFNβ treatment.

The present study was also conducted to identify marker genes for IFNβ responsiveness in MS. The separate analysis at Pre versus 3 months, at Pre versus 6 months or at 3 months versus 6 months did not identify the gene expression profile characteristic of IFNβ responders (unpublished observations). However, when all the data sets at three time points (Pre, 3 months and 6 months) were combined, discernible differences were found between IFNβ responders (R) and nonresponders (NR). A panel of IRGs including ISG15, SCYA2, TNFRSF1B and IFI27 were persistently upregulated in IFNβ responders, while they were downregulated to some extent in nonresponders by 6 months.

The class #5 genes composed of 78 genes were upregulated in MS compared with CN. They were comprised of proinflammatory and antiinflammatory cytokines, growth factors and their receptors (see all the datasets in Supplementary Table 1 online). These results suggest that not a simple Th1-mediated but more complex immunoregulatory mechanism is involved in the pathogenesis of MS.[35] Importantly, substantial numbers of upregulated genes in peripheral blood T cells of MS were expressed at high levels in the lesions of MS brain. The corresponding genes include IL-12p40, IL-10, SCYA3 (CCL3, MIP1-α), SCYB1 (CXCL1, GRO1), granulocyte colony-stimulating factor (GCSF), SCYB9 (CXCL9, MIG), platelet-derived growth factor receptor-alpha (PDGFRA), transforming growth factor-beta 2 (TGFB2), and insulin-like growth factor-II (IGF-II).[14,36-43]

At the initial stage of this work, we noticed that top 30 differentially expressed genes of $CD3^+$ T cells between untreated MS patients and CN subjects included a battery of apoptosis signaling-related genes pivotal for regulation of T cell development, such as nuclear receptor subfamily 4, group A, member 2 (NR4A2) and transcription

factor 8 (TCF8).[22] NR4A2 in the class #5 upregulated in MS encodes an orphan member of the steroid-thyroid hormone receptor superfamily transcription factors designated Nurr1. Nurr1 is induced in human T cells during apoptosis[44] and the members of this family positively regulate clonal deletion of self-reactive T cells in the thymus.[45] In contrast to our observations, a previous microarray analysis showed that NR4A2 expression is downregulated in unfractionated PBMC of MS patients compared with the levels in healthy subjects,[20] although the following study from the same group indicated a significant upregulation of NR4A2 in PBMC of both MS and SLE.[46] We verified upregulation of NR4A2 mRNA levels in $CD3^+$ T cells of MS versus CN by quantitative real-time RT-PCR analysis (data not shown). It is worthy to note that Nurr1 activates the transcription of osteopontin,[47] a Th1 cytokine possibly involved in progression of inflammatiory demyelination in MS.

Microarray analysis of T cell gene expression profile in MS identified an aberrant expression of key regulators for drug metabolism, whose role has not previously been proposed in MS.[48] A panel of cytochrome P450 (CYP) family enzymes, including CYP4B1, CYP3A4, CYP1A2, CYP8B1, CYP17, CYP2E, CYP4F2, and CYP2A6, were over-expressed in MS T cells (see Supplementary Table 1 online). Of note, the CYP family regulates Ca^{2+} influx in activated T cells.[49] On the other hand, various ABC transporters, including ABCD2, ABCE1, ABCG2 and ABCF2 in the class #2, ABCF1, ABCB10 and ABCC5 in the class #3, and ABCB7, ABCB1 and ABCB6 in the class #4 were downregulated in MS T cells (Supplementary Table 1 online). The ABC transporter superfamily regulates the transport of amino acids, ions, sugars, lipids and drugs across the cell membrane by consuming the energy derived from ATP hydrolysis. ABCB1 and ABCG2 expressed on brain endothelial cells act as a main transporter in the blood-brain barrier (BBB) that determines bioavailability of corticosteroids and mitoxantrone in the brain.[50] The precise pathological implication for the opposing changes in CYP family enzymes and ABC transporters in the T cells of MS remains to be further investigated.

Previous studies showed that autoreactive T cells are clonally expanded and activated in MS.[51-54] However, the frequency of potentially pathogenic autoreactive Th1 cells in total PBMC is very low, ranging from 1/200 to 1/10,000. The present study on transcriptional analysis of peripheral blood $CD3^+$ T cells has disclosed a number of differences in gene expression levels between MS and CN, but they were not simply consistent with a Th1 bias. These observations suggest that molecular changes observed in our study do not reflect a small population of autoreactive Th1 cells but involve broader range of T cell populations. A recent study showed that a mutation in the ZAP70 gene that dysregulates the proximal post-TCR signaling cascade causes development of autoimmune arthritis,[55] raising the possibility that development of certain clinicopathological variants of MS might be caused by unidentified mutations and polymorphisms shared by all T cell populations.

In conclusion, T cell gene expression profiling would provide us a novel insight into the molecular pathogenesis and tailor-made treatment of MS.

Acknowledgements

The authors thank Dr. Hikoaki Fukaura, Department of Neurology, Hokkaido University Graduate School of Medicine, Sapporo, Japan for their invaluable help.

This work was supported by grants from the Ministry of Health, Labour and Welfare of Japan.

Reference

1. Compston A, Coles A. Multiple sclerosis. Lancet 2002;359:1221-1231.

2. Panitch HS, Hirsch RL, Schindler J, Johnson KP. Treatment of multiple sclerosis with gamma interferon: Exacerbations associated with activation of the immune function. Neurology 1987;37:1097-1102.

3. The IFNB Multiple Sclerosis Study Group. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. Neurology 1993;43:655-661.

4. Jacobs LD, Cookfair DL, Rudick RA, et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. Ann Neurol 1996;39:285-294.

5. Jacobs LD, Beck RW, Simon JH, et al. Intramuscular interferon beta-1a therapy initiated during a first demyelinating event in multiple sclerosis. N Engl J Med 2000;343:898-904.

6. Misu T, Fujihara K, Nakashima I, et al. Pure optic-spinal form of multiple sclerosis in Japan. Brain 2002;125:2460-2468.

7. Waubant E, Vukusic S, Gignoux L, et al. Clinical characteristics of responders to interferon therapy for relapsing MS. Neurology 2003;61:184-189.

8. Lucchinetti C, Brück W, Parisi J, et al. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. Ann Neurol 2000;47:707-717.

9. Staudt LM. Gene expression physiology and pathophysiology of the immune system. Trends Immunol 2001;22:35-40.

10. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000;403:503-511.

11. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999-2009.

12. Geschwind DH. DNA microarrays: translation of the genome from laboratory to clinic. Lancet Neurol 2003;2:275-282.

13. Steinman L, Zamvil S. Transcriptional analysis of targets in multiple sclerosis.

Nature Rev Immunol 2003;3:483-492.

14. Lock C, Hermans G, Pedotti R, et al. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. Nature Med 2002;8:500-508.
15. Mycko, M.P., Papoian, R., Boschert, U., Raine, C.S. and Selmaj, K.W. (2003) cDNA microarray analysis in multiple sclerosis lesions: detection of genes associated with disease activity. Brain, 126, 1048-1057.
16. Graumann U, Reynolds R, Steck AJ, Schaeren-Wiemers N. Molecular changes in normal appearing white matter in multiple sclerosis are characteristic of neuroprotective mechanisms against hypoxic insult. Brain Pathol 2003;13:554-573.
17. Tajouri L, Mellick AS, Ashton KJ, et al. Quantitative and qualitative changes in gene expression patterns characterize the activity of plaques in multiple sclerosis. Mol Brain Res 2003;119:170-183.
18. van Boxel-Dezaire AHH, van Trigt-Hoff SCJ, Killestein J, et al. Contrasting responses to interferon β-1b treatment in relapsing-remitting multiple sclerosis: does baseline interleukin-12p35 messenger RNA predict the efficacy of treatment? Ann Neurol 2000;48:313-322.
19. Stürzbecher S, Wandinger KP, Rosenwald A, et al. Expression profiling identifies responder and non-responder phenotypes to interferon-β in multiple sclerosis. Brain 2003;126:1219-1429.
20. Achiron A, Gurevich M, Friedman N, et al. Blood transcriptional signatures of multiple sclerosis: unique gene expression of disease activity. Ann Neurol 2004;55: 410-417.
21. Koike F, Satoh J, Miyake S, et al. Microarray analysis identifies interferon β-regulated genes in multiple sclerosis. J Neuroimmunol 2003;139:109-118.
22. Satoh J, Nakanishi M, Koike F, et al. Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. Neurobiol Dis 2004, in press.

23. McDonald WI, Compston A, Edan G, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the international panel on the diagnosis of multiple sclerosis. Ann Neurol 2001;50:121-127.

24. Phillips J, Eberwine JH. Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells. Methods 1996;10:283-288.

25. Baldi P, Long AD. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics 2001;17:509-519.

26. Pomeroy SL, Tamayo P, Gaasenbeek M, et al. Prediction of central nervous system embryonal tumour outcome based on gene expression. Nature 2002;415:436-442.

27. Santee SM, Owen-Schaub LB. Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization. J Biol Chem 1996;271: 21151-21159.

28. Kim OS, Park EJ, Joe E-H, Jou I. JAK-STAT signaling mediates gangliosides-induced inflammatory responses in brain microglial cells. J Biol Chem 2002;277: 40594-40601.

29. Opdenakker G, Nelissen I, Van Damme J. Functional roles and therapeutic targeting of gelatinase B and chemokines in multiple sclerosis. Lancet Neurol 2003;2:747-756.

30. Dorner BG, Scheffold A, Rolph MS, et al. MIP-1α, MIP-1β, RANTES and ATAC/lymphotactin function together with IFN-γ as type 1 cytokines. Proc Natl Acad Sci USA 2002;99:6181-6186.

31. Rudick RA, Lee J-C, Simon J, et al. Defining interferon β response status in multiple sclerosis patients. Ann Neurol 2004;56:548-555.

32. Taniguchi T, Takaoka A. The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr Opin Immunol 2002;14:111-116.

33. Bisbal C, Martinand C, Silhol M, et al. Cloning and characterization of a RNase L

inhibitor. A new component of the interferon-regulated 2-5A pathway. J Biol Chem 1995;270:13308-13317.

34. Nourbakhsh M, Hauser H. Constitutive silencing of IFN-β promoter is mediated by NRF (NF-κB-repressing factor), a nuclear inhibitor of NF-κB. EMBO J 1999;18:6415-6452.

35. Hemmer B, Archelos JJ, Hartung H-P. New concepts in the immunopathogenesis of multiple sclerosis. Nature Rev Neurosci 2002;3:291-301.

36. Windhagen A, Newcombe J, Dangond F, et al. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. J Exp Med 1995;182:1985-1996.

37. Peress NS, Perillo E, Seidman RJ. Glial transforming growth factor (TGF)-β isotypes in multiple sclerosis: differential glial expression of TGF-β1, 2 and 3 isotypes in multiple sclerosis. J Neuroimmunol 1996;71:115-123.

38. Balashow KE, Rottman JB, Weiner HL, Hancock WW. $CCR5^+$ and $CXCR3^+$ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. Proc Natl Acad Sci USA 1999;96:6873-6878.

39. Gveric D, Cuzner ML, Newcombe J. Insulin-like growth factors and binding proteins in multiple sclerosis plaques. Neuropathol Appl Neurobiol 1999;25:215-225.

40. Simpson JE, Newcombe J, Cuzner ML, Woodroofe MN. Expression of the interferon-γ-inducible chemokines IP-10 and Mig and their receptor, CXCR3, in multiple sclerosis. Neuropathol Appl Neurobiol 2000;26:133-142.

41. Maeda Y, Solanky M, Menonna J, et al. Platelet-derived growth factor-α receptor-positive oligodendroglia are frequent in multiple sclerosis lesions. Ann Neurol 2001;49:776-785.

42. Hulshof S, Montagne L, De Groot CJ, Van Der Valk P. Cellular localization and expression patterns of interleukin-10, interleukin-4, and their receptors in multiple sclerosis lesions. Glia 2002;38:24-35.

43. Filipovic R, Jakovcevski I, Zecevic N. GRO-α and CXCR2 in the human fetal brain

and multiple sclerosis lesions. Dev Neurosci 2003;25:279-290.

44. Okabe T, Takayanagi R, Imasaki K, et al. cDNA cloning of a NGFI-B/nur77-related transcription factor from an apoptotic human T cell line. J Immunol 1995;154:3871-3879.

45. Zhou T, Cheng J, Yang P, et al. Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. J Exp Med 1996;183:1879-1892.

46. Mandel M, Gurevich M, Pauzner R, et al. Autoimmunity gene expression portrait: specific signature that intersects or differentiates between multiple sclerosis and systemic lupus erythematosus. Clin Exp Immunol 2004;138:164-170.

47. Lammi J, Huppunen J, Aarnisalo P. Regulation of the osteopontin gene by the orphan nuclear receptor NURR1 in osteoblasts. Mol Endrocrinol 2004;18:1546-1557.

48. Nguyen LT, Ramanathan M, Weinstock-Guttman B, et al. Detection of cytochrome P450 and other drug-metabolizing enzyme mRNAs in peripheral blood mononuclear cells using DNA arrays. Drug Metab Dispos 2000;28:987-993.

49. Aussel C, Breittmayer JP, Ticchioni M, et al. Regulation of T cell activation by cytochrome P450 inhibitors. Cell Immunol 1994;155:436-445.

50. Zhang W, Petrovic J-M, Andrade MF, et al. The expression and functional characterization of ABCG2 in brain endothelial cells and vessels. FASEB J 2003;17: 2085-2087.

51. Zhang J, Markovic-Plese S, Lacet B, et al. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. J Exp Med 1994;179:97-984.

52. Bieganowska KD, Ausubel LJ, Modabber Y, et al. Direct ex vivo analysis of activated, Fas-sensitive autoreactive T cells in human autoimmune disease. J Exp Med 1997;185:1585-1594.

53. Illés Z, Kondo T, Yokoyama K, et al. Identification of autoimmune T cells among in vivo expanded $CD25^+$ T cells in multiple sclerosis. J Immunol 1999;162:1811-1817.

54. Bielekova B, Goodwin B, Richert N, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. Nature Med 2000;6:1167-1175.
55. Sakaguchi N, Takahashi T, Hata H, et al. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature 2003;426: 454-460.

Figure Legends

Fig 1. Hierarchical clustering analysis of 286 genes differentially expressed between untreated MS patients and control subjects. The gene expression profile of peripheral blood $CD3^+$ T cells was studied in 72 untreated MS patients and 22 age- and sex-matched healthy control (CN) subjects on cDNA microarray spotted with 1,258 genes of various functional classes. Hierarchical cluster analysis was performed on a set of 286 genes differentially expressed between MS and CN (see Supplementary Table 1 online) as a discriminator following a standard x standard algorithm on GeneSpring. This analysis separated MS (purple) from CN (dark blue), and classified the former into four subgroups named A (green), B (light blue), C (red) and D (yellow). The 286 genes were also categorized into five classes numbered #1 (pink) to #5 (light blue). The results are expressed in a matrix format, with each row representing the gene expression level (GEL) of a single gene in all the subjects, and each column representing GEL of the 286 genes in an individual subject. The figure is shown by a pseudo-color, with red expressing upregulation, green expressing downregulation, and the color intensity representing the magnitude of the deviation from GEL 1.0 as shown on the upper right.

Fig 2. Principal component analysis (PCA) of 286 genes differentially expressed between untreated MS patients and control subjects. The gene expression profile of peripheral blood $CD3^+$ T cells was studied in 72 untreated MS patients and 22 age- and sex-matched healthy control (CN) subjects on an 1,258 cDNA microarray. Principal component analysis (PCA) was performed on GeneSpring to reduce all of the variance in the original dataset of 286 differentially expressed genes between MS and CN to three dimensions that account for a significant fraction of the variance. This analysis verified a clear separation of the CN group (dark blue) and four MS subgroups named A (green), B (light blue), C (red) and D (yellow) that were established by hierarchical clustering analysis.