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T cell gene expression profiling identifies distinct subgroups of Japanese multiple sclerosis patients

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Abstract

To clarify the molecular background underlying the heterogeneity of multiple sclerosis (MS), we characterized the gene expression profile of peripheral blood CD3$^+$ T cells isolated from MS and healthy control (CN) subjects by using a cDNA microarray. Among 1258 cDNAs on the array, 286 genes were expressed differentially between 72 untreated Japanese MS patients and 22 age- and sex-matched CN subjects. When this set was used as a discriminator for hierarchical clustering analysis, it identified four distinct subgroups of MS patients and five gene clusters differentially expressed among the subgroups. One of these gene clusters was overexpressed in MS versus CN, and particularly enhanced in the clinically most active subgroup of MS. After 46 of the MS patients were treated with interferon-beta (IFNβ-1b) for two years, IFNβ responders were clustered in two of the four MS subgroups. Furthermore, the IFNβ responders differed from nonresponders in the kinetics of IFN-responsive genes at 3 and 6 months after starting IFNβ treatment. These results suggest that T-cell gene expression profiling is valuable to identify distinct subgroups of MS associated with differential disease activity and therapeutic response to IFNβ.

Keywords: Gene expression profile; Hierarchical clustering analysis; IFNβ responder; Microarray; Multiple sclerosis; T cells

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process whose development is triggered by a complex interplay of both genetic and environmental factors (Compston and Coles, 2002). Intravenous administration of interferon-gamma (IFNγ) to MS patients in a previous clinical trial provoked acute relapses accompanied by activation of the systemic immune response, indicating a central role of proinflammatory T helper type 1 (Th1) lymphocytes in the immunopathogenesis of MS (Panitch et al., 1987). In contrast, treatment with interferon-beta (IFNβ) produced a

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beneficial effect on MS patients with a reduction of the relapse rate by approximately 30% (The IFNB Multiple Sclerosis Study Group, 1993; Jacobs et al., 1996; Saida et al., 2005). Recent studies indicated that an early initiation of IFNβ delays the conversion to clinically definite MS in the patients who experienced a first demyelinating event (Jacobs et al., 2000).

MS exhibits a great range of phenotypic variability. It is classified into relapsing–remitting MS (RRMS), secondary progressive MS (SPMS), or primary progressive MS (PPMS) with respect to the disease course, conventional MS (CMS) or opticospinal MS (OSMS) in terms of the lesion distribution (Saida et al., 2005), and IFNβ responder or nonresponder based on the therapeutic response to IFNβ (Waubant et al., 2003). MS brain lesions show a remarkable heterogeneity in the degree of inflammation, complement activation, antibody deposition, demyelination and remyelination, oligodendrocyte apoptosis, and axonal degeneration (Lucchinetti et al., 2000). These observations suggest that MS is a kind of neurological syndrome caused by different immunopathological mechanisms leading to the final common pathway that provokes inflammatory demyelination. Therefore, it is not surprising to find that individual MS patients show highly variable responses to IFNβ treatment. Currently, very little is known about the molecular background underlying clinical and pathological heterogeneity of MS.

DNA microarray technology is a novel approach that allows us to systematically monitor the expression of a large number of genes in disease-affected tissues (Staudt, 2001). This approach has discovered therapeutically relevant targets and prognostic markers for cancers (Alizadeh et al., 2000; van de Vijver et al., 2000), and has given new insights into the complexity of molecular interactions promoting the autoimmune process in MS (Steinman and Zamvil, 2003). Importantly, the comprehensive gene expression profiling of MS brain tissues and peripheral blood lymphocytes identified a battery of genes deregulated in MS, whose role has not been previously predicted in its pathogenesis (Lock et al., 2002; Graumann et al., 2003; Tajouri et al., 2003; Stürzbecher et al., 2003; Achiron et al., 2004). However, most of previous studies have focused on gene expression in heterogeneous populations of unfractionated lymphocytes and brain cells. Recently, by using microarray we showed that IFNβ treatment elevates the expression of 7 IFN-responsive genes in highly purified peripheral blood $CD3^+$ T cells of 13 Japanese RRMS patients (Koike et al., 2003). More recently, we found that the majority of differentially expressed genes in $CD3^+$ T cells between 72 untreated MS patients and 22 healthy control (CN) subjects were categorized into apoptosis signaling-related genes (Satoh et al., 2005).

To extend our previous studies, we conducted hierarchical clustering analysis of differentially expressed genes between MS and CN in peripheral blood $CD3^+$ T cells. Here we report that T-cell gene expression profiling classifies a heterogeneous population of Japanese MS into four subgroups that differ in the disease activity and therapeutic response to IFNβ, suggesting that this analysis could be applied for designing tailor-made treatment of MS.

2. Subjects and methods

2.1. The study population

The Research Group for IFNβ treatment of Japanese MS, sponsored by the Ministry of Health, Labour and Welfare of Japan, conducted the present study. It enrolled 72 clinically active Japanese MS patients, including 65 RRMS and 7 SPMS cases composed of 55 women and 17 men with the mean age of 36.1 ± 10.3 years, and 22 healthy control (CN) subjects composed of 16 women and 6 men with the mean age of 38.6 ± 12.3 years. The members of this research group (SK, KN, KY, KO, TK, TF and TY), all of who are certified neurologists, diagnosed individual cases according to the established criteria (McDonald et al., 2001), and followed up the patients for at least two years after entry. The patients showed the mean Expanded Disability Status Scale (EDSS) score of 2.8 ± 2.0 upon entry. No patients had a history of treatment with interferons, glatiramer acetate or mitoxantrone before enrollment, or received corticosteroids and other immunosuppressants during at least one month before blood sampling. MS patients were divided into two groups according to their own determination upon entry: one treated with IFNβ and the other without IFNβ. The IFNβ-treated group included 46 patients who started to receive an administration of 8 million units of IFNβ-1b (Betaferon, Schering, Osaka, Japan) for two years given subcutaneously on alternate days, while the IFNβ-untreated group included 26 patients who were followed up without IFNβ treatment for successive two years. From the IFNβ-treated group, blood samples were taken at three time points: before starting IFNβ treatment (designated Pre) and at 3 and 6 months after starting the treatment. In the IFNβ-untreated group, they were collected twice: at enrollment and at 6 months after the enrollment. In case of acute relapse, the patients in both groups were given intravenous methylprednisolone pulse (IVMP) following the standard protocol, although none received glatiramer acetate, mitoxantrone, or other immunosuppressants. The samples obtained during clinically obvious relapses or episodes of infection were omitted. Written informed consent was obtained from all the subjects. The present study was approved by the Ethics Committee of National Center of Neurology and Psychiatry (NCNP).

2.2. IFN β responder/nonresponder score

To evaluate the therapeutic response to IFNβ, we monitored the following six parameters during four years spanning two years before and after initiation of IFNβ

treatment; the number of clinical relapse, the day of IVMP treatment, the day of hospitalization, EDSS score, the number of lesions on T2-weighted MRI, and the patient's satisfaction on the treatment (Table 1). When compared before and after IFNβ treatment, these parameters have given three ranks and scores; good (+1), intermediate (0), and poor (−1). The total score was calculated for each patient, ranging from the maximum value of +6 to the minimum value of −6. The patients with the total score equal to or greater than +3 were considered as being the responder (R), the score from 0 to +2 as one with the undetermined response (UD), and the score equal to or smaller than −1 as the nonresponder (NR) (Table 1).

2.3. cDNA microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1258 cDNA immobilized on a poly-L-lysine-coated slide glass. They were composed of well annotated genes of various functional classes, including cytokines/growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, signal transducers, cell cycle regulators and housekeeping genes (Hitachi Life Science, Kawagoe, Saitama, Japan; http://www.hitachi.co.jp/LS). Peripheral blood mononuclear cells (PBMC) were isolated from 30 ml of heparinized blood by centrifugation on a Ficoll density gradient. They were labeled with anti-CD3 antibody-coated magnetic microbeads (#130-050-101, Miltenyi Biotec, Auburn, CA), and $CD3^+$ T cells were separated by AutoMACS (Miltenyi Biotec). The remaining cells after the positive selection of $CD3^+$ T cells were harvested as $CD3^-$ non-T cell fraction as described previously (Koike et al., 2003; Satoh et al., 2005). Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen, Valencia, CA). Five micrograms of purified RNA was in vitro amplified, and the antisense RNA (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 °C for 10 h 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.

2.4. 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 (Baldi and Long, 2001). The error rate of this test smaller than 0.25 following the Bonferroni correction was considered as significant. Hierarchical clustering analysis and principal component analysis (PCA) were performed on a set of 286 genes differentially expressed between MS and CN groups, which were selected

Table 1
IFNβ responder/nonresponder score

Category	The parameters	Rank and score of the therapeutic response		
		Poor	Intermediate	Good
#1	Number of relapse after 2 years/number of relapse before 2 years	≥1.5	1.5–0.5	≤0.5
	Score	(−1)	0	(+1)
#2	Number of IVMP treatment after 2 years/number of IVMP treatment before 2 years	≥1.5	1.5–0.5	≤0.5
	Score	(−1)	0	(+1)
#3	Day of hospitalization after 2 years/day of hospitalization before 2 years	≥1.5	1.5–0.5	≤0.5
	Score	(−1)	0	(+1)
#4	EDSS score before treatment-EDSS score in 2 years after treatment	≤ −0.5	0.5–(−0.5)	≥0.5
	Score	(−1)	0	(+1)
#5	Number of lesions on T2-weighted MRI in 2 years after treatment/number of lesions on T2-weighted MRI before treatment	≥1.2	1.2–0.8	≤0.8
	Score	(−1)	0	(+1)
#6	Patient's satisfaction	Unsatisfied	Neither satisfied nor unsatisfied	Satisfied
	Score	(−1)	0	(+1)

The total responder/nonresponder score of six categories ranges from the maximum value of +6 to the minimum value of −6. The patients with the score equal to +3 or greater than +3 were classified as responder (R), the score ranging from 0 to +2 as undetermined (UD), and the score equal to −1 or smaller than −1 as nonresponder (NR). Abbreviations: IVMP, intravenous methylprednisolone pulse.

as a discriminator for a standard × standard algorithm on GeneSpring 7.2 (Agilent Technologies, Palo Alto, CA). The differences in clinical parameters among MS subgroups were evaluated by multiple comparison test following the Bonferroni correction.

3. Results

3.1. Microarray analysis identified 286 genes differentially expressed in peripheral blood T cells between MS and control subjects

Among 1258 genes on the microarray, 286 genes were expressed differentially in peripheral blood $CD3^+$ T cells between 72 untreated MS patients and 22 CN subjects. Among them, 78 genes were upregulated, while 208 genes downregulated in MS versus CN (Supplementary Table 1 online for all datasets). We also conducted the microarray analysis of $CD3^-$ non-T cells, 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 and CN (data not shown).

3.2. Hierarchical clustering analysis identified four distinct subgroups of MS and five gene classes

Hierarchical clustering analysis was performed on $CD3^+$ T-cell samples of 72 untreated MS patients and 22 CN subjects, by using the set of 286 differentially expressed genes described above as a discriminator. This unsupervised approach, which arranged the genes and samples with a similar expression pattern to make a cluster in the dendrogram, identified four distinct

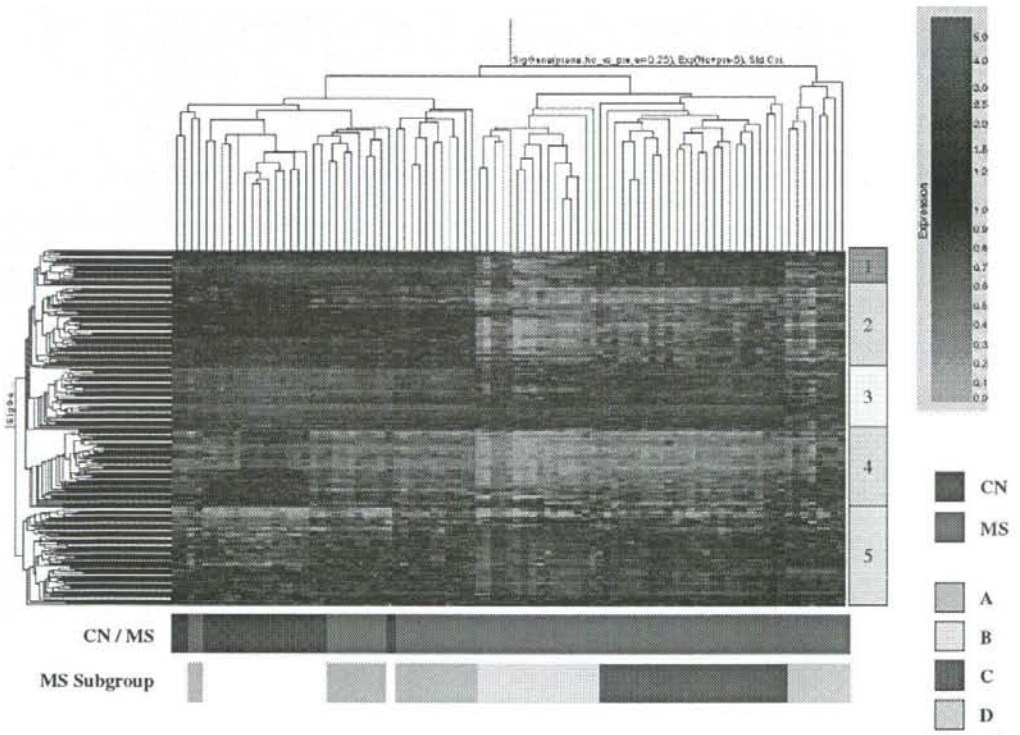

Fig. 1. Hierarchical clustering analysis of 286 genes differentially expressed between untreated MS patients and control subjects. The gene expression profile of peripheral blood $CD3^+$ T cells was studied in 72 untreated MS patients and 22 age- and sex-matched healthy control (CN) subjects, by using a 1258 cDNA microarray. Hierarchical clustering analysis was performed by selecting a set of 286 genes differentially expressed between MS and CN as a discriminator. 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 286 genes in an individual subject. The matrix 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. Hierarchical clustering 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 categorized into five classes numbered #1 (pink) to #5 (light blue).

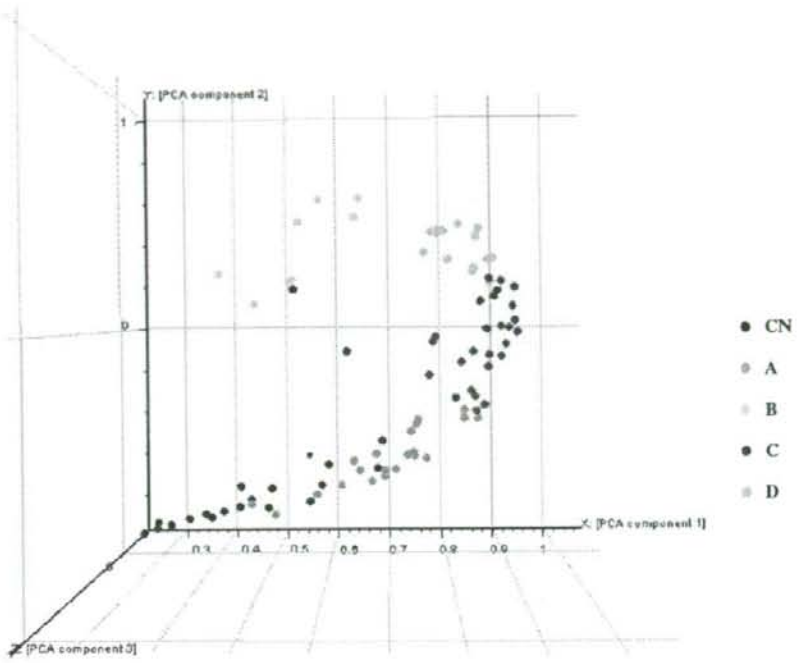

Fig. 2. Principal component analysis of 286 discriminator genes. Principal component analysis (PCA), which reduces all of the variance in the original dataset to three dimensions accounting for a significant fraction of the variance, verified a clear separation of the CN group (dark blue) and four MS subgroups named A (green), B (light blue), C (red) and D (yellow) identified by hierarchical clustering analysis.

subgroups of MS, clearly separated from the CN group (Fig. 1). We operationally designated each subgroup of MS as A, B, C and D, following the relative location in the dendrogram (Fig. 1). Principal component analysis (PCA) verified a clear discrimination of four MS subgroups and CN group (Fig. 2). Among 94 subjects examined, two MS patients and three CN subjects were considered as being unclassifiable (UC). In contrast, the clustering analysis of $CD3^-$ non-T cells did not clearly separate MS subgroups from CN (data not shown). 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 for all datasets). The remaining five, 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). Upregulation of several class #5 genes in MS was validated by quantitative real-time RT-PCR analysis (data not shown).

3.3. Association of MS subgroups with gene clusters

Expression of the class #5 genes were elevated in all MS subgroups, whereas the classes #1 to #4 genes were downregulated in all of them, although the present study could not identify the marker genes specific for each MS subgroup. The subgroup A showed the gene expression pattern that is the most similar to CN. The similarity was supported by a partial overlap between A and CN in PCA (Fig. 2), and by the observations that one CN subject was incorporated in A, while two MS patients of A were included in CN (Fig. 1). Notably, the subgroup B showed the greatest upregulation of class #5 genes and the most prominent suppression of classes #1 to #4 genes (Fig. 1).

The class #5 genes ($n=78$) contain nine chemokines (11.5%), including CCL1, CCL3, CCL13, CCL18, CCL24, CXCL1, CXCL2, CXCL9, and CXCL14. In contrast, the classes #1 to #4 genes ($n=203$) contained only two chemokines (1.0%), such as CXCL5 and CXCL10. These observations suggest that the class #5 gene cluster is highly enriched in chemokine genes.

Fig. 3. Clinical characteristics of microarray-determined four MS subgroups. MS patients were classified into four distinct subgroups named A, B, C, and D by hierarchical clustering analysis. The bar indicates the data of individual patients. The number of relapse, the day of IVMP treatment, the day of hospitalization, and the number of lesions on T2-weighted MRI represent the data of 2 years before enrollment. Abbreviations: EDSS, Expanded Disability Status Scale; IVMP, intravenous methylprednisolone pulse; R/NR, responder/nonresponder.

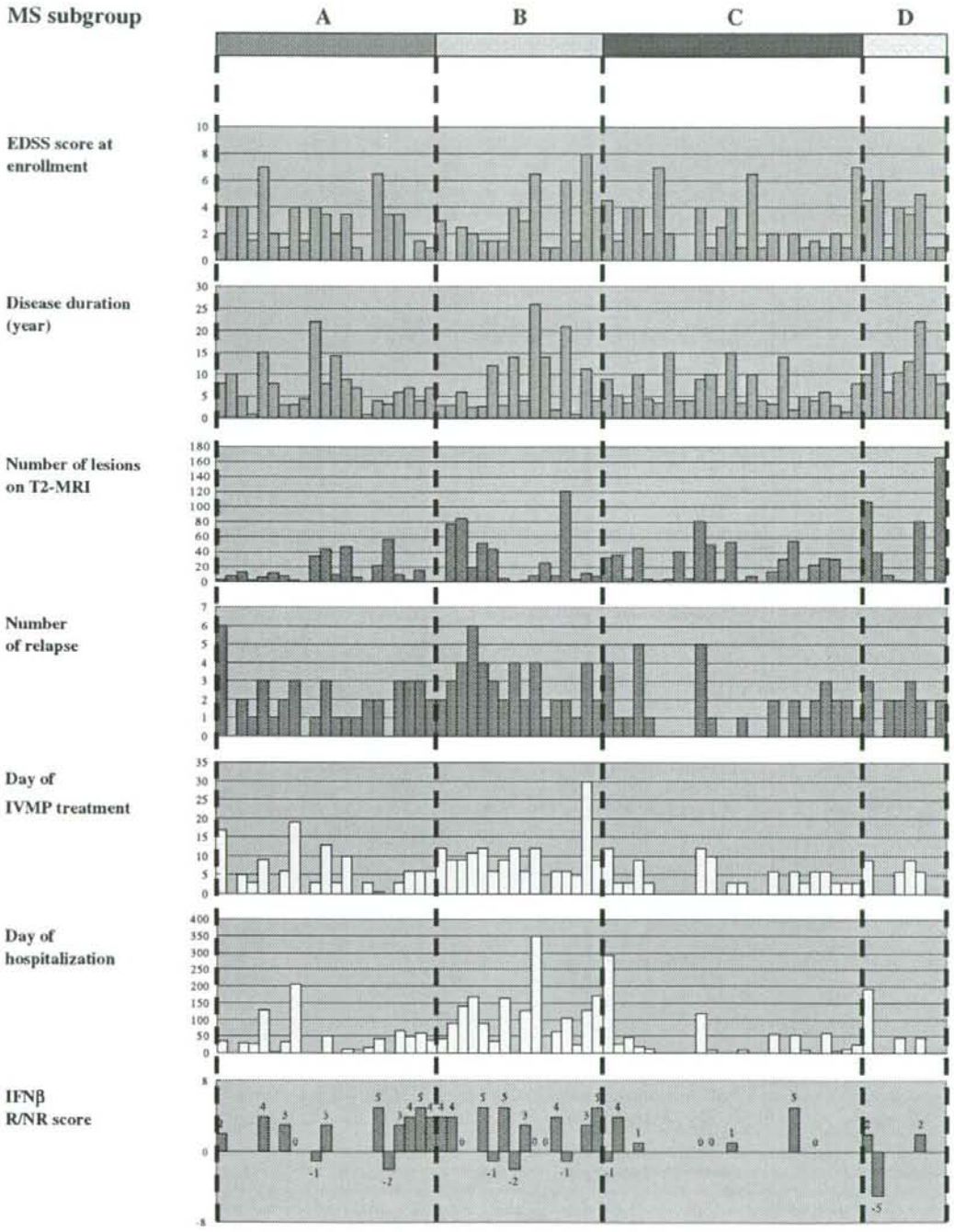
MS subgroup
A
B
C
D
EDSS score at enrollment
Disease duration (year)
Number of lesions on T2-MRI
Number of relapse
Day of IVMP treatment
Day of hospitalization
IFNβ R/NR score

Table 2
The therapeutic response to IFNβ in microarray-determined four MS subgroups

	Total	A	B	C	D	UC
IFNβ-treated patients (*n*)	46	14	14	11	5	2
Age of IFNβ-treated patients (average, SD)	34.9±9.2	33.2±7.6	36.5±10.4	33.1±8.3	36.2±13.3	41.5
Male to female ratio of IFNβ-treated patients	8 to 38	1 to 13	3 to 11	3 to 8	0 to 5	1 to 1
IFNβ responder/nonresponder score (average, SD)	1.9±2.6	2.5±2.3	2.1±2.6	1.3±2.1	−0.3±4.0	3
Dropout during a follow-up (*n*)	7	2	0	3	2	0
IFNβ responder (*n*, %)	19 (41.3%)	8 (57.1%)	8 (57.1%)	2 (18.2%)	0 (0%)	1 (50%)
IFNβ nonresponder (*n*)	7	2	3	1	1	0
Undetermined group (*n*)	13	2	3	5	2	1
The patients with IFNβ-related adverse effects (*n*, %)	29 (63.0%)	8 (57.1%)	9 (64.3%)	7 (63.6%)	4 (80%)	1 (50%)
Increase in the number of lesions on T2-weighted MRI during a follow-up (average, SD)	1.7±9.7	−2.0±7.1	2.8±6.6	7.6±15.8	−0.7±8.1	−3.5
The patients satisfied with IFNβ treatment (*n*, %)	17 (37.0%)	8 (57.1%)	6 (42.9%)	2 (18.2%)	0 (0%)	1 (50%)
The patients neither satisfied nor unsatisfied with IFNβ treatment (*n*)	21	4	7	7	2	1
The patients unsatisfied with IFNβ treatment (*n*)	8	2	1	2	3	0

Among 72 MS patients, 46 patients were treated with IFNβ for two years after enrollment. The therapeutic response was evaluated by IFNβ responder/nonresponder score shown in Table 1. Abbreviations: UC, unclassifiable.

3.4. Clinical characteristics of microarray-determined MS subgroups

Next, we investigated clinical characteristics of four MS subgroups (Supplementary Table 2 online and Fig. 3). No statistically significant differences were found among the subgroups in the age, disease duration, EDSS score, and the number of lesions on T2-weighted MRI at enrollment. However, there was a trend that the subgroup D showed a greater EDSS score and had a larger number of MRI lesions, suggestive of an advanced stage of the disease (Supplementary Table 2 online). The female outnumbered the male in all the subgroups. The male to female ratio was relatively higher in C, while no male patient was included in D. The patients with RRMS outnumbered those with SPMS in all the subgroups, although there was a mild bias for SPMS in B. The number of relapse, the day of IVMP treatment, and the day of hospitalization during preceding two years before enrollment were the largest and longest in subgroup B, and this difference was statistically significant, when compared between subgroups B and C ($p=0.0128$, 0.0183, and 0.0329 for each parameter) (Supplementary Table 2 online and Fig. 3). These observations indicate that the subgroup B included the patients who were the clinically most active before starting IFNβ.

In all MS subgroups, the conventional form of MS (CMS) greatly outnumbered non-CMS, the latter was composed of the opticospinal form (OSMS) and multifocal recurrent myelitis without optic nerve involvement. No obvious association was identified between a particular MS subgroup and the spinal cord involvement. However, 5 of 6 patients having the lesions restricted to the cerebrum (CBR) were included in subgroup C (Supplementary Table 2 online). These observations suggest that the status of T-cell gene expression might affect the lesion distribution in this subgroup.

3.5. IFN β responders were clustered in subgroups A and B

Based on the patient's own determination at enrollment, 72 MS patients were separated into two groups; 46 who started to receive IFNβ treatment for following two years, and 26 who were followed up without IFNβ treatment for successive two years (Supplementary Table 3 online). All the IFNβ-treated patients were evaluated by the IFNβ responder/nonresponder score (Table 1) at the end of the two year-treatment. They were classified into 19 IFNβ responders, 7 nonresponders, 13 undetermined subjects, and 7 dropouts (Table 2). The difference in the score among the 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 2). However, there existed a trend that IFNβ responders were clustered either in subgroup A or B. Because the subgroup A contains the greatest proportion of IFNβ responders (57.1%), the patients of A were judged as being the most IFNβ responsive (Table 2). All the responders of A expressed a satisfaction on IFNβ treatment. The patients of the subgroup B also showed a good response equivalent to A (57.1%), although the number of satisfied patients was 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. The patients of C showed a trend for great increase in the number of MRI lesions during IFNβ treatment, consistent with the poor response to IFNβ (Table 2). A battery of IFNβ treatment-related adverse effects, including skin reactions, flu-like symptoms, leukocytopenia, depression, and amenorrhea, were observed in more than 50% of IFNβ-treated patients in all the subgroups (Table 2). Seven patients of the IFNβ-treated group discontinued the treatment: five due to adverse effects, one due to a severe relapse, and another by a personal reason.

We also studied T-cell gene expression profile of IFNβ-treated MS patients at 3 or 6 months after starting the

treatment. Although hierarchical clustering analysis classified these patients into several subgroups, they did not match with the subgroup A, B, C, or D determined at pretreatment (data not shown). Furthermore, no significant association was identified between these new clusters and the response to IFNβ. These observations suggest that T-cell gene expression profiling at pretreatment is the most valuable to predict the clinical outcome, whereas the analysis after starting IFNβ treatment is less informative.

3.6. Temporal profile of IFN-responsive gene expression in the first six months discriminated responders and nonresponders

Finally, we investigated the temporal expression profile of the genes with IFN-responsive promoter elements named IFN-responsive genes (IRGs) following IFNβ treatment. As we previously reported (Koike et al., 2003), IFNβ treatment for 6 months enhanced the expression of a battery of IRGs in T cells (Fig. 4). A remarkable difference was found between IFNβ responders (R) and nonresponders (NR) in the kinetics of several IRGs, such as IFN-stimulated protein 15 (ISG15), small inducible cytokine A2 (SCYA2, CCL2, or MCP-1), TNF receptor subfamily member 1B (TNFRSF1B, TNFRp75), and IFNα-inducible protein 27 (IFI27) (Fig. 5). The IFNβ responders exhibited a pattern of persistent upregulation during 6 months of the treatment. In contrast, the nonresponders showed a seesaw pattern, i.e. higher upregulation at 3 months than the responders, followed by substantial downregulation at 6 months. The differences between R and NR in the kinetics of both TNFRSF1B and IFI27 levels from 3 to 6 months were statistically significant ($p=0.0092$ and 0.0307, respectively) (Fig. 5). These observations suggest that IFNβ nonresponders also well respond to IFNβ at 3 months, but they could not maintain the responsiveness until 6 months.

4. Discussion

To elucidate the molecular basis underlying clinicopathological variability of MS, we conducted a comprehensive study that combines T-cell gene expression profiling and clinical characteristics of Japanese MS patients. Hierarchical clustering analysis of 286 genes differentially expressed between 72 untreated MS patients and 22 CN subjects classified a clinically heterogeneous population of MS into four distinct subgroups, named A, B, C, and D, and identified five gene classes numbered #1 to #5. The class

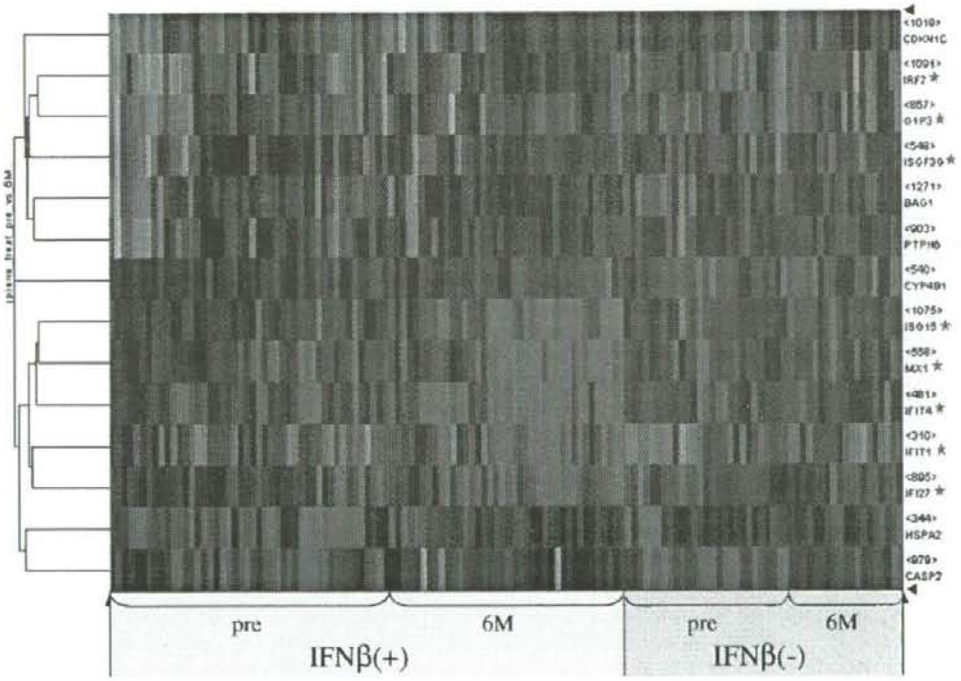

Fig. 4. Induction of IFN-responsive genes in IFNβ-treated MS patients. Seventy-two MS patients were divided into IFNβ-treated group (IFNβ+; $n=46$) and untreated group (IFNβ−; $n=26$). A cluster of known IFN-responsive genes (IRGs) indicated by the star were significantly upregulated exclusively in IFNβ-treated patients at 6 months after starting the treatment.

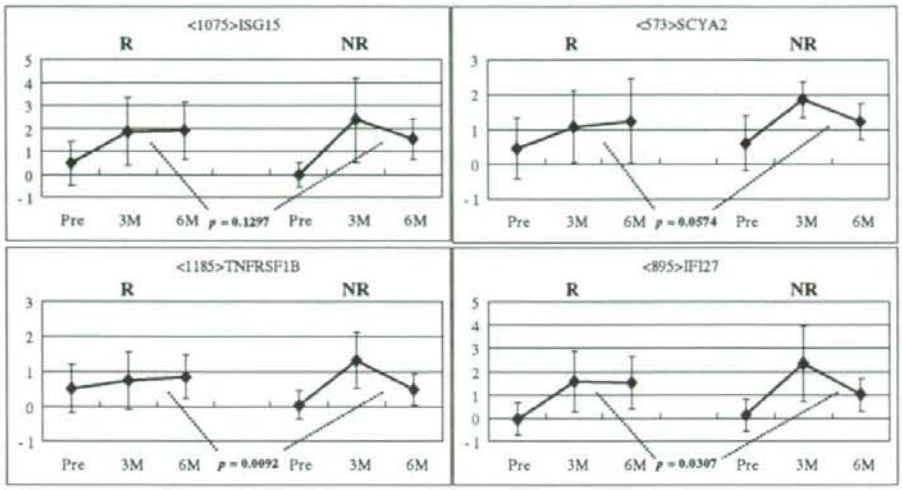

Fig. 5. Temporal profile of induction of IFN-responsive genes in IFNβ responders and nonresponders during IFNβ treatment. T-cell gene expression profiling was examined in 46 IFNβ-treated MS patients at three time points: before starting IFNβ treatment (Pre), and at 3 months (3M) and 6 months (6M) after starting the treatment. Based on the IFNβ responder/nonresponder score, they were separated into 19 IFNβ responders (R: the left) and 7 nonresponders (L: the right) as shown in Table 2. The temporal expression profile of IFN-responsive genes (IRGs), such as IFN-stimulated protein 15 (ISG15), small inducible cytokine A2 (SCYA2), TNF receptor subfamily member 1B (TNFRSF1B), and IFNα-inducible protein 27 (IFI27), is shown with the statistical differences in the kinetics during 3 to 6 months between R and NR. The vertical axis represents the gene expression level (GEL), while the horizontal axis indicates the time course.

#5 genes containing nine chemokines were upregulated exclusively in MS. The most clinically active subgroup B showed the highest upregulation of the class #5 genes. These observations suggest that the higher disease activity of B is in part attributable to overproduction of chemokines which promote lymphocyte and macrophage trafficking into the CNS (Balashow et al., 1999; Opdenakker et al., 2003). Recently, using this database as a training set for support vector machine (SVM) analysis of T cell gene expression, we found that the great majority of active RRMS patients were classified into MS subgroups, while clinically stable patients without obvious relapses for several years were occasionally classified into CN group (unpublished observations).

The class #5 genes upregulated in MS T cells include various cytokines, growth factors and their receptors, whose expression was detected at high levels in demyelinating lesions of MS, such as IL-12p40 (Windhagen et al., 1995), IL-10 (Hulshof et al., 2002), granulocyte colony-stimulating factor (GCSF) (Lock et al., 2002), platelet-derived growth factor receptor-alpha (PDGFRA) (Maeda et al., 2001), transforming growth factor-beta 2 (TGFB2) (Peress et al., 1996), and insulin-like growth factor-II (IGF-II) (Gveric et al., 1999). The class #5 genes also contain many apoptosis-signaling regulators pivotal for T cell development. It is worthy to note that nuclear receptor subfamily 4, group A, member 2 (NR4A2) in the class #5 was the most strongly upregulated gene in MS T cells. NR4A2 encodes an orphan member of the steroid–thyroid hormone receptor superfamily transcription factors designated Nurr1. Nurr1 is induced in T cells during apoptosis (Okabe et al., 1995) and the members of this family regulate clonal deletion of self-reactive T cells in the thymus (Zhou et al., 1996). Nurr1 activates the transcription of osteopontin (Lammi et al., 2004), a Th1 cytokine that plays a key role in progression of inflammatory demyelination in MS (Steinman and Zamvil, 2003). In contrast, a previous study showed that NR4A2 is downregulated in unfractionated PBMC of MS patients (Achiron et al., 2004), although the following study from the same group indicated a significant upregulation of NR4A2 in PBMC of both MS and SLE patients (Mandel et al., 2004). We validated upregulation of NR4A2 mRNA levels in MS T cells by quantitative real-time RT-PCR analysis (unpublished observations).

The present study suggests that the microarray-based classification of MS is useful to predict therapeutic response to IFNβ. The proportion of IFNβ responders greatly differed among MS subgroups: 57.1% in A, 57.1% in B, 18.2% in C and 0% in D. Furthermore, the responders are significantly different from the non-responders in the kinetics of IFN-responsive genes (IRGs). A panel of IRGs were upregulated persistently in IFNβ responders, whereas they were downregulated to some extent in nonresponders by 6 months. The precise reason for downregulation of IRGs in nonresponders after a long-term treatment remains unknown, because our study did not determine neutralizing antibody (NAb) development in individual patients. Unexpectedly, the patients of subgroup B exhibited a good response to IFNβ, despite its highest clinical activity. A recent study showed that IFNβ res-

ponders are characterized by higher relapse rates during the year prior to initiation of IFNβ treatment (Waubant et al., 2003), supporting our observations. In contrast, the patients of C with the poor response to IFNβ showed a trend for great increase in the number of MRI lesions during IFNβ treatment. A different study indicated that the number of on-treatment new T2 MRI lesions correlates with poor response to IFNβ-1a (Rudick et al., 2004), being consistent with our study. Although the subgroup D did not include any IFNβ responders, the number of MRI lesions did not increase much during the treatment, suggesting that this subgroup undergoes a neurodegenerative process independent of active inflammation (Steinman, 2001).

A number of differentially expressed genes between MS and CN include those having IFN-responsive elements in the promoter regions: p300 (EP300) and IFNα receptor 1 (IFNAR1) in the class #1, CXCL10, ATP-binding cassette (ABC) subfamily E member 1 (ABCE1 or RNS4I) (Bisbal et al., 1995), IFNγ-inducible protein 16 (IFI16), and STAT1 in the class #2, myxovirus resistance 2 (MX2) in the class #3, IFN-regulatory factors IRF9 and IRF2 in the class #4, and IFNα-16 (IFNA16) and CXCL9 (MIG) in the class #5. These observations suggest that T cells of MS patients have a constitutive defect in regulation of IFN signaling. We previously showed that CXCL9 expression was suppressed in T cells of 13 RRMS patients by a long-term treatment with IFNβ (Koike et al., 2003), suggesting that IFNβ produces a beneficial effect on MS by correcting the preexisting disturbance in the IFN-signaling pathway.

Finally, T-cell gene expression profiling identified an aberrant expression of key regulators for drug metabolism, whose role has not previously been proposed in MS (Nguyen et al., 2000). A panel of cytochrome P450 (CYP) family, which regulates Ca^{2+} influx in activated T cells (Aussel et al., 1994), were overexpressed in MS T cells. On the other hand, a wide range of ABC transporters in the classes #2, #3, and #4 were downregulated in MS T cells (see Supplementary Table 1 for all datasets). 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. The downregulated genes in MS include ABCB1 and ABCG2 expressed on brain endothelial cells, which act as a main transporter in the blood–brain barrier and determine bioavailability of corticosteroids and mitoxantrone in the brain (Zhang et al., 2003). The clinicopathological relevance of opposing changes in CYP family enzymes and ABC transporters to MS remains to be further investigated.

In conclusion, T-cell gene expression profiling is highly valuable to identify distinct subgroups of MS associated with differential disease activity and therapeutic response to IFNβ. This approach could be applied for designing tailor-made treatment of MS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2006.02.004.

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Microarray analysis identifies a set of CXCR3 and CCR2 ligand chemokines as early IFNβ-responsive genes in peripheral blood lymphocytes *in vitro*: an implication for IFNβ-related adverse effects in multiple sclerosis

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Abstract

Background: A substantial proportion of multiple sclerosis (MS) patients discontinue interferon-beta (IFNβ) treatment due to various adverse effects, most of which emerge at the early phase after initiation of the treatment and then diminish with time. At present, the molecular mechanism underlying IFNβ-related adverse effects remains largely unknown. The aim of this study is to identify a comprehensive list of early IFNβ-responsive genes (IRGs) in peripheral blood mononuclear cells (PBMC) that may play a key role in induction of adverse effects.

Methods: Total RNA of PBMC exposed to 50 ng/ml recombinant human IFNβ for 3 to 24 hours *in vitro* was processed for cDNA microarray analysis, followed by quantitative real-time RT-PCR analysis.

Results: Among 1,258 genes on the array, IFNβ elevated the expression of 107 and 87 genes, while it reduced the expression of 22 and 23 genes at 3 and 24 hours, respectively. Upregulated IRGs were categorized into conventional IFN-response markers, components of IFN-signaling pathways, chemokines, cytokines, growth factors, and their receptors, regulators of apoptosis, DNA damage, and cell cycle, heat shock proteins, and costimulatory and adhesion molecules. IFNβ markedly upregulated CXCR3 ligand chemokines (SCYB11, SCYB10 and SCYB9) chiefly active on effector T helper type 1 (Th1) T cells, and CCR2 ligand chemokines (SCYA8 and SCYA2) effective on monocytes, whereas it downregulated CXCR2 ligand chemokines (SCYB2, SCYB1 and IL8) primarily active on neutrophils.

Conclusion: IFNβ immediately induces a burst of gene expression of proinflammatory chemokines *in vitro* that have potential relevance to IFNβ-related early adverse effects in MS patients *in vivo*.

Background

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) white matter mediated by an autoimmune process, whose development is triggered by a complex interplay of both genetic and environmental factors [1]. Administration of interferon-gamma (IFNγ) induced acute relapses, along with activation of the systemic immune response [2], suggesting that T-lymphocytes producing proinflammatory T helper type 1 (Th1) cytokines play a pivotal role in the immunopathogenesis of MS. In contrast, interferon-beta (IFNβ) significantly reduces the frequency of clinical exacerbations and delays the progression of disability in relapsing-remitting MS (RRMS), accompanied by a reduction in the number of new brain lesions on MRI [3,4]. Furthermore, an early initiation of IFNβ delays the conversion to clinically definite MS in the patients who experienced a first demyelinating event [5]. Although the precise mechanism underlying therapeutic effects of IFNβ on MS remains to be fully elucidated, previous studies proposed several possibilities, including the inhibition of Th1 cell development [6], induction of Th2 immune deviation [7], restoration of function of the disrupted blood-brain barrier [8], and downregulation of IFNγ-induced expression of class II major histocompatibility complex (MHC) molecules [9].

Although clinical benefits of IFNβ in MS are meaningful, approximately one-third of the patients receiving IFNβ therapy suffered from a higher or identical annual relapse rate on treatment [10]. New lesion formation on MRI during the treatment correlates with poor response to IFNβ [11]. Furthermore, a substantial proportion of the patients discontinued IFNβ treatment because of various adverse effects, including skin reactions, flu-like symptoms, leukocytopenia, liver dysfunction, depression and amenorrhea [12]. The molecular mechanisms accounting for IFNβ-related adverse effects remain unknown, although most of these emerge at the early phase after initiation of the treatment, and then diminish with time [12]. At present, no biologically relevant markers capable of predicting either therapeutic or detrimental responses of IFNβ in MS are available [13].

DNA microarray technology is a novel approach that allows us to systematically monitor the expression of a large number of genes. It has given new insights into the complexity of molecular interactions promoting the autoimmune process in MS [14]. IFNβ induces a complex pattern of gene regulation in peripheral blood mononuclear cells (PBMC) of MS [15]. Recently, we studied the gene expression profile of $CD3^+$ T cells isolated from PBMC of 13 Japanese MS patients before and after treatment with IFNβ-1b by analyzing a custom cDNA microarray containing a set of well-annotated, immunologically relevant genes. IFNβ upregulates the expression of 7 IFNβ-responsive genes (IRGs) during the treatment [16]. A following study performed on RRMS patients receiving IFNβ-1a supported our observations [17]. More recently, we found that the vast majority of genes expressed in $CD3^+$ T cells differentially between 72 untreated MS patients and 22 healthy controls are categorized into apoptosis regulators [18]. Regarding the gene expression profile of IFNβ responders in MS, baseline levels of IL-12p35 mRNA are significantly lower in the responders [19]. Downregulation of IL-8 expression in PBMC during IFNβ treatment distinguishes the responders from nonresponders in RRMS [20]. IFNβ responders differ from nonresponders in the kinetics of expression of IRGs at 3 and 6 months after starting the treatment [21]. A three-dimensional model of gene triplets detected by RT-PCR analysis predicts IFNβ response status in RRMS [22]. However, all of previous observations do not clearly illustrate the molecular basis of complex biological effects of IFNβ in MS. Furthermore, no databases of immediate early IRGs in PBMC are currently available.

The present study is designed to identify a comprehensive list of immediate early IRGs in PBMC with potential relevance to IFNβ-related early adverse effects in MS.

Methods

Treatment of peripheral blood lymphocytes with IFNβ

PBMC were isolated from heparinized blood by centrifugation on a Ficoll density gradient. PBMC were suspended at 5×10^6 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium). The cells were incubated in a 5%CO_2/95% air incubator at 37°C for 3 hours to characterize the immediate response or for 24 hours to detect the early response, in the culture medium with or without inclusion of 50 ng/ml recombinant human IFNβ (a specific activity of = 2×10^7 units/mg, PeproTech, London, UK), 50 ng/ml recombinant human IFNγ (a specific activity of = 2×10^7 units/mg, PeproTech), 50 ng/ml recombinant human TNFα (a specific activity of = 2×10^7 units/mg, PeproTech), or 50 ng/ml recombinant human IL-1β (a specific activity of = 1×10^7 units/mg, PeproTech). They were then processed for RNA preparation as described previously [16,18,21]. Written informed consents were obtained from the subjects involved in the present study according to the form approved by the Ethics Committee of National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan. The samples of the subjects #1, #2 and #4 were processed for both microarray and real-time RT-PCR analysis, while those of the subject #3 were studied only by real-time RT-PCR analysis.

Table 1: Primers utilized for real-time RT-PCR analysis

Genes	GenBank accession No.	Sense primers	Antisense primers	PCR product (bp)
ISG15 (G1P2)	NM_005101 (M13755)	5'aagcccctgagcaccgtgttcat3'	5'ttgatcctgctcggatgctggtg3'	102
SCYB10 (CXCL10, IP-10)	NM_001565 (X02530)	5'tcgatgcagtgcttccaaggatgg3'	5'ccttcctacaggagtagtagcagc3'	162
SCYA8 (CCL8, MCP2)	NM_005623 (Y10802)	5'tctgtgctgaccccaaggagagat3'	5'taatgtcacactgcacctgggga3'	164
SCYA2 (CCL2, MCP1)	NM_002982 (S71513)	5'ctagctttccccagacaccctgtt3'	5'ccaggggtagaactgtggttcaag3'	197
SCYB2 (CXCL2, GRO2)	NM_002089	5'cccgcatcgcccatggttaagaaa3'	5'tcttctgttcctgtaaggcagggc3'	131
FOS	NM_005252	5'gagctggtgcattacagagaggag3'	5'ggacttgagtccacacatggatgc3'	140
RGS14	NM_006480	5'tgacagctacccaacagtccagga3'	5'agggattgggggtgagcttgttga3'	222
G3PDH	NM_002046	5'ccatgttcgtcatgggtgtgaacca3'	5'gccagtagaggcagggatgatgttc3'	251

Abbreviations: ISG15, interferon-stimulated gene 15; SCYB10, small inducible cytokine subfamily B, member 10; SCYA8, small inducible cytokine subfamily A, member 8; SCYA2, small inducible cytokine subfamily A, member 2; SCYB2, small inducible cytokine subfamily B, member 2; FOS, cellular oncogene c-fos; RGS14, regulator of G-protein signaling 14; and G3PDH, glyceraldehyde-3-phosphate dehydrogenase

Quantitative real-time RT-PCR analysis

DNase-treated total RNA was processed for cDNA synthesis using oligo(dT)$_{12-18}$ primers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified by PCR in LightCycler ST300 (Roche Diagnostics, Tokyo, Japan) using SYBR Green I dye and the primer sets listed in Table 1. To calibrate the concentration of mRNA levels in test cDNA samples, serially-diluted purified PCR products generated by conventional RT-PCR (a 10-fold dilution from 1 pg/ml to 0.0001 pg/ml) were amplified in parallel. The levels of expression of target genes were standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene detected in the identical cDNA samples. The assays were performed in triplicate measurements of the same sample and the results were expressed as the average with standard error.

cDNA microarray analysis

The present study utilized a custom microarray containing duplicate spots of 1,258 cDNA immobilized on a poly-L-lysine-coated slide glass (Hitachi Life Science, Kawagoe, Saitama, Japan) [16,18,21]. They were prepared by PCR of well-annotated genes, selected from cytokines, growth factors and their receptors, apoptosis regulators, oncogenes, transcription factors, cell cycle regulators and housekeeping genes. The complete gene list is shown in Additional file 1. Five μg of purified RNA was *in vitro* amplified and antisense RNA (aRNA) was processed for microarray analysis. aRNA derived from IFNβ-treated PBMC was labeled with a fluorescent dye Cy5, while aRNA of untreated PBMC was labeled with Cy3 by reverse transcriptase reaction. The arrays were hybridized at 62°C for 17 hours in the hybridization buffer containing equal amounts of Cy3- or Cy5-labeled cDNA, and they were then scanned at two different photomultiplier tube (PMT) gains by the ScanArray 5000 scanner (GSI Lumonics, Boston, MA). The data were analyzed by using the QuantArray software (GSI Lumonics). The fluorescence intensities (FI) of individual spots were quantified following global normalization between Cy3 and Cy5 signals. The average of FI of duplicate spots was calculated, then the ratio of FI of Cy5 signal versus FI of Cy3 signal exceeding 2.0 was defined as significant upregulation, whereas the ratio smaller than 0.5 was considered as substantial downregulation. The impact of inter-experiment variability was validated by analyzing a scatter plot (see Additional file 2).

The IFN-regulated expression of the genes we identified was verified by computerized search of PubMed database and IFN Stimulated Gene (ISG) database [23].

Results

Microarray analysis identified immediate early IFNβ-responsive genes in PBMC

Among 1,258 genes on the array, IFNβ treatment for 3 hours elevated the expression of 107 genes in PBMC isolated from a 46 year-old healthy man (the subject #1), while it reduced the expression of 22 genes (see Additional files 3 and 4 for the complete list). IFNβ treatment for 24 hours upregulated 87 genes and downregulated 23 genes (see Additional files 5 and 6 for the complete list). Sixty-nine genes were upregulated at both 3 and 24 hours, while only two genes such as FOS and IL1A were downregulated at both. The IRGs upregulated at both time points contained 11 *in vivo* IRGs reported previously by us [16], including IFIT1 (IFI56), ISG15 (G1P2), IFIT4 (IFI60), IFI27, G1P3 (IFI6-16), IRF7, ABCB2 (TAP1), ATF3, IFITM1 (IFI17), SULT1C1, and TNFAIP6, whose expression was elevated in T cells and non-T cells *ex vivo*, isolated from 13 RRMS patients during IFNβ treatment for 3 to 6 months. Top 20 most significant genes, either

Table 2: Top 20 upregulated genes in PBMC following exposue to IFNβ

No.	Cy5/Cy3 ratio	Symbol	GenBank	Gene name
at 3 hours				
1	188.50	IFIT1	X03557	interferon-induced protein with tetratricopeptide repeats 1 (IFIS6)
2	149.82	SCYB11	AF030514	chemokine (C-X-C motif) ligand 11 (CXCL11, IP-9, I-TAC)
3	81.81	ISG15	M13755	interferon-stimulated gene ISG15 (G1P2)
4	67.84	IFIT4	AF083470	interferon-induced protein with tetratricopeptide repeats 4 (IFI60)
5	66.38	MX1	M30817	myxovirus resistance protein 1 (MXA)
6	64.43	SCYB10	X02530	chemokine (C-X-C motif) ligand 10 (CXCL10, IP-10)
7	55.01	SCYA8	Y10802	chemokine (C-C motif) ligand 8 (CCL8, MCP2)
8	36.88	SCYB9	X72755	chemokine (C-X-C motif) ligand 9 (CXCL9, MIG)
9	35.14	TNFSF10	U37518	tumor necrosis factor superfamily, member 10 (TRAIL)
10	32.86	MX2	M30818	myxovirus resistance protein 2 (MXB)
11	21.99	IFI27	X67325	interferon alpha-inducible protein 27
12	21.98	G1P3	X02492	interferon alpha-inducible protein (IFI6-16), isoform a
13	19.26	ISG20	U88964	interferon-stimulated gene ISG20
14	19.12	STAT1	M97935	signal transducer and activator of transcription 1, isoform alpha
15	13.71	IRF7	U53830	interferon-regulatory factor 7, isoform alpha
16	13.44	CCNA1	U66838	cyclin A1
17	12.72	IL6	M14584	interleukin 6
18	10.86	IL1RN	X53296	interleukin 1 receptor antagonist, isoform 1
19	10.47	SCYA2	S71513	chemokine (C-C motif) ligand 2 (CCL2, MCP1)
20	9.91	STAT2	M97934	signal transducer and activator of transcription 2
at 24 hours				
1	193.19	SCYA8	Y10802	chemokine (C-C motif) ligand 8 (CCL8, MCP2)
2	124.77	IFIT1	X03557	interferon-induced protein with tetratricopeptide repeats 1 (IFIS6)
3	107.21	IFI27	X67325	interferon alpha-inducible protein 27
4	70.51	ISG15	M13755	interferon-stimulated gene ISG15 (G1P2)
5	53.22	SCYB11	AF030514	chemokine (C-X-C motif) ligand 11 (CXCL11, IP-9, I-TAC)
6	38.07	MX1	M30817	myxovirus resistance protein 1 (MXA)
7	37.95	IFIT4	AF083470	interferon-induced protein with tetratricopeptide repeats 4 (IFI60)
8	31.56	SCYB10	X02530	chemokine (C-X-C motif) ligand 10 (CXCL10, IP-10)
9	23.65	G1P3	X02492	interferon alpha-inducible protein (IFI6-16), isoform a
10	23.10	TNFSF10	U37518	tumor necrosis factor superfamily, member 10 (TRAIL)
11	21.31	MX2	M30818	myxovirus resistance protein 2 (MXB)
12	20.68	PDGFRL	D37965	platelet-derived growth factor receptor-like protein
13	15.95	CD80	M27533	CD80 antigen (B7-1)
14	13.99	IRF7	U53830	interferon-regulatory factor 7, isoform alpha
15	13.93	IL1RN	X53296	interleukin 1 receptor antagonist, isoform 1
16	13.31	ISG20	U88964	interferon-stimulated gene ISG20
17	11.81	IFITM1	J04164	interferon induced transmembrane protein 1 (IFI17)
18	8.67	SCYA2	S71513	chemokine (C-C motif) ligand 2 (CCL2, MCP1)
19	8.54	IL6	M14584	interleukin 6
20	8.51	IL8RB	L19593	interleukin 8 receptor beta (CXCR2)

Top 20 upregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by exposure to 50 ng/ml recombinant human IFNβ for 3 or 24 hours are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name. In vivo IRGs in T cells and non-T cells of RRMS patients reported previously (Ref. 16) are underlined.

upregulated or downregulated, are listed in Table 2 and Table 3, respectively. All of top 20 upregulated genes were found as known IRGs identified in various cell types by searching through PubMed and ISG databases.

The upregulated IRGs in the complete lists (see Additional files 3 and 5) were classified into several functional categories following; (i) conventional IFN-response markers (n = 12), (ii) components of classical and Toll-like receptor (TLR)-dependent IFN-signaling pathways (n = 12), (iii) chemokines and their receptors (n = 11), (iv) cytokines, growth factors and their receptors (n = 17), (v) apoptosis, DNA damage, and cell cycle regulators (n = 29), (vi) heat shock proteins (n = 9), and (vii) costimulatory and adhesion molecules (n = 7) (Table 4). The chemokine and chemokine receptor group included both CXC and CC chemokines and their receptors, such as SCYB11 (CXCL11, I-TAC), SCYB10 (CXCL10, IP-10), SCYA8 (CCL8, MCP2), SCYB9 (CXCL9, MIG), SCYA2 (CCL2, MCP1), CCR5, SCYA4 (CCL4, MIP1B), IL8RB (CXCR2),

Table 3: Top 20 downregulated genes in PBMC following exposue to IFNβ

No.	Cy5/Cy3 ratio	Symbol	GenBank	Gene name
at 3 hours				
1	0.29	RGS14	NM_006480	regulator of G-protein signaling 14
2	0.30	FOS	NM_005252	cellular oncogene c-fos
3	0.31	SCYB2	NM_002089	chemokine (C-X-C motif) ligand 2 (GRO2, MIP2A)
4	0.41	PPARG2	U63415	peroxisome proliferative activated receptor gamma, isoform 2
5	0.41	TNFSF11	NM_003701	tumor necrosis factor ligand superfamily, member 11 (RANKL), isoform 1
6	0.42	ABCC4	NM_005845	ATP-binding cassette, subfamily C, member 4 (MRP4)
7	0.42	MERTK	NM_006343	c-mer proto-oncogene tyrosine kinase
8	0.42	TGFBR3	NM_003243	transforming growth factor beta receptor III (betaglycan)
9	0.44	ATP2B1	J04027	ATPase, Ca++ transporting, plasma membrane 1, isoform 1b
10	0.45	SCYB1	NM_001511	chemokine (C-X-C motif) ligand 1 (GRO1, MGSA)
11	0.45	IL8	NM_000584	interleukin 8 (SCYB8, CXCL8)
12	0.45	TRAF5	NM_004619	TNF receptor-associated factor 5, variant 1
13	0.47	CD3G	NM_000073	CD3G antigen, gamma polypeptide
14	0.47	IL1A	NM_000575	interleukin 1 alpha
15	0.47	TCFL5	NM_006602	transcription factor-like 5
16	0.48	LAT	AF036905	linker for activation of T cells, isoform b
17	0.48	HNMT	U08092	histamine N-methyltransferase, isoform 1
18	0.48	SLC3A1	NM_000341	solute carrier family 3, member 1
19	0.49	MGST1	NM_145764	microsomal glutathione S-transferase 1, variant 1d
20	0.49	TNFSF8	NM_001244	tumor necrosis factor ligand superfamily, member 8 (CD30L)
at 24 hours				
1	0.18	ABCC3	AF083552	ATP-binding cassette, subfamily C, member 3, isoform MRP3
2	0.20	FOS	NM_005252	cellular oncogene c-fos
3	0.30	ALDH2	NM_000690	aldehyde dehydrogenase 2 family
4	0.30	TPST1	NM_003596	tyrosylprotein sulfotransferase 1
5	0.31	CD9	NM_001769	CD9 antigen
6	0.34	NRG1	NM_013962	neuregulin 1, isoform GGF2
7	0.35	TLR5	NM_003268	toll-like receptor 5
8	0.36	PTGS1	NM_000962	prostaglandin-endoperoxide synthase 1 (COX1), isoform 1
9	0.37	MGST2	NM_002413	microsomal glutathione S-transferase 2
10	0.38	ITGAM	NM_000632	integrin, alpha M (CD11B, MAC-1, CR3A)
11	0.38	SCYA24	NM_002991	chemokine (C-C motif) ligand 24 (eotaxin 2)
12	0.38	CYP27A1	NM_000784	cytochrome P450, family 27, subfamily A, polypeptide 1 (CTX)
13	0.39	ALDH1A1	NM_000689	aldehyde dehydrogenase 1 family, member A1
14	0.39	MYCL1	NM_005376	v-myc myelocytomatosis viral oncogene homolog 1 (LMYC)
15	0.41	ATP6AP2	NM_005765	ATPase, H+ transporting, lysosomal accessory protein 2 (ATP6M8-9)
16	0.44	PRKCG	NM_002739	protein kinase C gamma
17	0.45	ATP2B1	J04027	ATPase, Ca++ transporting, plasma membrane 1, isoform 1b
18	0.46	AP1S2	NM_003916	adaptor-related protein complex 1, sigma 2 subunit
19	0.47	NR6A1	NM_001489	nuclear receptor subfamily 6, group A, member 1 (RTR), isoform 2
20	0.47	GCG	NM_173916	glucagon

Top 20 downregulated genes in PBMC of the subject #1 (a 46 year-old healthy man) by exposure to 50 ng/ml recombinant human IFNβ for 3 or 24 hours are listed with Cy5/Cy3 signal intensity ratio, gene symbol, GenBank accession number, and gene name.

SCYA3 (CCL3, MIP1A), SCYA19 (CCL19, MIP3B) and SCYA13 (CCL13, MCP4). It is worthy to note that both CXCR3 ligand chemokines (SCYB11, SCYB10 and SCYB9) and CCR2 ligand chemokines (SCYA10 and SCYA2) were clustered in top 20 genes greatly elevated at 3 and 24 hours of IFNβ treatment (Table 2). With respect to top 20 downregulated genes, four genes such as SCYB2 (CXCL2, GRO2), SCYB1 (CXCL1, GRO1), IL8 (SCYB8, CXCL8), and SCYA24 (eotaxin-2) were categorized into the chemokine group (Table 3). Among them, SCYB2, SCYB1 and IL8, whose expression was reduced immediately at 3 hours of IFNβ treatment, belong to CXCR2 ligand chemokines. Although the analysis in the present study was a single microarray for each sample design, the results from two additional subjects, including a 28 year-old healthy man (the subject #2) and a 27 year-old woman with RRMS who was a dropout of IFNβ treatment due to induction of frequent severe relapses (the subject #4), verified the observations of immediate early induction of CXCR3 ligand and CCR2 ligand chemokine genes in PBMC by exposure to IFNβ, supporting the reproducibility of these results (see Additional file 7).

Table 4: Functional classification of IFNβ-upregulated genes in PBMC

Categories	Number of genes	Gene symbols (alternative symbols or names)
1 Conventional IFN-response markers	12	IFIT1 (IFI56), ISG15 (G1P2), IFIT4 (IFI60), MX1 (MXA), MX2 (MXB), IFI27, G1P3 (IFI6-16), ISG20, IFI16, IFITM1 (IFI17), IFITM3 (1-8U), ABCB2 (TAP1)
2 Components of IFN-signaling pathways	12	STAT1, IRF7, STAT2, JAK2, IRF2, ISGF3G (IRF9), MYD88, IRF8, STAT3, JAK3, IRF1, TLR3
3 Chemokines and receptors	11	SCYB11 (CXCL11, I-TAC), SCYB10 (CXCL10, IP-10), SCYA8 (CCL8, MCP2), SCYB9 (CXCL9, MIG), SCYA2 (CCL2, MCP1), CCR5, SCYA4 (CCL4, MIP1B), IL8RB (CXCR2), SCYA3 (CCL3, MIP1A), SCYA19 (CCL19, MIP3B), SCYA13 (CCL13, MCP4)
4 Cytokines, growth factors, and receptors	17	IL6, ILRN (IL-1 receptor antagonist), IL1R2, IL15RA, IL15, SPP1 (osteopontin), CSF1, IL12RB2, TNF (TNFA), IL2RB, IFNG, NTRK1 (TRKA), PDGFRL, TNFAIP6, KITLG (SCF), IL10, IL3RA
5 Apotosis, DNA damage, and cell cycle regulators	29	TNFSF10 (TRAIL), CASP10, BAG1, TNFRSF6 (FAS), CASP4, TRADD, GZMA, CASP7, RIPK2, MAD, RIPK1, CFLAR (FLIP), RELA, STK3, CASP1, TNFSF6 (FASL), PARP4, TANK (I-TRAF), POLE2, LMNB1, E2F2, CCNA1 (cyclin A1), CDKN1A (p21), PPP1R15A (GADD34), CASP3, CDKN1C (p57), CDK5R2 (p39), TERF1, NBS1 (nibrin)
6 Heat shock proteins	9	HSPA6 (HSP70B'), HSJ2 (HSPF4), HSPA1A (HSP70-1), HSPA1B (HSP70-2), HSPCA (HSP90A), HSPA5 (GRP78), HSPA1L (HSP70-HOM), HSPA8 (HSC70), HSPB1 (HSP27)
7 Costimulatory and adhesion molecules	7	CD80 (B7-1), SELL (selectin L), TNFRSF5 (CD40), CD163, CD86 (B7-2), HLA-DRA, FCER1G

The upregulated IRGs in PBMC listed in Additional files 3 and 5 were classified into seven functional categories.

Real-time RT-PCR analysis validated IFNβ-regulated expression of IRGs identified by microarray analysis

Although the microarray we utilized contains total 64 spots of the G3PDH gene (see Additional file 1), G3PDH was neither identified as a significantly upregulated nor a downregulated gene in the microarray analysis, suggesting that G3PDH represents a reliable housekeeping gene in gene expression analysis of PBMC following treatment with IFNβ. Therefore, quantitative real-time RT-PCR analysis was performed by evaluating the levels of expression of target genes standardized against those of G3PDH detected in the identical cDNA samples. It verified the key observations of microarray analysis, such as marked upregulation of ISG15, the prototype of IRGs (Figure 1a–c), and great elevation of SCYB10, SCYA8 and SCYA2 (Figures 2, 3, 4a–c) in PBMC at both 3 and 24 hours of IFNβ treatment. Furthermore, the quantitative analysis confirmed substantial downregulation of FOS at both time points (Figure 5a–c), and RGS14 and SCYB2 predominantly at 3 hours (Figures 6, 7a–c). Exposure of PBMC to IFNγ greatly elevated the expression of SCYB10 and SCYA2, and to a lessor extent, ISG15 and SCYA8 at both time points (Figures 1, 2, 3, 4d), suggesting a functional overlap in induction of CXCR3 ligand and CCR2 ligand chemokines between type I and type II IFN signaling pathways. In contrast, TNFα and IL-1β the prototype of proinflammatory cytokines, did not at all elevate the levels of expression of ISG15, SCYB10 or SCYA8 (Figures 1, 2, 3e, f), while IL-1β significantly ($p = 0.041$ at 3 hours and $p = 0.004$ at 24 hours by two-sided paired t-test) but TNFα only marginally ($p = 0.2102$ at 3 hours and $p = 0.0825$ at 24 hours by two-sided paired t-test) upregulated SCYA2 expression (Figure 4e, f). Treatment with IFNγ, TNFα or IL-1β reduced the levels of FOS and RGS14 substantially at 24 hours (Figures 5, 6d–f). IFNγ reduced the expression of SCYB2, whereas TNFα and IL-1β elevated its levels at both time points, suggesting differential regulation of SCYB2 gene expression in PBMC by exposure to distinct cytokines (Figure 7d–f). The IFNβ-regulated gene expression pattern was similar among PBMC derived from three distinct healthy subjects #1, #2 and #3, supporting the reproducibility of these observations (Figures 1, 2, 3, 4, 5, 6, 7a–c).

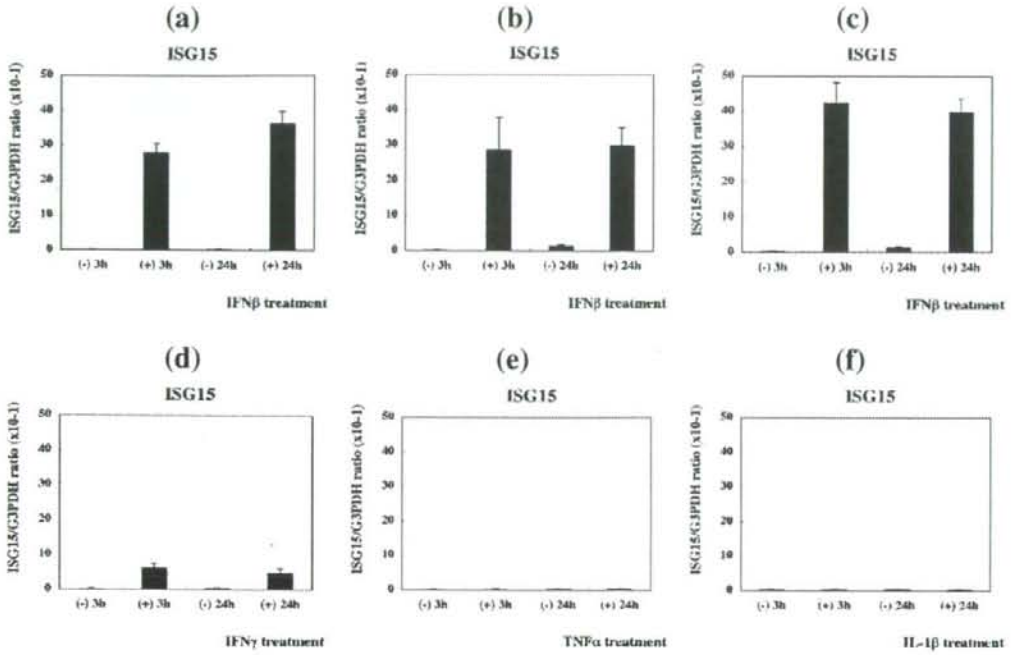

Figure 1

Real-time RT-PCR analysis of ISG15 expression in PBMC. PBMC derived from three distinct healthy subjects numbered #1 (a 46 year-old man), #2 (a 28 year-old man), and #3 (a 42 year-old woman) were incubated for 3 hours or 24 hours in the culture medium with (+) or without (-) inclusion of recombinant human IFNβ, IFNγ, TNFα or IL-1β at a concentration of 50 ng/ml each. cDNA was processed for real-time PCR analysis using specific primers listed in Table 1. The levels of expression of ISG15 are standardized against those of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene detected in identical cDNA samples. The assays were performed in triplicate measurements of the same sample, and the results were expressed as the average with standard error. The panels represent the expression of ISG15 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

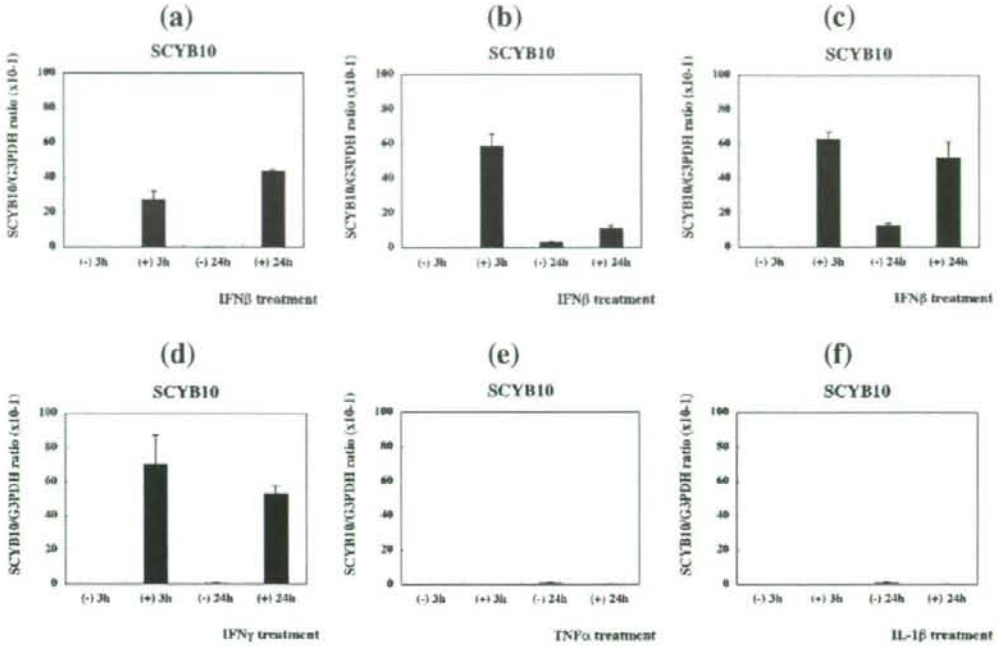

Figure 2
Real-time RT-PCR analysis of SCYB10 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYB10 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.

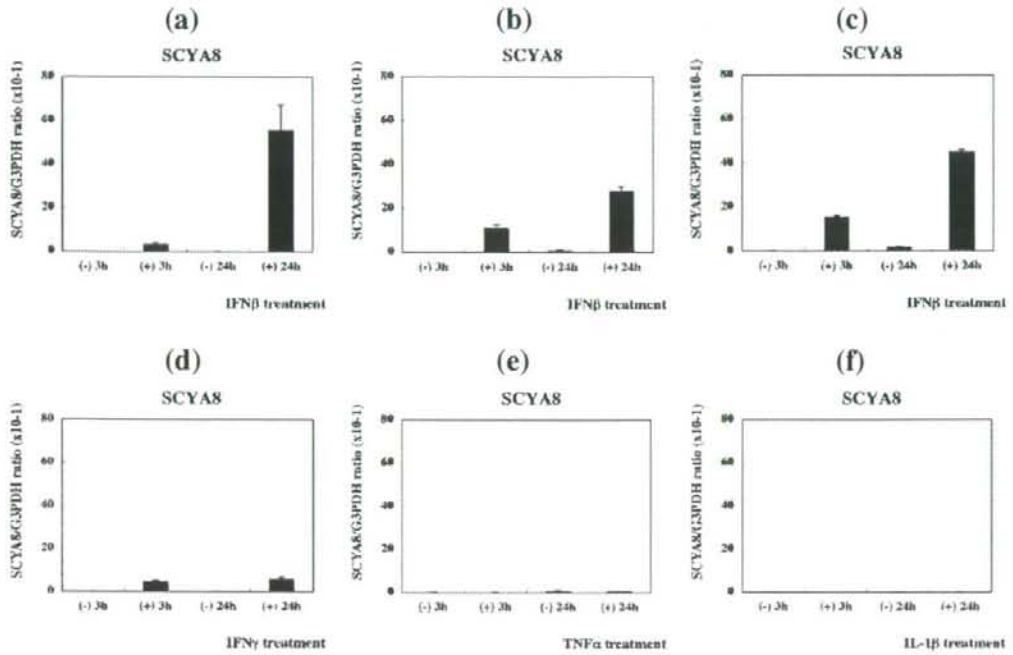

Figure 3

Real-time RT-PCR analysis of SCYA8 expression in PBMC. See the footnote of Figure 1. The panels represent the expression of SCYA8 in (a) #1, IFNβ; (b) #2, IFNβ; (c) #3, IFNβ; (d) #1, IFNγ; (e) #1, TNFα; and (f) #1, IL-1β.