doi:10.1093/brain/awp228 Brain 2009: 132; 3353–3365 | 3353

BRAIN
A JOURNAL OF NEUROLOGY

A type I interferon signature in monocytes is associated with poor response to interferon-β in multiple sclerosis

M. Comabella,[1] J. D. Lünemann,[2,†] J. Río,[1] A. Sánchez,[3] C. López,[1] E. Julià,[1] M. Fernández,[1] L. Nonell,[1] M. Camiña-Tato,[1] F. Deisenhammer,[4] E. Caballero,[5] M. T. Tortola,[5] M. Prinz,[6] X. Montalban[1,*] and R. Martin[7,*,‡]

1 Centre d'Esclerosi Múltiple de Catalunya, CEM-Cat, Unitat de Neuroimmunologia Clínica, Hospital Universitari Vall d'Hebron (HUVH), Departament de Medicina de la Universitat Autònoma de Barcelona, 08035 Barcelona, Spain
2 Laboratory of Viral Immunobiology, Christopher H. Browne Center for Immunology and Immune Diseases, The Rockefeller University, NY 10021-6399, USA
3 Departament d'Estadística, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain
4 Clinical Department of Neurology, Innsbruck Medical University, A-6020 Innsbruck, Austria
5 Servei de Microbiologia, HUVH, 08035 Barcelona, Spain
6 Institute of Neuropathology, Universität Freiburg, D-79106 Freiburg, Germany
7 Institució Catalana de Recerca i Estudis Avançats (icrea), 08035 Barcelona, Spain

*These authors contributed equally to this work.

†Present address: Viral Immunobiology,
Institute of Experimental Immunology,
University Hospital of Zürich,
8091 Zürich,
Switzerland

‡Present address: Institute for Neuroimmunology and Clinical multiple sclerosis Research,
Center for Molecular Neurobiology Hamburg (ZMNH),
University Medical Center Eppendorf,
20251 Hamburg,
Germany

Correspondence to: Manuel Comabella,
Unitat de Neuroimmunologia Clínica,
CEM-Cat. Edif. EUI 2ª planta,
Hospital Universitari Vall d'Hebron,
Pg. Vall d'Hebron 119-129,
08035 Barcelona,
Spain
E-mail: mcomabel@ir.vhebron.net

The effect of interferon-beta in multiple sclerosis is modest and many patients do not respond to treatment. To date, no single biomarker reliably correlates with responsiveness to interferon-β in multiple sclerosis. In the present study, genome-wide expression profiling was performed in peripheral blood mononuclear cells from 47 multiple sclerosis patients treated with interferon-β for a minimum of 2 years and classified as responders and non-responders based on clinical criteria. A validation cohort of 30 multiple sclerosis patients was included in the study to replicate gene-expression findings. Before treatment,

Received April 24, 2009. Revised June 30, 2009. Accepted July 23, 2009. Advance Access publication September 9, 2009

interferon-β responders and non-responders were characterized by differential expression of type I interferon-induced genes with overexpression of the type interferon-induced genes in non-responders. Upon treatment the expression of these genes remained unaltered in non-responders, but was strongly upregulated in responders. Functional experiments showed a selective increase in phosphorylated STAT1 levels and interferon receptor 1 expression in monocytes of non-responders at baseline. When dissecting this type I interferon signature further, interferon-β non-responders were characterized by increased monocyte type I interferon secretion upon innate immune stimuli via toll-like receptor 4, by increased endogenous production of type I interferon, and by an elevated activation status of myeloid dendritic cells. These findings indicate that perturbations of the type I interferon signalling pathway in monocytes are related to lack of response to interferon-β, and type I interferon-regulated genes may be used as response markers in interferon-β treatment.

Keywords: multiple sclerosis; monocytes; gene-expression signature; interferon-beta

Abbreviations: EBV = Epstein Barr virus; HHV-6 = human herpesvirus 6; IFN = interferon; IFNβ = interferon-beta; PBMC = peripheral blood mononuclear cells; TLR = toll-like receptor

Introduction

Interferons (IFNs) are inducible cytokines with potent antiviral and anti-proliferative effects. IFN-beta (IFNβ), a type I IFN, is the most commonly used treatment for relapsing-remitting multiple sclerosis (RRMS). It decreases relapse rates by ~30%, reduces brain magnetic resonance imaging (MRI) activity, and slows progression of disability (The Interferon β Multiple Sclerosis Study Group, 1993; Jacobs *et al*., 1996; PRISMS, 1998). The effects of IFNβ are modest, and a considerable fraction of patients do not respond to treatment (Rio *et al*., 2002). Criteria to classify patients into responders and non-responders to IFNβ are usually applied after 1 or 2 years follow-up. Hence many patients are treated with IFNβ without benefit and at high socioeconomic cost. Furthermore, patients on ineffective therapy accumulate further disability and often suffer treatment-related side effects. It is, therefore, highly desirable to identify biomarkers that allow early identification of treatment failure or ideally even predict non-responder status. Although several markers have previously been shown to be of potential use to this end (Baranzini *et al*., 2005; Soilu-Hanninen *et al*., 2005; Minagar *et al*., 2007), to date there is no definitive biomarker that allows one to predict the response to IFNβ in multiple sclerosis.

We employed oligonucleotide microarrays to identify differentially expressed genes associated with the response to IFNβ treatment in RRMS patients. Our results show that peripheral blood mononuclear cells (PBMs) from patients who will subsequently become non-responders to treatment are characterized by a type I IFN signature that was selectively altered in monocytes.

Materials and methods

Study design and clinical assessment

This is a prospective study of RRMS patients receiving treatment with IFNβ. Patients were included in a follow-up protocol collecting basal and longitudinal clinical data (Rio *et al*., 2006). The study was approved by the local ethics committee and patients gave their informed consent.

Definition of response to IFNβ therapy

Clinical criteria of response to IFNβ were applied after 2 years of treatment. Patients were classified as responders when there was no increase in the Expanded Disability Status Scale (EDSS) score and no relapses during the follow-up period. Patients were labelled as non-responders when they experienced one or more relapses and an increase of at least one point in the EDSS score that persisted for a minimum of two consecutive visits separated by a 6-month interval (Rio *et al*., 2006). These stringent clinical criteria were applied in order to discriminate clearly between responders and non-responders and avoid patients having overlapping clinical responses to treatment. Patients with intermediate phenotypes of responses, i.e. presence of relapses and increase of less than one point in the EDSS score or absence of relapses with increase in the EDSS were not included in the study.

Patients

Forty-seven RRMS patients, 29 (61.7%) responders and 18 (38.3%) non-responders, were included as part of the original cohort. Thirty RRMS, 15 (50.0%) responders and 15 (50.0%) non-responders comprised the validation cohort. None of the patients had ever received treatment with IFNβ or other immunosuppressive therapy before study entry. No patient had exacerbations or received treatment with corticosteroids during the month before study entry.

Sample collection and microarrays

PBMC were collected from patients before and during IFNβ treatment by Ficoll-Isopaque density gradient centrifugation (Gibco BRL, Life Technologies LTD, UK) and stored in liquid nitrogen until used. Gene-expression profiling was performed with oligonucleotide microarrays at baseline in all the patients and after 3 months of treatment in 35 patients (21 responders and 14 non-responders). Total RNA was extracted from PBMC using an RNeasy kit (Quiagen, Santa Clarita, USA) and hybridized to GeneChips (Affymetrix Human Genome U133A Plus 2.0 arrays) according to the manufacturer's protocol (Affymetrix Inc, Santa Clara, CA, USA) (Supplementary Material S1).

Statistical analysis of microarray data

A schematic flow chart summarizing the main steps performed in the analysis of microarray data is represented in Fig. 1. Analysis of the expression data was carried out by using the Bioconductor packages

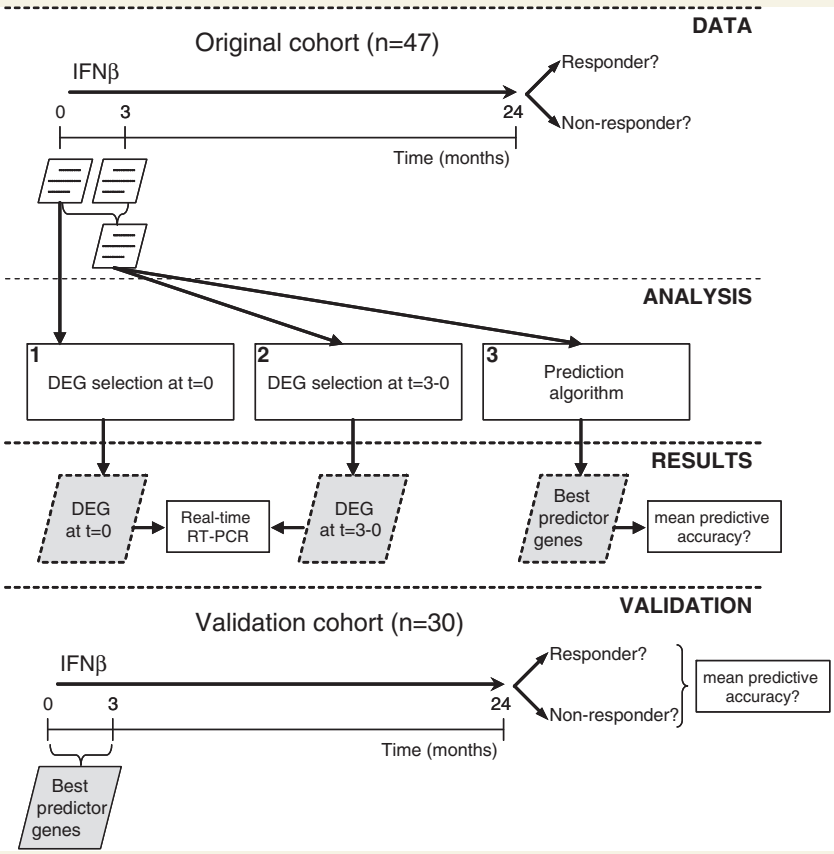


Figure 1 Flow chart summarizing the different steps undertaken in the processing of microarray data. Patients were followed for at least 24 months after initiation of IFNβ treatment and then classified into responders and non-responders based on stringent clinical criteria. In the original cohort, data from patients were obtained at baseline ($t = 0$) and after 3 months of treatment ($t = 3$). Analysis was performed before treatment ($t = 0$) comparing differentially expressed genes between responders and non-responders (**1**), and after 3 months of treatment comparing differences in gene-expression levels between 3 months and baseline samples ($t = 3–0$) in responders and non-responders (**2**). Additionally, we applied a prediction algorithm to analyse whether changes in gene expression induced by IFNβ in the first 3 months of treatment could predict therapeutic outcome after 24 months (**3**). Lists of genes that discriminate between responders and non-responders were obtained at baseline (Supplementary Table S1) and after 3 months of treatment (Supplementary Table 3), and changes in their expression were confirmed by real time RT–PCR (shown in Supplementary Tables 2 and 4, respectively). A list of the best predictive genes of response to IFNβ ($n = 8$; depicted in Fig. 2) was obtained and applied to the original cohort to compute mean predictive accuracy. Finally, a validation cohort also followed for at least 24 months after initiation of IFNβ treatment and classified into responders and non-responders based on the same clinical criteria was used to replicate the gene-expression signature and response prediction observed in the original cohort. To do this, changes in the gene expression of the eight best predictive genes obtained in the original cohort were used to calculate mean predictive accuracy in the validation cohort. DEG: differentially expressed genes.

for the R programming environment. All chips were explored one by one and checked for quality. After that, data obtained from .CEL files were preprocessed using the RMA method (Irizarry *et al.*, 2003). These preprocessed values were the basis for all the analyses. To avoid genes with low signal and little variation in their expression among all patients, a two-step non-specific filtering was carried out: first, genes not reaching a minimum mean signal in all groups were left out of the analysis; second, from the selected genes, only those having variability (standard deviation was chosen as the comparison parameter) between all arrays greater than a certain percentile were kept for further analysis. Both thresholds were chosen after studying the behaviour of the expression data for each step of the analysis. The selection of differentially expressed genes between responders and non-responders at baseline and in the first 3 months of treatment with IFNβ was based on a linear model analysis with empirical Bayes moderation of the variance estimates. In order to deal with the multiple testing issues derived from the fact that many tests are performed simultaneously, as a linear model is generated for each gene, *P*-values were adjusted to obtain strong control over the false discovery rate, which is defined as the expected proportion of Type 1 or false positives among the rejected hypothesis using Benjamini and Hochberg's method (1995).

Studies on the prediction of response to treatment were performed based on changes in gene expression induced by IFNβ in the first 3 months of treatment. Thus, the difference in the expression levels between 3 months and baseline was computed for each gene in responders and non-responders. A prediction algorithm was generated building and comparing several predictors in an iterated process which

was cross-validated in order to avoid well known problems such as overfitting or selection bias (Barrier *et al*., 2005). In this way, data were partitioned randomly in 10 balanced sets and successively one of the partitions was considered as validation set whereas the other 9 parts were considered as training set. For each training set predictors were constructed and applied to the validation set. This procedure was repeated several times with an increasing subset of genes ranging from 2 to 100. In this process, we considered a maximum of 100 genes, given that our purpose was to find a small list of genes that could help to predict response to treatment. Genes to be included in the predictors were selected using an *F*-test. The following candidate predictors were evaluated: diagonal linear discriminant analysis, *k*-nearest neighbour with one, three, five and seven neighbours, support vector machine with several parameters, nearest shrunken centroids and random forest. The selection of best predictor genes was done taking the subset with the number of genes that had associated the smallest estimated misclassification error. We then calculated the definitive list by using all data and selecting again the best number of genes founded in the feature selection step. The list of best predictor genes selected from the original cohort was applied to the validation cohort to calculate mean predictive accuracy.

The identification of relevant pathways in differentially expressed genes between responders and non-responders was performed with the package 'sigPathway' implemented in R (Tian *et al*., 2005).

Validation of microarray data with real-time quantitative reverse transcription polymerase chain reaction

Real-time polymerase chain reaction (PCR) was used to validate differentially expressed genes obtained with microarrays at baseline and in the first months of treatment.

Total RNA was taken from the same samples that had been used for the microarrays. cDNA was synthesized from 150 ng of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), combined with TaqMan® Universal PCR Master Mix and loaded in duplicates into Taqman® Low-density Arrays containing TaqMan® Gene Expression Assays specific for the genes selected to validate microarray data. The housekeeping gene 18S rRNA was used as an endogenous control. Taqman arrays were run on the ABI PRISM® 7900HT system (Applied Biosystems).

The threshold cycle (C_T) value for each reaction, and the relative level of gene expression for each sample were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Briefly, 18S rRNA was employed for the normalization of the quantity of RNA used. Its C_T value was subtracted from that of the specific genes to obtain a ΔCT value. The difference ($\Delta\Delta$CT) between the ΔCT values obtained for the responders (calibrators) and the ΔCT values for the non-responders was determined. The relative quantitative value was then expressed as $2^{-\Delta\Delta CT}$, representing the fold change in gene expression normalized to the endogenous control and relative to the calibrators.

Detection of basal and IFNβ-induced STAT1 phosphorylation by flow cytometry in different PBMC populations

The methodology for the detection of STAT1 phosphorylation by flow cytometry was adapted from that described by Lesinski and Dhodapkar (Lesinski *et al*., 2004; Dhodapkar *et al*., 2007). PBMC obtained at baseline were treated with and without recombinant IFNβ-1a (10 000 IU/ml) in serum-free conditions at 37°C for 20 min. Cells were fixed immediately and permeabilized with 90% methanol for 30 min on ice. Cells were washed and stained with a PE-coupled mouse anti-human phospho STAT1 antibody (BD Biosciences, San Jose, CA, USA) and an IgG1-PE isotype control antibody as well as with directly coupled monoclonal antibodies specific for CD3, CD14 and CD19 (all BD Biosciences) as per the manufacturer's protocol. Data were acquired on a BD-LSRII flow cytometer and analysed using FlowJo software (TreeStar, Ashland, OR, USA). The geometric mean fluorescence intensity of the IgG1-PE isotype control antibody was subtracted from the geometric mean fluorescence intensity of the phospho STAT1 antibody.

mRNA levels of negative regulators of type I IFN-induced signalling pathway

The mRNA levels of protein inhibitor of activated STAT1 (*PIAS1*), and suppressors of cytokine signalling 1 (*SOCS1*) and 3 (*SOCS3*) were determined at baseline by real time RT–PCR using TaqMan probes and 18S rRNA as endogenous control in a ABI Prism 7000 Sequence Detection system (Applied Biosystems). Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method, as described earlier.

Cell-surface quantification of activation markers and IFN receptors 1 (IFNAR1) and 2 (IFNAR2) in different cell populations

The following monoclonal antibodies were obtained from Pharmingen (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD64, CD86, CD38, phycoerythrin (PE)-conjugated mouse anti-human CD80, HLA-ABC, CD25, CD83, CD14, allophycocyanin (APC)-conjugated mouse anti-human CD14, CD19, CD11c and allophycocyanin-Cyanine 7 (APC-Cy7)-conjugated mouse anti-human CD4. Peridinine chlorophyll protein (PerCP)-conjugated mouse anti-human CD3, HLA-DR and a cocktail of FITC-labelled anti-CD3, CD14, CD16, CD19, CD20 and CD56 (lineage cocktail) were obtained from Becton Dickinson (Mountain View, CA, USA). PE-conjugated mouse anti-human CD86 was obtained from Caltag Laboratories (Burlingame, CA, USA). FITC-conjugated mouse anti-human IFNAR1 and IFNAR2 were purchased from R&D Systems (Gaithersburg, MD, USA) and PBL InterferonSource (Piscataway, NJ, USA.), respectively. FITC-conjugated mouse IgG1, IgG2a, PE-conjugated mouse IgG1, APC-conjugated mouse IgG1, APC-Cy7-conjugated mouse IgG1, were obtained from Pharmingen. PerCP-conjugated mouse IgG1 and IgG2a were purchased from Becton Dickinson.

To determine expression of activation markers at baseline, PBMC were stained with monoclonal antibodies against CD14, CD64, CD80, CD83, CD86, HLA-ABC and HLA-DR for monocytes, CD19 and CD38 for B cells, CD3, CD4 and CD25 for T cells, CD11c, HLA-DR, CD80, CD83, CD86 and lineage cocktail for dendritic cells (DC), or the corresponding isotype controls.

Cells were analysed using a dual laser FACSCanto (Becton Dickinson) flow cytometer equipped with FACSDiva software.

Lymphocytes and monocytes were gated based on forward and side light scatter properties. To analyse DC, cells were first gated based on forward and side light scatter properties. Myeloid dendritic cells were identified as lineage$^-$HLADR$^+$CD11c$^+$ cells, whereas plasmacytoid DC were lineage$^-$HLADR$^+$CD11c$^-$. Results are presented as percentage of positive cells and as mean fluorescence intensity.

The expression of the surface IFN receptors 1 and 2 (IFNAR1 and IFNAR2) was determined at baseline in T cells, B cells, monocytes, myeloid DC and plasmacytoid DC. Surface immunostaining and data analysis were performed as described earlier.

Toll-like receptor agonist stimulation and IFN quantification

Production of type I and II IFNs was examined at baseline in responders and non-responders after Toll-like receptor (TLR) agonist stimulation. In total, 2×10^5 PBMC per well were seeded in 96-well plates in 10% human AB-serum and stimulated with TLR3- [Poly(I:C); 25 μg/ml], TLR4- (LPS; 1 μg/ml), TLR7- (3M-13; 1 μg/ml), TLR8- (3M-2; 1 μg/ml) and TLR9- (CpG-C; 500 mM) agonists in duplicates for 48 h. Supernatants were analysed for type I (IFNα2) and type II (IFNγ) IFN concentrations using a standard sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (Mabtech, Cincinnati, OH, USA).

Quantification of type I IFN bioactivity

Type I IFN bioactivity was determined in serum samples at baseline using the iLite™ Human Interferon Alpha Kit (Neutekbio, Galway, Ireland) according to the manufacturer's recommendations. This bioassay uses a stable transfected cell line derived from a pro-monocytic human cell line (U937). These cells have been characterized as human pro-monocytic and express constitutively MHC Class II antigens on the cell surface. The assay is designed in such a way that no cell culture is needed and detects the luciferase generated bioluminescence intensity, which is proportional to the amount of type I IFN activity (IU/ml) in the sample. A standard curve with different dilutions of IFNα-2b was used for quantification of type IFN bioactivity.

Epstein Barr virus and human herpes virus 6 serologies and viral genome detection

Epstein Barr virus (EBV) and human herpes virus 6 (HHV-6) replication was evaluated at baseline in the study patients. IgG and IgM serologies for EBV were performed using standard tittered immunofluorescence methods for antibodies to viral capsid antigen. HHV-6 antibodies (IgG and IgM) were determined by a commercially available ELISA (Panbio, Brisbane, Australia).

In EBV and HHV-6 seropositive patients, detection of viral genome was performed by PCR in a SmartCycler II Real Time PCR System (Cepheid Inc., Sunnyvale, CA, USA). Briefly, DNA was isolated from 1×10^6 PBMC by means of the NucliSens® easyMAG™ automatic system (Biomerieux, Boxtel, The Netherlands). A quantitative PCR assay was used for quantification of EBV DNA viral genome load. The Artus® EBV RG PCR Kit (Qiagen, Hamburg, Germany) was utilized for specific amplification of the EBV genome following the manufacturer's recommendations. Detection of HHV-6 genome was carried out with a non-commercial qualitative PCR assay using primers and TaqMan probes as previously described (Watzinger *et al*., 2004).

Presence of neutralizing antibodies

The presence of neutralizing antibodies to IFNβ in serum samples was determined in all patients at baseline and after 12 and 24 months of treatment by means of the myxovirus A induction bioassay, as previously described (Gneiss *et al*., 2006). Titres of $\geqslant$20 neutralizing units at 12 or 24 months were considered to be a positive test result.

Results

Clinical and MRI description of the responders and non-responders to IFNβ included in the study

Except for EDSS scores, responders and non-responders were comparable for all clinical variables within cohorts (Table 1). The baseline EDSS was higher in non-responders, a pattern that has been reported in previous studies (Byun *et al*., 2008). In the original cohort, non-responders showed higher inflammatory activity at baseline than responders, as reflected by the higher number of gadolinium (Gd)-enhancing lesions, although these results should be taken with caution owing to the small number of non-responders from whom MRI scans were available ($n = 5$) compared with responders ($n = 15$). As shown in Table 1, IFNβ was associated with a reduction in the number of active lesions after 1 year of treatment; however, as previously reported (Río *et al*., 2008), non-responders had a higher number of Gd-enhancing lesions and accumulated more new T2 lesions after 1 year of treatment.

Differentially expressed genes at baseline reveal a type I IFN gene-expression signature in non-responders

We first studied differentially expressed genes in RRMS patients before treatment with IFNβ. To exclude a confounding role of neutralizing antibodies against IFNβ, we measured neutralizing antibodies at baseline and following 12 and 24 months. Two patients in the non-responder group (11.1%) and one patient in the responder group (3.4%) developed neutralizing antibodies at 12 or 24 months (Fisher's Exact Test: $P = 0.549$). Since expression analyses focused on the baseline and 3 months treatment time points and since the presence of neutralizing antibodies was not associated with the response to IFNβ, all patients were analysed. We found 47 differentially expressed genes ($P < 0.05$) at baseline between responders and non-responders (Supplementary Table 1). The vast majority of these were overexpressed in patients subsequently non-responsive to IFNβ. Of note, functional annotation revealed the type I IFN-signalling pathway as the most significantly associated with the non-responder 'phenotype' (Table 2), and up to 25% of differentially expressed genes at baseline are known to be predominantly or selectively induced by type I IFNs (Der *et al*., 1998; Leszczyniecka *et al*., 2003; Barnes *et al*., 2004;

Table 1 Demographic, clinical and radiological characteristics of multiple sclerosis patients' responders and non-responders to IFNβ treatment

	Original cohort			Validation cohort		
	R	NR	*P*-values[a]	R	NR	*P*-values[a]
Baseline characteristics						
n (%)	29 (61.7)	18 (38.3)	–	15 (50.0)	15 (50.0)	–
Age (years)	34.1 (5.9)	35.1 (9.2)	0.463	32.7 (7.6)	35.5 (8.8)	0.412
Female/male (% female)	20/9 (69.0)	13/5 (72.2)	0.812	11/4 (73.3)	10/5 (66.7)	0.690
Duration of disease (years)	6.0 (5.9)	6.4 (4.8)	0.487	2.3 (1.5)	5.5 (4.6)	0.106
EDSS[b]	1.9 (1.3–2.8)	2.5 (1.9–3.0)	0.032	1.6 (1.0–2.0)	2.3 (1.5–3.5)	0.045
Number of relapses[c]	2.6 (1.2)	2.9 (1.7)	0.656	2.1 (0.7)	2.3 (0.9)	0.539
Number of Gd-enhancing lesions[d]	1.3 (1.4)	4.4 (3.2)	0.011	2.4 (3.8)	2.9 (3.6)	0.595
Type of IFNβ [*n* (%)]						
IFNβ 1a IM	10 (34.5)	6 (33.3)		3 (20.0)	6 (40.0)	
IFNβ 1b SC	10 (34.5)	6 (33.3)	0.987	5 (33.3)	4 (26.7)	0.486
IFNβ 1a SC	9 (31.0)	6 (33.3)		7 (46.7)	5 (33.3)	
MRI characteristics at 1-year[d]						
Number of Gd-enhancing lesions	0.07 (0.3)	1.0 (1.2)	0.087	0.0 (0.0)	0.6 (1.8)	0.740
Number of new T2 lesions	1.5 (1.8)	3.6 (1.1)	0.019	1.4 (2.8)	6.0 (8.0)	0.078

	Combined cohorts		
	R	NR	*P*-values[e]
Number of Gd-enhancing lesions	0.04 (0.2)	0.7 (1.6)	0.038
Number of new T2 lesions	1.4 (2.3)	5.2 (6.6)	0.043

Data are expressed as mean (standard deviation) unless otherwise stated.
a Refers to *P*-values obtained following comparisons between responders and non-responders within each cohort by means of Student's *t*-test or Mann–Whitney's test depending on the applicability conditions (age, duration, EDSS and number of relapses) and chi-square test (gender and type of IFNβ).
b Data are expressed as mean (interquartile range).
c Refers to the number of relapses in the two previous years.
d MRI data were available for a subgroup of 46 patients, 20 patients from the original cohort (15 responders and 9 non-responders) and 26 patients from the validation cohort (14 responders and 12 non-responders). IM: intramuscular. SC: subcutaneous. R: responders to IFNβ. NR: non-responders to IFNβ.
e Refers to *P*-values obtained following comparisons between responders and non-responders from the combined cohorts.

Table 2 Relevant pathways associated with the response to IFNβ obtained with 'sigPathway'

Gene Set Category	Pathway	Set size	NTk Stat	NTk *q*-value	NTk Rank	NEk* Stat	NEk* *q*-value	NEk* rank
humanpaths	IFNα,β response	37	−7.64	<0.0001	1.0	−2.89	0.0109	1.0
humanpaths	Nitric oxide	21	−6.45	<0.0001	2.0	−2.17	0.0815	2.0
KEGG	Toll-like_receptor_signaling_pathway	21	−5.02	<0.0001	3.0	−1.80	0.0783	5.0
humanpaths	Stress/Toxicity PathwayFinder	21	−4.33	<0.0001	6.0	−1.96	0.0906	3.0
humanpaths	Dendritic/Antigen Presenting Cell	37	−4.86	<0.0001	4.0	−1.60	0.0747	7.5
GO:0006954	inflammatory response	27	−4.25	<0.0001	7.0	−1.67	0.0851	6.0
humanpaths	Glucocorticoid Signaling I	20	−3.09	0.0028	11.5	−1.84	0.0897	4.0
GO:0005125	cytokine activity	22	−4.68	<0.0001	5.0	−1.39	0.0743	12.0
humanpaths	Toll-Like Receptor Signaling Pathway	20	−4.16	<0.0001	8.0	−1.47	0.0772	9.5
humanpaths	cAMP/Ca^{2+} Signaling PathwayFinder	26	−3.09	0.0028	11.5	−1.60	0.0747	7.5
humanpaths	Cardiovascular Disease	20	−3.69	<0.0001	10.0	−1.47	0.0772	9.5
humanpaths	G-Protein Coupled Receptors Signaling PathwayFinder	22	−4.04	<0.0001	9.0	−1.28	0.0844	13.0
humanpaths	Signal Transduction in Cancer	22	−2.88	0.0052	13.0	−1.42	0.0771	11.0
humanpaths	JAK/STAT Signalling Pathway	20	−2.14	0.0389	14.0	−0.90	0.1429	14.0

Statistically significant pathways are found through a statistical hypothesis testing framework proposed by Tian *et al*. (2005) for determining whether a specified group of genes for a pathway has a coordinated association with a phenotype of interest. The two null hypotheses are the following: the genes in a gene set show the same pattern of associations with the phenotype compared with the rest of the genes (Q_1). The gene set does not contain any genes whose expression levels are associated with the phenotype of interest (Q_2). Then, two statistics are developed, one for each hypothesis, and to get statistical significance each statistic is compared against the distribution under the null hypothesis. Set Size is the number of genes in the original list contained in the corresponding pathway. NTk Stat is the value of the statistic corresponding to hypothesis Q_1. NTk *q*-value is the adjusted *P*-value corresponding to hypothesis Q_1. NTk Rank is the rank corresponding to hypothesis Q_1. NEk* Stat is the value of the statistic corresponding to hypothesis Q_2. NEk* *q*-value is the adjusted *P*-value corresponding to hypothesis Q_2. NEk* Rank is the rank corresponding to hypothesis Q_2. The global ranking is obtained by adding up rank corresponding to hypotheses Q_1 and Q_2

Kirou *et al*., 2004; Helbig *et al*., 2005). The majority of the differentially expressed genes were confirmed by PCR with values ranging from 1.6- to 5-fold overexpression in non-responders (Supplementary Table 2). These results point to pre-existing differences between responders and non-responders in the type I IFN pathway.

An activated type I IFN-signalling pathway is associated with lack of response to IFNβ

We next analysed changes in gene expression during the first 3 months of treatment. Supplementary Table 3 lists the 43 differentially expressed genes. Interestingly, the highest differences were obtained for genes predominantly or selectively induced by type I IFNs, and data were again confirmed by PCR in a subgroup of genes. For the majority of them, a strong and significant induction was observed in treatment responders as reflected by fold changes from 3 to over 120 (Supplementary Table 4). Conversely, in non-responders, changes in gene expression induced by IFNβ were much lower or absent and did not reach statistical significance for any gene. These findings indicate that non-responders have an activated type I IFN system in peripheral blood cells that is refractory to exogenous IFNβ.

Type I IFN-induced genes are among the best treatment response predictors

One objective of our study was to predict the response status to IFNβ after 24 months prior to or within the first 3 months of treatment. A prediction algorithm was applied comparing several predictors as described in the Methods. Figure 2A lists the eight best discriminating genes between responders and non-responders. Interestingly, five of the eight best predictor genes (*IFIT1-3*, *IFI44* and *OASL*) are selectively induced by type I IFNs. The remaining genes are: RASGEF1B, which encodes a small GTP-binding protein of the Ras superfamily; MARCKS, which codes for a protein which is a substrate for the protein kinase C and is highly expressed by macrophages; FADS1, which encodes a desaturase enzyme that regulates unsaturation of fatty acids.

A mean predictive accuracy of 78% was obtained with these eight genes (Fig. 2B) indicating that the clinical response to IFNβ can indeed be predicted early.

Replication of the type I IFN signature in a validation cohort

Analysis of microarray data in a validation cohort of 30 new RRMS patients revealed 99 differentially expressed genes ($P<0.05$) between responders and non-responders at baseline. Similar to our findings in the original cohort, many of the differentially expressed genes obtained in the validation cohort are induced by type I IFN. As shown in Supplementary Table 5, 25% of the original type I IFN signature was also observed in the validation cohort. Of note, up to 21% of differentially expressed genes at baseline in the validation cohort are known to be predominantly or selectively induced by type I IFNs (Supplementary Table 5), and the type I IFN-signalling pathway was again ranked in first position (NTk q-value <0.0001). Finally, the mean predictive accuracy obtained in the smaller validation cohort with the best predictor genes ($n=8$) was 63%, and thus comparable to the first cohort (Fig. 2C).

IFNβ non-responders show increased STAT1 phosphorylation and IFNAR1 expression in monocytes

Next we analysed which molecules of the type I IFN response pathway are involved in IFN-non-responsiveness. First, we examined phosphorylated STAT1 (p-STAT1) protein as a marker for the type I IFN response at the single-cell level. IFNβ stimulation of PBMC led to a substantial increase of p-STAT1 levels (Fig. 3A), and monocytes showed the highest IFNβ-induced p-STAT1 levels. There were no differences in IFNβ-induced p-STAT1 levels between groups indicating that the type I IFN receptor-signalling machinery is not impaired in non-responders (Fig. 3B, lower panels). However, p-STAT1 baseline levels were significantly higher in monocytes derived from non-responders compared with responders (Fig. 3B, upper panels), while no differences were observed in T and B cells. These results indicate that the type I IFN pathway in monocytes is fully activated prior to therapy with IFNβ in non-responders and cannot be activated further. Consistent with this notion, we found significantly elevated surface expression of IFNAR1 on monocytes of non-responders (Fig. 3C). In contrast, IFNAR2 was not elevated on monocytes, and neither IFNAR1 nor IFNAR2 expression were altered on other cell populations (Supplementary Fig. 1).

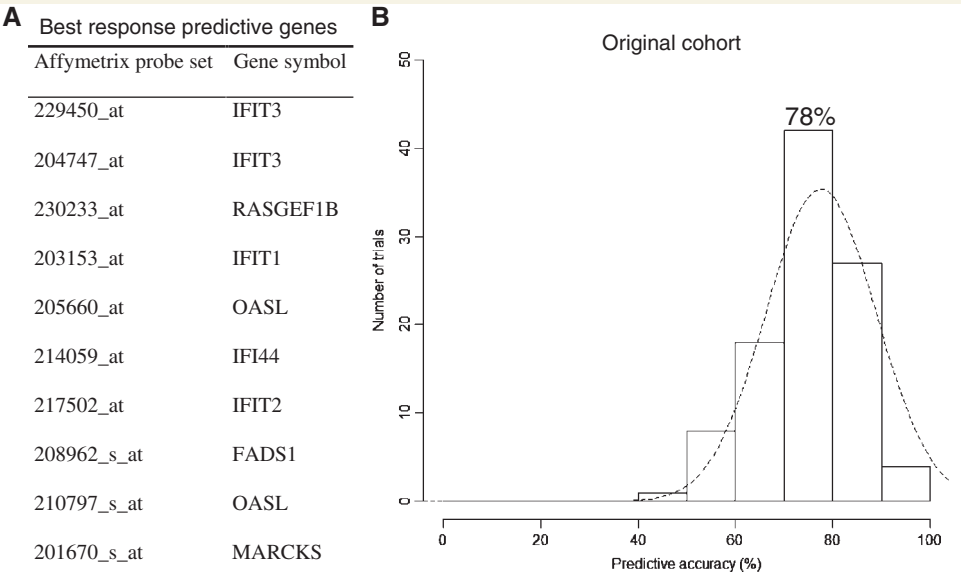
In further functional studies, we examined whether overexpression of type I IFN-responsive genes in non-responders is due to lower expression of negative type I IFN regulators or occurs in the context of global activation of monocytes alone or together with other immune cells. Baseline expression levels of cytokine regulators *SOCS1*, *SOCS3* and *PIAS1* were similar between responders and non-responders (Supplementary Table 6) and no differences of activation marker expression were observed between groups for monocytes or other PBMC populations (Supplementary Table 7).

Innate immune stimulation elicits higher type I IFN responses in IFNβ non-responders

Many of the genes that are transcribed at higher levels at baseline in non-responders (*IL1B*, *TNF*, *STAT1*, *CXCL10*, *PTX3*, *CCR1*) suggest increased activation of the innate immune system. Supporting this idea, cellular pathways related with the innate immune system, such as the TLR or the dendritic/antigen presenting cell-signalling pathways were overexpressed in IFNβ non-responders (Table 2). To test whether the activated IFN pathway in IFNβ non-responders was associated with increased innate immune activation via TLR agonists, we stimulated PBMC with

A Best response predictive genes

Affymetrix probe set	Gene symbol
229450_at	IFIT3
204747_at	IFIT3
230233_at	RASGEF1B
203153_at	IFIT1
205660_at	OASL
214059_at	IFI44
217502_at	IFIT2
208962_s_at	FADS1
210797_s_at	OASL
201670_s_at	MARCKS



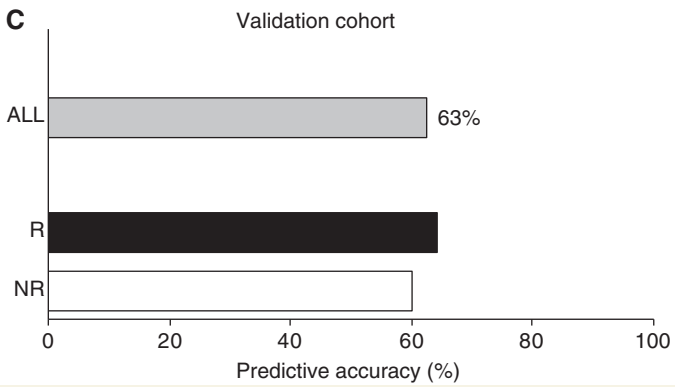


Figure 2 A set of predominantly type I IFN-induced genes are among the best predictors of response to IFNβ in the first 3 months of treatment. (**A**) List of genes discriminating best between responders and non-responders. A total of 6410 genes from the initial list of 54 675 passed the non-specific filtering in their expression between baseline and 3 months. After applying a prediction algorithm to this set of genes, the *k*-nearest neighbour with one neighbour and with eight genes (10 probe sets) were selected as the method and number of genes that had associated the lowest misclassification error ($\varepsilon = 0.3083$). This misclassification error was computed as the mean of 10 cross-validation steps in which nine-tenth of data were used as training data, i.e. to build a predictor, and the left out samples used as validation data, that is, to compute the misclassification error. (**B**) Histogram showing predictive accuracy of the best predictive genes after random partitions in the original cohort. To compute the histogram, an iterative method of 100 random divisions was applied to the original sample and filtered genes. Each random division selected a balanced set of three-fourth parts of the patients to build a predictor using the *k*-nearest neighbour method with one neighbour and eight genes (10 probe sets). This predictor was applied to the remaining one-fourth part of patients to compute mean predictive accuracy over the 100 trials. The mean predictive accuracy of all random divisions was 78%, which we used to generate a normal approximation to predictive data, plotted in the figure as a dotted curve. (**C**) Graph showing mean predictive accuracy in a totally independent validation cohort of 15 responders and 15 non-responders to treatment. The mean predictive accuracy obtained for the whole group was 63%, being similar in responders and non-responders.

classical TLR agonists and analysed the supernatants for IFNα and IFNγ levels. All TLR ligands elicited a robust IFN response (Fig. 4A). Interestingly, non-responders showed significantly higher levels of IFNα only following TLR4 stimulation via LPS, and this effect was most likely mediated by the monocyte lineage since TLR4 expression by plasmacytoid DC, one of the major cell sources of IFNα, is low or absent (Kadowaki *et al*., 2001).

Increased endogenous production of type I IFNs in non-responders to IFNβ

Based on the higher p-STAT1- and IFNAR1-expression levels in non-responders, we next addressed whether type I IFNs, which engage IFNAR1/R2 and induce the downstream events are elevated. Type I IFN bioactivity in serum was indeed significantly higher in IFNβ non-responders (Fig. 4B). While we do not know

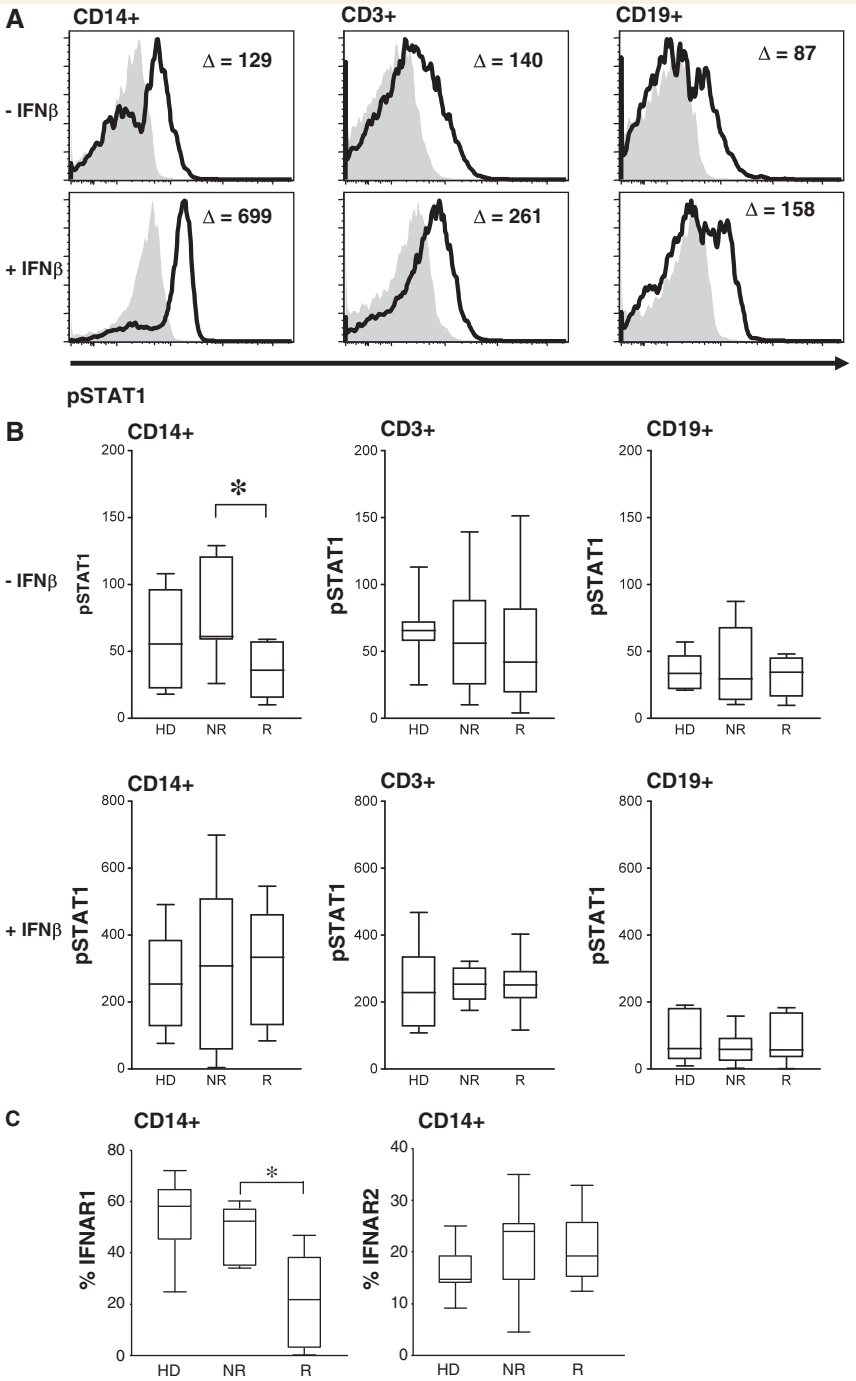


Figure 3 Selective increase of baseline STAT1 phosphorylation and IFNAR1 expression in CD14+ monocytes derived from IFNβ non-responders. (**A**) Baseline and IFNβ-induced levels of p-STAT1 protein were quantified by intracellular flow cytometry in CD14+ monocytes, CD3+ T cells and CD19+ B cells. IFNβ-1a stimulation led to a substantial increase of p-STAT1 levels in CD14+ monocytes, CD3+ T cell and CD19+ B cells. Monocytes showed the highest IFNβ induced p-STAT1 levels. (**B**) p-STAT1 baseline levels are significantly higher in monocytes derived from non-responders compared with responders (*$P = 0.01$, Mann–Whitney U-test). HD: healthy donors ($n = 10$). NR: non-responders ($n = 10$). R: responders ($n = 10$). (**C**) Boxplots showing mean percentage of positive cells expressing IFNAR1 and IFNAR2 in non-responders (NR, $n = 11$), responders (R, $n = 11$) and healthy donors (HD, $n = 9$). Baseline IFNAR1 expression by monocytes from non-responders is significantly higher compared with responders (*$P = 0.008$, Mann–Whitney U-test) and similar to healthy donors.

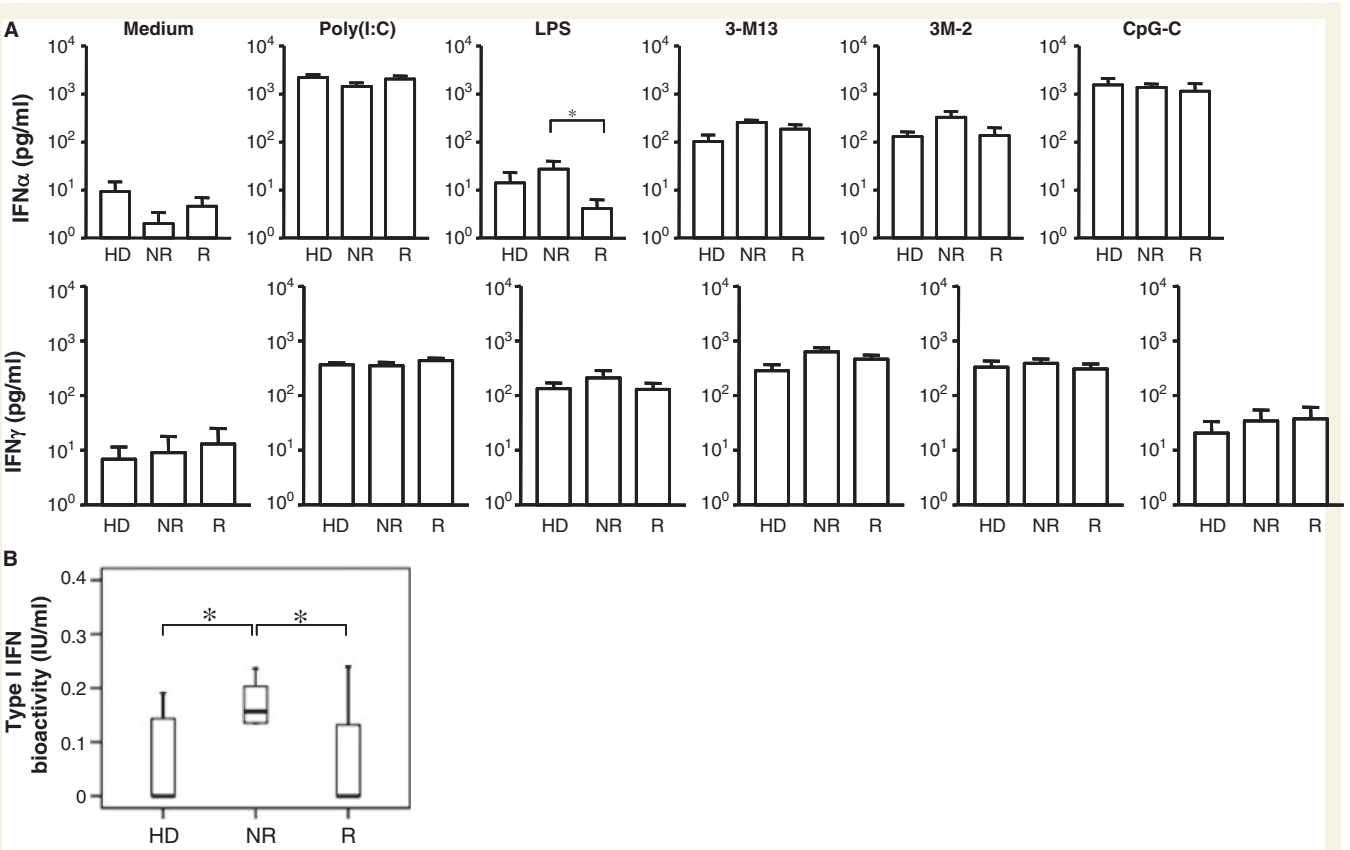


Figure 4 Type I IFN secretion upon innate immune stimulation and serum type I IFN bioactivity are increased in IFNβ non-responders. (**A**) Type I (IFNα2) and type II (IFNγ) IFN production in PBMC following TLR stimulation. PBMC were stimulated with TLR3- [Poly(I:C); 25 mg/ml], TLR4- (LPS; 1 mg/ml), TLR7- (3M-13; 1 mg/ml), TLR8- (3M-2; 1 mg/ml) and TLR9- (CpG-C; 500 mM) agonists in duplicates for 48 h. Cytokine levels in supernatants were quantified by ELISA. Results are expressed as mean values (SEM). IFNα2 production following LPS stimulation is substantially higher in non-responders compared with responders (*$P = 0.03$, Mann–Whitney U-test). There were no statistically significant differences in cytokine responses to other TLR agonists between healthy donors and multiple sclerosis patients and between IFNβ responders and non-responders. (**B**) Quantification of type I IFN bioactivity. A pro-monocytic cell line was transferred to a 96-well plate and then 25 µl of serum samples from multiple sclerosis patients and healthy donors were placed in individual wells and tested in duplicate for type I IFN bioactivity. Plates were read in a luminometer after incubation at 37°C for 17 h and type I IFN bioactivity quantified by extrapolation from a standard curve generated with IFNα-2b (range 1.5–25 IU/ml). Type I IFN bioactivity in non-responders is significantly higher compared with responders and healthy donors (*$P = 0.04$ for both groups, Mann–Whitney U-test). HD: healthy donors ($n = 10$). NR: non-responders ($n = 10$). R: responders ($n = 10$).

the natural stimuli leading to increased production of type I IFNs in non-responders, we excluded EBV or HHV-6, two viruses that are being discussed as aetiological factors in multiple sclerosis, as likely candidates. Neither responders nor non-responders showed detectable viral DNA levels in PBMC or IgM seropositivity at baseline, and IgG positivity for both viruses was similar (Supplementary Table 8). These results render it unlikely that differences in EBV or HHV-6 reactivation account for the differences of the type I IFN-signalling pathway in both groups.

Increased activation of myeloid dendritic cells in IFNβ non-responders

As part of their proinflammatory effects type I IFNs induce the activation and maturation of dendritic cells (DC), the most potent antigen-presenting cells. We therefore assessed the state of activation and maturation of myeloid and plasmacytoid DC. As shown in Fig. 5, myeloid dendritic cells from non-responders showed a significant increase in CD86 expression, whereas CD80 and CD83 and HLA-DR were expressed at similar levels (Supplementary Fig. 2).

Discussion

The present study aimed at identifying biomarkers that are associated with or even predictive of the response to IFNβ treatment in RRMS patients. IFNβ responders and non-responders were characterized by stringent criteria previously applied by our group (Río *et al*., 2006). Interestingly, IFNβ non-responders express a substantial fraction of type I IFN-regulated genes at elevated levels prior to IFNβ treatment. In contrast, the expression of type I IFN-regulated genes is low at baseline in responders,

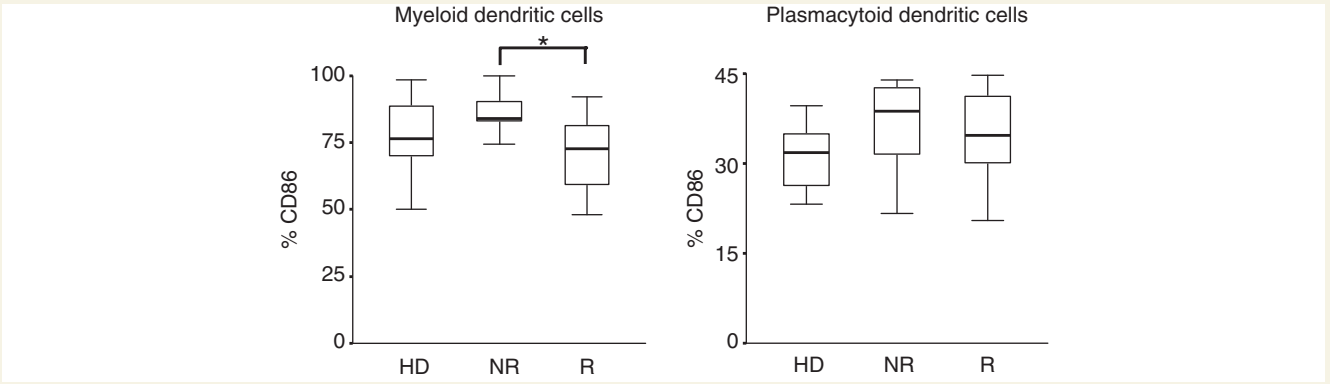


Figure 5 In treatment non-responders myeloid dendritic cells are more activated. Boxplots showing baseline expression of CD86 in myeloid and plasmacytoid dendritic cells. PBMC from responders and non-responders to IFNβ were stained with monoclonal antibodies against CD11c, HLA-DR, CD86 and lineage cocktail. Myeloid dendritic cells were identified as lineage$^-$HLADR$^+$CD11c$^+$ cells, whereas plasmacytoid dendritic cells were lineage$^-$HLADR$^+$CD11c$^-$. Results are expressed as mean percentage of positive cells for each marker. CD86 expression is increased in myeloid dendritic cells from non-responders compared with responders (*$P = 0.006$, Student's *t*-test). HD: healthy donors ($n = 9$). NR: non-responders ($n = 10$). R: responders ($n = 10$).

but is inducible during treatment. In addition, genes that are selectively induced by type I IFNs were the best predictors of the therapeutic outcome after 24 months of treatment. This type I IFN gene-expression signature was validated in an independent cohort. When dissecting the type I IFN signature further, IFNβ non-responders are characterized by elevated expression of IFNAR1 and increased type I IFN secretion of monocytes upon TLR4 stimuli, and by increased serum type I IFN bioactivity and elevated activation status of myeloid DC.

A type I gene expression IFN signature has been reported in several autoimmune disorders such as rheumatoid arthritis (van der Pouw Kraan *et al*., 2007), systemic lupus erythematosus (Han *et al*., 2003), dermatomyositis (Baechler *et al*., 2007), systemic sclerosis (Tan *et al*., 2006) and Sjögren's syndrome (Gottenberg *et al*., 2006). In multiple sclerosis, a recent study also observed the presence of a selective upregulation of the type I IFN-signalling pathway in a subgroup of RRMS patients (van Baarsen *et al*., 2006), and suggested that common aetiological factors and pathogenetic pathways operate in these autoimmune disorders. Here, we describe a constitutively activated type I IFN system in non-responders to IFNβ treatment. These data may indicate the uncoupling of regulatory mechanisms via the recently reported family of TAM receptor tyrosine kinases (Rothlin *et al*., 2007), which employ IFNAR1 and pSTAT1 to downregulate innate immune activation upon prior TLR signals, IFNAR1 engagement and STAT1 phosphorylation. Alternatively, they could indicate that the desensitization to further IFNα stimulation, which is mediated by IFNβ engagement (Severa *et al*., 2006, 2007), is not functional in non-responders. The lack of changes in gene expression upon exogenous IFNβ supports the latter hypothesis. Of note, the type I IFN pathway is selectively perturbed in the monocyte lineage in non-responders, as the increase in p-STAT1 levels and IFNAR1 expression was observed only in this cell population. Furthermore, the increased production of IFNα upon TLR4 stimulation was most likely mediated by monocytes. The fact that LPS stimulation of monocytoid DC primarily induces IFNβ and IFNλ, but not IFNα expression (Coccia *et al*., 2004) further argues for an uncoupling of the abovementioned desensitization to IFNα by secretion of IFNβ.

Monocytes are abundant in inflammatory multiple sclerosis brain lesions, and infiltrating myeloid DC as well as microglia are thought to drive brain inflammation (Heppner *et al*., 2005; Karni *et al*., 2006; Bailey *et al*., 2007). We excluded a deficient expression of negative regulators of the type I IFN pathway or reactivation of latent viral infections by EBV and HHV-6 as causes of the overexpression of type I IFN-responsive genes in monocytes of IFNβ non-responders. Furthermore, we did not find a broad activation of monocytes or other PBMC populations. Instead, our results indicate increased signalling via IFNAR upon increased endogenous type I IFNs, as shown by elevated type I IFN bioactivity and increased production of IFNα after TLR4 stimulation.

Based on the above findings it appears that the IFNβ non-responders represent a pathogenetically different phenotype of multiple sclerosis with clinically more severe course, altered monocyte function and an activated innate immune system. In these patients the administration of exogenous IFNβ failed to induce the expression of a number of type I IFN-regulated genes. At present, we cannot distinguish whether the presence of relapses and progression of disability in non-responders during the 2 years of treatment is due to lack of response to IFNβ, more active disease, pathogenetically distinct disease processes, or a combination of factors. We have identified a subgroup of patients who have a constitutively activated and less inducible type I IFN pathway, and these patients fail to respond to IFNβ treatment. Furthermore, for yet unknown reasons, the type I IFN pathway is dysregulated in monocytes, which may contribute to more active disease. The biological relevance of this observation is underscored by a recent study that documents that conditional genetic knockout of IFNAR1 in monocytes, but not in T cells,

B cells or CNS cells, leads to enhanced disease severity in the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (Prinz *et al*., 2008). It should be taken into account that, in the context of a type I IFN signature, type I IFNs may not be beneficial for multiple sclerosis, as they are known to activate DC (Radvanyi *et al*., 1999), enhance humoral immunity (Le Bon *et al*., 2001), and favour Th1 immune responses (Brinkmann *et al*., 1993). We show that at baseline in non-responders myeloid DC are more activated. In this context, the deleterious effect of type I IFNs is further supported by the recent finding of a strong upregulation of *BAFF* (B cell activating factor of the TNF family) induced by IFNβ in multiple sclerosis patients, thus providing a link between type I IFNs and enhanced humoral responses (Krumbholz *et al*., 2008).

In summary, two conclusions can be drawn from our observations. We believe that IFNβ non-responsiveness can be predicted early by the differential expression of a small number of type I IFN-inducible genes. Furthermore, it appears counterintuitive to administer IFNβ, a type I IFN, to patients with overexpression of type I IFN-responsive genes under basal conditions and relative unresponsiveness of the type I IFN pathway to the effects of IFNβ. Our data raise the important question whether neutralization of type I IFNs would be more appropriate in IFNβ non-responder multiple sclerosis patients as has been proposed for other autoimmune disorders characterized by a type I IFN signature (Stewart, 2003). Both points should be explored soon with respect to usefulness for clinical management and individualizing first-line therapies in multiple sclerosis. With respect to a historical perspective of multiple sclerosis therapy, IFNβ was the first anti-inflammatory therapy for multiple sclerosis that was approved over a decade ago. Over this long period, clinical experience with individual patients suggest that its modest efficacy is in part due to pathogenetic differences between patients that result in non-responsiveness in some and good clinical response in others. Our data indicate that the continuing exploration of the differential treatment responses to such an 'old' drug merits further studies and may eventually be useful for treatment individualization. Independent of IFNβ the latter aspect will rapidly gain importance with a growing number of effective drugs that are currently in late stages of clinical development.

Acknowledgements

The authors thank the 'Red Temática de Investigación Cooperativa (C03/06)', the 'Ajuts per donar Suport als Grups de Recerca de Catalunya (SGR 2005-1081)' and the NABINMS (Neutralising Antibodies in multiple sclerosis) funded by the EEC. R. Martin was a member of the Institució Catalana de Recerca i Estudis Avançats (ICREA). The Institute for Neuroimmunology and Clinical multiple sclerosis Research is supported by the Gemeinnützige Hertie Stiftung.

Supplementary material

Supplementary material is available at *Brain* online.

References

Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Espe KJ, Novitzke J, et al. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. Mol Med 2007; 13: 59–68.

Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. Nat Immunol 2007; 8: 172–80.

Baranzini SE, Mousavi P, Rio J, Caillier SJ, Stillman A, Villoslada P, et al. Transcription-based prediction of response to IFNbeta using supervised computational methods. PLoS Biol 2005; 3: e2.

Barnes BJ, Richards J, Mancl M, Hanash S, Beretta L, Pitha PM. Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection. J Biol Chem 2004; 279: 45194–207.

Barrier A, Lemoine A, Boelle PY, Tse C, Brault D, Chiappini F, et al. Colon cancer prognosis prediction by gene expression profiling. Oncogene 2005; 24: 6155–64.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B 1995; 57: 289–300.

Brinkmann V, Geiger T, Alkan S, Heusser CH. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. J Exp Med 1993; 178: 1655–63.

Byun E, Caillier SJ, Montalban X, Villoslada P, Fernández O, Brassat D, et al. Genome-wide pharmacogenomic analysis of the response to interferon beta therapy in multiple sclerosis. Arch Neurol 2008; 65: 337–44.

Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, et al. Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells. Eur J Immunol 2004; 34: 796–805.

Der SD, Zhou A, Williams BRG, Silverman RH. Identification of genes differentially regulated by interferon α, β, or γ using oligonucleotide arrays. Proc Natl Acad Sci USA 1998; 95: 15623–8.

Dhodapkar KM, Banerjee D, Connolly J, Kukreja A, Matayeva E, Veri MC, et al. Selective blockade of the inhibitory Fc{gamma} receptor (Fc{gamma}RIIB) in human dendritic cells and monocytes induces a type I interferon response program. J Exp Med 2007; 204: 1359–69.

Gneiss C, Tripp P, Reichartseder F, Egg R, Ehling R, Lutterotti A, et al. Differing immunogenic potentials of interferon beta preparations in multiple sclerosis patients. Mult Scler 2006; 12: 731–7.

Gottenberg JE, Cagnard N, Lucchesi C, Letourneur F, Mistou S, Lazure T, et al. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. Proc Natl Acad Sci USA 2006; 103: 2770–5.

Han GM, Chen SL, Shen N, Ye S, Bao CD, Gu YY. Analysis of gene expression profiles in human systemic lupus erythematosus using oligonucleotide microarray. Genes Immun 2003; 4: 177–86.

Helbig KJ, Lau DT, Semendric L, Harley HA, Beard MR. Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. Hepatology 2005; 42: 702–10.

Heppner FL, Greter M, Marino D, Falsig J, Raivich G, Hövelmeyer N, et al. Experimental autoimmune encephalomyelitis repressed by microglial paralysis. Nat Med 2005; 11: 146–52.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003; 4: 249–64.

Jacobs LD, Cookfair DL, Rudick RA, Herndon RM, Richert JR, Salazar AM, et al. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). Ann Neurol 1996; 39: 285–94.

Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different

toll-like receptors and respond to different microbial antigens. J Exp Med 2001; 194: 863–9.

Karni A, Abraham M, Monsonego A, Cai G, Freeman GJ, Hafler D, et al. Innate immunity in multiple sclerosis: myeloid dendritic cells in secondary progressive multiple sclerosis are activated and drive a proinflammatory immune response. J Immunol 2006; 177: 4196–5202.

Kirou KA, Lee C, George S, Louca K, Papagiannis IG, Peterson MG, et al. Coordinate overexpression of interferon-alpha-induced genes in systemic lupus erythematosus. Arthritis Rheum 2004; 50: 3958–67.

Krumbholz M, Faber H, Steinmeyer F, Hoffmann LA, Kümpfel T, Pellkofer H, et al. Interferon-beta increases BAFF levels in multiple sclerosis: implications for B cell autoimmunity. Brain 2008; 131: 1455–63.

Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 2001; 14: 461–70.

Lesinski GB, Kondadasula SV, Crespin T, Shen L, Kendra K, Walker M, et al. Multiparametric flow cytometric analysis of inter-patient variation in STAT1 phosphorylation following interferon Alfa immunotherapy. J Natl Cancer Inst 2004; 96: 1331–42.

Leszczyniecka M, Su ZZ, Kang DC, Sarkar D, Fisher PB. Expression regulation and genomic organization of human polynucleotide phosphorylase, hPNPase(old-35), a Type I interferon inducible early response gene. Gene 2003; 316: 143–56.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402–8.

Minagar A, Adamashvili I, Kelley RE, Gonzalez-Toledo E, McLarty J, Smith SJ. Saliva soluble HLA as a potential marker of response to interferon-beta1a in multiple sclerosis: a preliminary study. J Neuroinflammation 2007; 4: 16.

Prinz M, Schmidt H, Mildner A, Knobeloch KP, Hanisch UK, Raasch J, et al. Distinct and nonredundant in vivo functions of ifnar on myeloid cells limit autoimmunity in the central nervous system. Immunity 2008; 28: 675–86.

PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. Lancet 1998; 352: 1498–504.

Radvanyi LG, Banerjee A, Weir M, Messner H. Low levels of interferon-alpha induce CD86 (B7.2) expression and accelerates dendritic cell maturation from human peripheral blood mononuclear cells. Scand J Immunol 1999; 50: 499–509.

Rio J, Nos C, Tintoré M, Borras C, Galan I, Comabella M, et al. Assessment of different treatment failure criteria in a cohort of relapsing-remitting multiple sclerosis patients treated with interferon beta: implications for clinical trials. Ann Neurol 2002; 52: 400–6.

Rio J, Nos C, Tintoré M, Tellez N, Galan I, Pelayo R, et al. Defining the response to interferon-beta in relapsing-remitting multiple sclerosis patients. Ann Neurol 2006; 59: 344–52.

Río J, Rovira A, Tintoré M, Huerga E, Nos C, Tellez N, et al. Relationship between MRI lesion activity and response to IFN-beta in relapsing-remitting multiple sclerosis patients. Mult Scler 2008; 14: 479–84.

Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell 2007; 131: 1124–36.

Severa M, Remoli ME, Giacomini E, Ragimbeau J, Lande R, Uzé G, et al. Differential responsiveness to IFN-alpha and IFN-beta of human mature DC through modulation of IFNAR expression. J Leukoc Biol 2006; 79: 1286–94.

Severa M, Remoli ME, Giacomini E, Annibali V, Gafa V, Lande R, et al. Sensitization to TLR7 agonist in IFN-beta-preactivated dendritic cells. J Immunol 2007; 178: 6208–16.

Soilu-Hanninen M, Laaksonen M, Hanninen A, Eralinna JP, Panelius M. Downregulation of VLA-4 on T cells as a marker of long term treatment response to interferon beta-1a in multiple sclerosis. J Neuroimmunol 2005; 167: 175–82.

Stewart TA. Neutralizing interferon alpha as a therapeutic approach to autoimmune diseases. Cytokine Growth Factor Rev 2003; 14: 139–54.

Tan FK, Zhou X, Mayes MD, Gourh P, Guo X, Marcum C, et al. Signatures of differentially regulated interferon gene expression and vasculotrophism in the peripheral blood cells of systemic sclerosis patients. Rheumatology 2006; 45: 694–702.

The Interferon β 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: 655–61.

Tian L, Greenberg SA, Kong SW, Altschuler J, Kohane IS, Park PJ. Discovering statistically significant pathways in expression profiling studies. Proc Natl Acad Sci USA 2005; 102: 13544–9.

van Baarsen LG, van der Pouw Kraan TC, Kragt JJ, Baggen JM, Rustenburg F, Hooper T, et al. A subtype of multiple sclerosis defined by an activated immune defense program. Genes Immun 2006; 7: 522–31.

van der Pouw Kraan TC, Wijbrandts CA, van Baarsen LG, Voskuyl AE, Rustenburg F, Baggen JM, et al. Rheumatoid arthritis subtypes identified by genomic profiling of peripheral blood cells: assignment of a type I interferon signature in a subpopulation of patients. Ann Rheum Dis 2007; 66: 1008–14.

Watzinger F, Suda M, Preuner S, Baumgartinger R, Ebner K, Baskova L, et al. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. J Clin Microbiol 2004; 42: 5189–98.