Implication of the Toll-Like Receptor 4 Pathway in the Response to Interferon-β in Multiple Sclerosis

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Objective: Interferon-beta (IFNβ) has demonstrated beneficial effects reducing disease activity in multiple sclerosis (MS) patients, but a relatively large proportion of patients do not respond to treatment. Here we aimed to investigate the roles of the Toll-like receptor 4 (TLR4) and the type I IFN pathways in the response to IFNβ in MS patients.
Methods: The expression levels of several components of the TLR4 and the type I IFN pathways were determined by flow cytometry and real-time polymerase chain reaction (PCR) in peripheral blood mononuclear cells (PBMCs) from a cohort of 85 MS patients treated for at least 2 years with IFNβ and classified into responders, intermediate responders, and nonresponders based on their clinical response to treatment. Thirty-two healthy controls were also included in the study for comparison purposes.
Results: Compared to responders and controls, PBMCs from nonresponders and intermediate responders were characterized by increased baseline expression levels of endogenous IFNβ and elevated IFN receptor 1 (IFNAR1) expression in monocytes. Furthermore, the capacity of IFNβ to induce its own expression was deficient in cells from nonresponders compared with responders. Baseline expression of the interleukin-1 receptor-associated kinase 3 (*IRAK3*), a negative regulator of TLR4 signaling primarily expressed in monocytes, was found to be significantly decreased in IFNβ responders compared with nonresponders.
Interpretation: These findings provide evidence of the involvement of the TLR4 and type I IFN signaling pathways in the response to IFNβ.

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Interferon-beta (IFNβ) is the most widely prescribed treatment for relapsing-remitting multiple sclerosis (RRMS) and has shown positive effects on reducing disease activity.[1–3] However, IFNβ is only partially effective and a significant proportion of MS patients do not respond to treatment.[4] In a previous study, we showed that peripheral blood mononuclear cells (PBMCs) from patients who will show a lack of response to IFNβ were characterized by a baseline overexpression of type I IFN responsive genes.[5] The type I IFN pathway appeared to be selectively altered in monocytes from a subgroup of IFNβ-treated patients whose lack of response was determined by applying stringent clinical criteria after 2 years of treatment.[5] However, these findings could indicate a true lack of response to IFNβ, the presence of more active disease in patients who continue to have relapses and disability progression despite treatment, or a combination of both factors.

Toll-like receptors (TLRs) are transmembrane proteins that recognize pathogen-associated molecular patterns and play important roles in the regulation of immune function and inflammation.[6,7] Among the TLRs, Toll-like receptor 4 (TLR4) signals through myeloid differentiation

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primary response gene 88 (MYD88)-dependent and MYD88-independent pathways, leading to the production of proinflammatory cytokines such as interleukin-1 beta (IL1β) and tumor necrosis factor-alpha (TNFα) and type I IFNs like IFNβ, respectively.[8] TLR4 is abundantly expressed in myelomonocytic subpopulations of leukocytes, and its upregulation in peripheral blood monocytes has been found to correlate with the severity of disorders such as cerebral infarction and type 1 diabetes mellitus.[9,10] Based on these findings, and taking into account the close relationship existing between the TLR4 and the type I IFN pathways,[11] in the present study we aimed to investigate the roles of the TLR4 and the type I IFN pathways in disease activity and the response to IFNβ in MS patients.

Patients and Methods

Study Design and Clinical Assessment

This is a prospective study of RRMS patients treated with IFNβ at the outpatient clinic of the Centre d'Esclerosi Múltiple de Catalunya (CEM-Cat, Barcelona, Spain). All patients were included in a follow-up protocol collecting basal and longitudinal clinical data, as previously described.[12] The study was approved by the local ethics committee, and all patients gave their informed consent.

Criteria of Response to IFNβ Therapy

Clinical criteria of response to IFNβ were applied after 2 years of treatment. Patients were labeled as responders when there was no increase in the Expanded Disability Status Scale (EDSS) score and no relapses over the follow-up period. Patients were classified as nonresponders when they experienced during the follow-up period 1 or more relapses and an increase of at least 1 point in the EDSS score that persisted for a minimum of 2 consecutive scheduled visits separated by a 6-month interval.[12] Patients were labeled as intermediate responders when there was, during the follow-up period, presence of relapses with an increase of less than 1 point in the EDSS score, or absence of relapses with an increase in the EDSS score of 1 point or higher.

Patients

Eighty-five RRMS patients were included in the study. Of these, 49 (57.6%) were responders, 18 (21.2%) nonresponders, and 18 (21.2%) intermediate responders to IFNβ. None of these patients had ever received treatment with IFNβ or other immunosuppressive therapy before study entry. No patient had clinical exacerbations or received corticosteroid treatment during the month before initiation of IFNβ. A control group of 32 individuals was also included in the study.

Sample Collection and Cell Surface Quantification of TLR4, TLR2, and IFN Receptor 1 by Flow Cytometry in Monocytes

PBMC from RRMS patients and healthy controls were isolated in the same conditions by Ficoll-Isopaque density gradient centrifugation (Gibco BRL, Life Technologies Ltd., Paisley, UK) and stored in liquid nitrogen until used. Cell surface expression of TLR4, TLR2, and IFN receptor 1 (IFNAR1) in monocytes was determined by flow cytometry in RRMS patients at baseline and after 12 months of IFNβ treatment and in healthy controls. For this, PBMCs were thawed and stained with allophycocyanin (APC)-conjugated mouse anti-human CD14 (Pharmingen, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IFN-α/βR1 (IFNAR1) (R&D Systems, Gaithersburg, MD), and phycoerythrin (PE)-conjugated mouse anti-human TLR4 and TLR2 (eBioscience, San Diego, CA), or the corresponding isotype controls (FITC-conjugated mouse immunoglobulin G1 [IgG1] and PE-conjugated mouse IgG2a; Pharmingen). Cells were analyzed using a dual laser FACSCanto (Becton Dickinson, Mountain View, CA) flow cytometer equipped with FACSDiva software. Monocytes were gated based on forward and side light scatter properties and also on their CD14 positivity. Median fluorescence intensity of monocytes expressing TLR4, TLR2, and IFNAR1 was calculated by subtracting the antibody control signal from the specific signal.

Determination of Serum Levels of Heat Shock Protein 70

Peripheral blood was collected by standard venipuncture and allowed to clot spontaneously for 30 minutes. Serum was isolated by centrifugation and stored frozen at -80°C until used. Baseline levels of heat shock protein 70 (HSP70) (total) were measured in MS patients and healthy controls by means of an enzyme immunoassay (EIA) using a commercially available kit (HSP70 high sensitivity EIA kit, EKS-715; Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions. Recombinant HSP70 standards ranged from 0 to 12.5ng/mL. Serum samples and standards were tested in duplicate, and concentrations of HSP70 were determined by interpolation from a lineal regression standard curve generated from the range of recombinant human HSP70. The intraassay and interassay coefficients of variation were determined to be $<10\%$.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) from frozen PBMC samples of RRMS patients at baseline and healthy controls. RNA was retrotranscribed with the High Capacity cDNA Retrotranscription Kit (Applied Biosystems, Foster City, CA) and gene expression levels of interferon, beta 1, fibroblast (*IFNB1*), chemokine (C-X-C motif) ligand 10 (*CXCL10*), interleukin 1 β (*IL1B*), *TNF*, *MYD88*, and toll-like receptor adaptor molecule 1 (*TICAM1*) were determined by real-time polymerase chain reaction (PCR) absolute quantification. In brief, fragments covering the commercial TaqMan expression assay of 6 genes were amplified by conventional PCR from human total RNA, using the corresponding forward primer with a NheI restriction site and the reverse primer with a XmaI site (primer information is shown in Supporting Table 1). The products of $CXCL10_{(96–245)}$, $IL1B_{(490–649)}$, $TNF_{(368–536)}$, $MYD88_{(456–582)}$, and $TICAM1_{(1869–2026)}$, were cloned into a pCI vector (Promega,

Mannheim, Germany) using the NheI/XmaI restriction enzymes to create the following constructs: pCI-*CXCL10*$_{(96–245)}$, pCI-*IL1B*$_{(490–649)}$, pCI-*TNF*$_{(368–536)}$, pCI-*MYD88*$_{(456–582)}$, and pCI-*TICAM1*$_{(1869–2026)}$. The product of *IFNB1*$_{(611–751)}$ was inserted into a pCR®2.1-TOPO® vector using the TOPO TA Cloning® system according to the manufacturer's protocol in order to create the pCR®2.1-TOPO®-*IFNB1*$_{(611–751)}$ construct.

The mass of 1 construct copy was obtained by multiplying the plasmid length (bp) with the Avogadro number. Calculi were made to achieve 3×10^6 copies/2μl of the construct as the first standard, which was subjected to a 10-fold serial dilution (3×10^6 to 3×10^1) with RNase free water. Real-time PCR was carried out using the ABI-Applied Biosystems 7900 HT Thermal Cycler (Applied Biosystems) in 384 optical PCR plates. Each reaction contained 2μl standard/complementary DNA (cDNA) template, 5μl of TaqMan Universal PCR Master Mix, No AmpErase®, UNG, 0.5μl of corresponding TaqMan® Gene expression assay (Applied Biosystems), and 2.5μl of RNase-free water following the standard PCR program suggested by the manufacturer. Samples were determined in duplicates. Analysis was performed with the absolute quantification application of the software SDS 2.3 (Applied Biosystems) in order to obtain the number of messenger RNA (mRNA) copies of the specific gene for each sample.

mRNA expression levels of tyrosine kinase 2 (TYK2), protein tyrosine phosphatase, nonreceptor type 6 (PTPN6, also known as SHP1), protein tyrosine phosphatase, nonreceptor type 11 (PTPN11, also known as SHP2), sterile alpha and TIR motif containing 1 (SARM1), interleukin-1 receptor-associated kinase 3 (IRAK3), suppressor of IKBKE 1 (SIKE), single immunoglobulin and toll-interleukin 1 receptor (TIR) domain (SIGIRR), and receptor-interacting serine-threonine kinase 3 (RIPK3) were determined by real-time PCR relative quantification in RRMS patients at baseline and healthy controls. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was employed for the normalization of the quantity of RNA used. Its threshold cycle (Ct) was subtracted from that of the specific genes to obtain a ΔCt value.

IFNβ Induction Experiments

PBMC from responders, nonresponders, and healthy controls were cultured for 24 hours in the presence or absence of 100IU/ml of IFNβ-1b (Betaferon). After cell culture, mRNA expression levels of *IFNB1* were determined by real-time PCR absolute quantification, as described above.

Experimental Autoimmune Encephalomyelitis Induction in TLR4-Deficient Mice

Eight-week-old to 10-week-old TLR4-deficient mice (B6.B10ScN-Tlr4lps-del/JthJ) and age-matched and sex-matched C57Bl/6J wild-type mice purchased from Jackson Laboratory (Bar Harbor, ME) were used. Experiments were done according to the European Union regulations and approved by our institutional Ethics Committee on Animal Experimentation. Disease induction, proliferation assays, and histopathology were performed as described in Supporting Methods.

Statistical Analysis

The Kruskal-Wallis test was used to analyze differences among groups at baseline in serum levels of HSP70, protein expression levels of TLR4, TLR2, and IFNAR1, and mRNA expression levels of TLR4 downstream genes and key regulators belonging to the TLR4 and type I IFN pathways. If significant differences ($p < 0.05$) were found, a Mann-Whitney test was then used to test for significant differences between 2 groups. In MS patients, comparisons of TLR4, TLR2, and IFNAR1 expression levels before and after 12 months of IFNβ treatment were assessed by a Wilcoxon signed ranks test. Differences in *IFNB1* expression between groups before and after IFNβ induction were assessed by a paired *t* test. Linear association between clinical and radiological variables and levels of measured molecules were determined by the Spearman rank correlation coefficient. Bonferroni correction was used to correct the alpha level for multiple comparisons in correlation analyses (alpha = 0.003) and comparisons of expression levels of key regulators of the TLR4 and type I IFN pathways (alpha = 0.006). Statistical analysis was performed by using the SPSS 15.0 package (SPSS, Inc., Chicago, IL) for MS Windows.

Results

Figure 1 exemplifies the components of the TLR4, TLR2, and type I IFN pathways interrogated in the study.

Clinical and Magnetic Resonance Imaging Description of MS Patients Included in the Study

Except for EDSS scores, responders, intermediate responders, and nonresponders were comparable for all clinical and radiological variables (Table 1). A trend toward increased baseline EDSS was observed in nonresponders compared with responders, a pattern already reported in previous studies.[5,13]

Baseline TLR4 Expression by Monocytes Is Decreased in MS Patients Irrespective of the Clinical Response to IFNβ

We first evaluated the roles of the TLR4 pathway in the disease and the response to IFNβ treatment by determining TLR4 expression by flow cytometry at baseline in monocytes from MS patients and controls. As shown in Figure 2A, TLR4 expression was significantly decreased in the whole MS group compared with the control group ($p = 0.019$). However, TLR2 expression by monocytes, which was selected as specificity control of TLR4 expression, was similar between MS patients and controls ($p = 0.402$; see Fig 2B). When the MS group was stratified based on the response to IFNβ treatment, TLR4 and TLR2 expression was comparable between responders, intermediate responders, and nonresponders ($p > 0.05$ for all comparisons; see Fig 2A, B).

Correlations between baseline clinical and radiological variables in the whole MS group and TLR4 and TLR2 expression in monocytes revealed a statistically significant negative association between TLR4 levels and EDSS scores

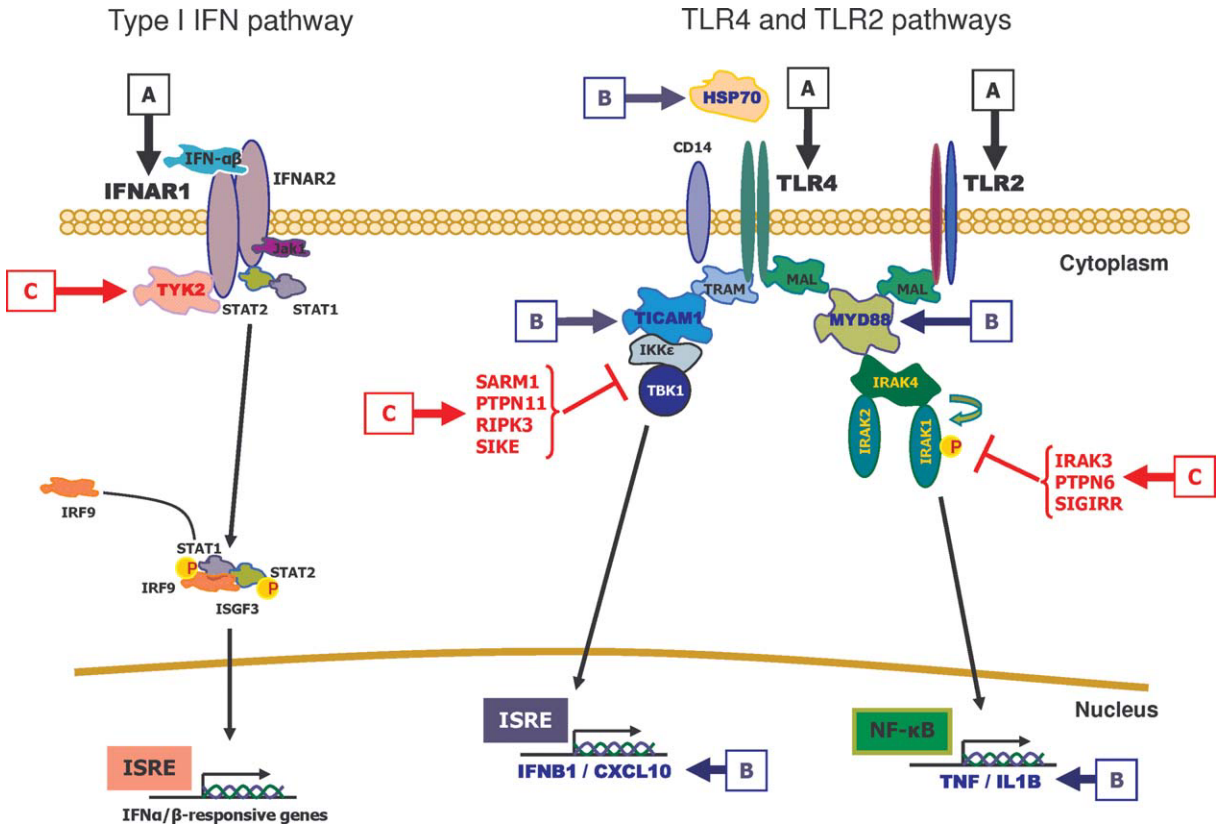


FIGURE 1: Schematic representation of the components of the TLR4, TLR2, and type I IFN pathways investigated in the study. Molecules measured in the study were grouped into 3 different levels: A: Expression of the surface receptors TLR4, TLR2, and IFNAR1 was determined by flow cytometry in monocytes; B: Serum levels of the TLR4 ligand HSP70 were measured by ELISA, and mRNA expression levels of TLR4 downstream genes such as *TICAM1*, *MYD88*, *IFNB1*, *CXCL10*, *TNF*, and *IL1B* were determined by real-time PCR absolute quantification; and C: mRNA expression levels of key regulators belonging to the type I IFN pathway like *TYK2*, and to the TLR4 pathway such as *SARM1*, *PTPN11*, *RIPK3*, *SIKE*, *IRAK3*, *PTPN6*, and *SIGIRR* were evaluated by real-time PCR relative quantification. CXCL10 = chemokine (C-X-C motif) ligand 10; HSP70 = heat shock protein 70; IFNAR1 and IFNAR2 = interferon receptors 1 and 2, respectively; IFNB1 = interferon beta; IKKϵ = inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon; IL1B = interleukin 1 beta; IRAK1–4 = interleukin-1 receptor-associated kinases 1–4; IRF9 = interferon regulatory factor 9; ISGF3 = interferon-stimulated gene factor 3; ISRE = interferon-stimulated responsive genes; MAL = T-cell differentiation protein; MYD88 = myeloid differentiation primary response (88); NF-κB = nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; PTPN6 and PTPN11 = protein tyrosine phosphatases, nonreceptor types 6 and 11, respectively; RIPK3 = receptor-interacting serine-threonine kinase 3; SARM1 = sterile alpha and TIR-containing motif 1; SIGIRR = single immunoglobulin and TIR domain; SIKE = suppressor of IKBKE1; STAT1 and STAT2 = signal transducers and activators of transcription 1 and 2, respectively; TBK1 = TANK-binding kinase 1; TICAM1 = Toll-like receptor adaptor molecule 1; TIR = toll-interleukin 1 receptor; TLR2 and TLR4 = Toll-like receptors 2 and 4, respectively; TNF = tumor necrosis factor; TRAM = translocation associated membrane protein 1; TYK2 = tyrosine kinase 2.

($r = -0.40$, $p = 0.0004$) (Table 2). Following stratification of the MS group into different response phenotypes, none of the correlations reached the threshold for statistical significance after Bonferroni correction (see Table 2).

Taking into account the differences observed in TLR4 expression between MS patients and controls, and the significant association obtained between TLR4 expression and EDSS scores in the MS group, we aimed to further investigate the role of TLR4 in the disease by inducing experimental autoimmune encephalomyelitis (EAE) in mice deficient for TLR4. As shown in Supplementary Figure 1A, both TLR4-deficient and wild-type mice developed EAE and no significant differences in the disease onset or clinical severity were observed between the two groups. Histopathology revealed similar degrees of inflammation and demyelination in TLR4 deficient and wild-type mice, and T cell proliferative responses did not differ between groups (Supporting Fig 1B, C).

TLR4 Expression Is Induced by IFNβ Treatment in Responders and Nonresponders to Treatment

We next evaluated the effect of IFNβ on TLR4 expression by monocytes after 12 months of treatment. As shown in Figure 2C, IFNβ treatment was associated with a strong induction in TLR4 expression in the whole MS group ($p = 4.3 \times 10^{-5}$). However, this effect was similarly

TABLE 1: Demographic and Baseline Clinical and Radiological Characteristics of MS Patients and Healthy Controls Included in the Study

Baseline Characteristics	HC	MS[a]	p[b]	R	IR	NR	p[c]
n	32	85	—	49	18	18	—
Age (yr)	33.9 (9.9)	34.3 (8.3)	0.814	33.7 (7.8)	33.8 (8.4)	36.3 (9.6)	0.514
Female/male (% women)	26/6 (81.3)	66/19 (77.6)	0.672	36/13 (73.5)	14/4 (77.8)	16/2 (88.9)	0.406
Duration of disease (yr)	—	4.3 (4.4)	—	3.6 (3.7)	4.4 (4.6)	6.1 (5.8)	0.123
EDSS[d]	—	2.0 (1.5-2.5)	—	1.9 (1.5-2.5)	2.2 (1.5-2.6)	2.4 (1.9-3.5)	0.055
Number of relapses[e]	—	1.5 (0.7)	—	1.5 (0.8)	1.5 (0.7)	1.5 (0.5)	1.000
Number of Gd-enhancing lesions[f]	—	2.6 (5.3)	—	1.9 (3.3)	4.8 (9.0)	1.4 (1.9)	0.112
T2LL[f]	—	0.7 (0.7)	—	0.7 (0.7)	0.8 (0.9)	0.8 (0.7)	0.640
Type of IFNβ, n (%)	—		—				
IFNβ 1a IM	—	25 (29.4)	—	14 (28.6)	6 (33.3)	5 (27.8)	
IFNβ 1b SC	—	24 (28.2)	—	13 (26.5)	4 (22.2)	7 (38.9)	0.815
IFNβ 1a SC	—	36 (42.4)	—	22 (44.9)	8 (44.4)	6 (33.3)	

Data are expressed as mean (standard deviation) unless otherwise stated.
[a]Refers to the whole MS group.
[b]Refers to p values obtained following comparisons between the whole MS group and healthy controls by means of Student t test (age) and chi-square test (gender).
[c]Refers to p values obtained following comparisons between responders, intermediate responders, and nonresponders by means of an ANOVA test (age, duration, EDSS, number of relapses, number of Gd-enhancing lesions, and T2LL) and chi-square test (gender and type of IFNβ).
[d]Data are expressed as mean (interquartile range).
[e]Refers to the number of relapses in the 2 previous years.
[f]MRI data were available for 72 patients, 47 responders, 18 intermediate responders, and 7 nonresponders.
ANOVA = analysis of variance; EDSS = Expanded Disability Status Scale; HC = healthy controls; IFNβ = interferon beta; IM = intramuscular; IR = intermediate responders to IFNβ; MRI = magnetic resonance imaging; MS = multiple sclerosis; NR = nonresponders to IFNβ; R = responders to IFNβ; SC = subcutaneous; T2LL = T2 lesion load expressed as percentage of total brain content.

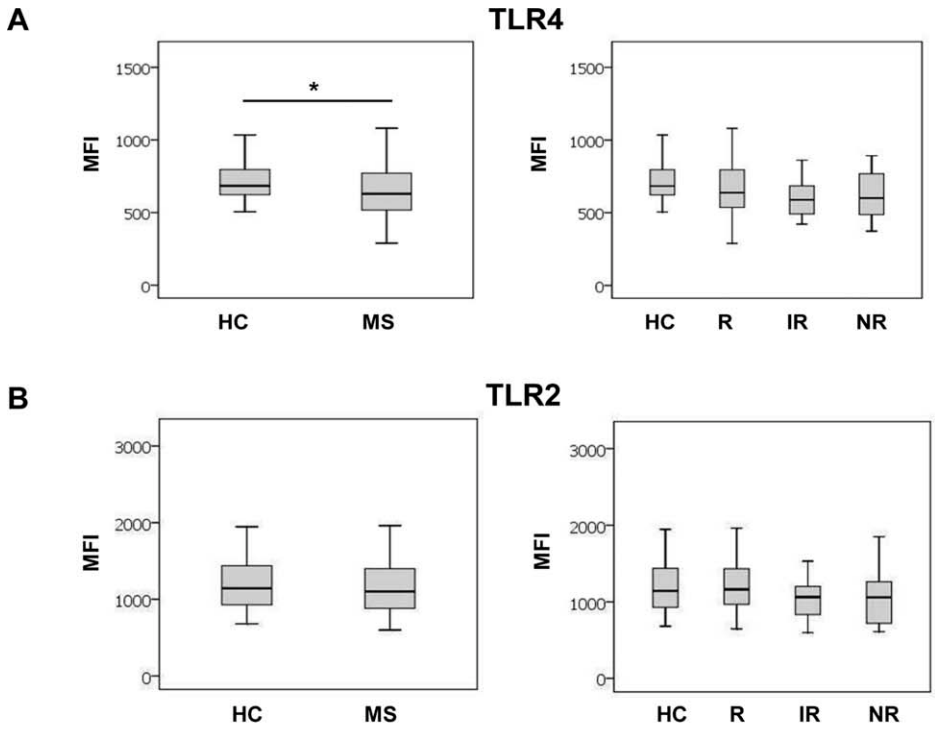
observed in all treated patients, and reached statistical significance for responders ($p = 0.019$) and intermediate responders ($p = 0.001$). Although TLR4 expression was also induced by IFNβ treatment in nonresponders, the difference did not reach statistical significance ($p = 0.091$; see Fig 2C).

The effect of IFNβ was not specific for TLR4, as similar induction levels by IFNβ were observed for TLR2 in the whole MS group ($p = 2.0 \times 10^{-7}$) and in responders ($p = 0.001$), intermediate responders ($p = 0.001$), and nonresponders ($p = 0.018$) (see Fig 2C).

Baseline Endogenous IFNβ Expression Levels Are Increased in Patients Who Will Show a Lack of Response to Treatment

Following, we measured the baseline levels of several components of the TLR4 pathway in MS patients stratified by the clinical response to IFNβ treatment and controls. First, serum levels of HSP70, a ligand for TLR4, were measured by an enzyme immunoassay and found to be similar between the different groups ($p > 0.05$ for all group comparisons; Fig 3A).

Next, we determined the mRNA expression levels of TLR4 downstream molecules by real-time PCR absolute quantification. Expression levels of the genes coding for the adaptor proteins MYD88 and TICAM1 were comparable between controls, responders, intermediate responders, and nonresponders ($p > 0.05$; see Fig 3B, C). However, expression levels of *IFNβ1* and *IL1B*, genes induced through MYD88-independent and MYD88-dependent pathways, respectively, were significantly higher in IFNβ nonresponders compared with responders (*IFNB1*: $p = 0.005$; *IL1B*: $p = 0.019$) and intermediate



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Groups	BL	IFN	p-values
	Expression levels		
	TLR4		
MS	673.1(217.5)	782.3 (253.2)	$4.3x10^{-5}$
R	696.7 (235.3)	760.7 (246.9)	0.019
IR	622.6 (178.5)	834.4 (293.5)	0.001
NR	642.2 (185.7)	797.8 (219.7)	0.091
	TLR2		
MS	1156.8 (332.7)	1416.8 (403.1)	$2.0 x10^{-7}$
R	1189.3 (349.2)	1362.6 (377.2)	0.001
IR	1080.4 (251.1)	1470.5 (430.0)	0.001
NR	1128.1 (397.1)	1611.5 (475.7)	0.018

FIGURE 2: Baseline and IFNβ-induced TLR4 and TLR2 expression levels in CD14+ monocytes from MS patients classified by their response to IFNβ treatment and healthy controls. Box plots showing (A) TLR4 and (B) TLR2 expression in monocytes at baseline in the whole MS group, patients with different response phenotypes and healthy controls. TLR4 and TLR2 expression in CD14+ cells was determined by flow cytometry, as described in Patients and Methods. Monocytic TLR4 expression is decreased in MS patients compared with healthy controls (*$p = 0.019$). (C) Table showing changes in TLR4 and TLR2 expression induced by IFNβ after 12 months of treatment. Data are expressed as mean values (standard deviation) of MFI levels. BL = baseline; HC = healthy controls (n = 32); IFN = 1-year interferon beta (IFNβ) treatment; IR = intermediate responders to IFNβ (n = 18); MFI = median fluorescence intensity; MS = whole multiple sclerosis (MS) group (n = 71); NR = nonresponders to IFNβ (n = 8); R = responders to IFNβ (n = 45); TLR = Toll-like receptor.

responders (*IFNB1*: $p = 0.033$; *IL1B*: $p = 0.014$; see Fig 3B, C), and differences reached marginal significance when compared with controls ($p = 0.05$ for both genes). Interestingly, intermediate responders were also characterized by elevated *IFNB1* expression levels, and differences became significant when compared with responders ($p = 0.033$; see

TABLE 2: Summary of Correlations between Clinical and Radiological Variables and Expression Levels of TLR4, TLR2, and IFNAR1 at Baseline

Characteristics	MS	R	IR	NR
TLR4				
Duration of disease	−0.22 (0.062)	−0.18 (0.228)	−0.36 (0.143)	−0.15 (0.729)
EDSS	**−0.40 (0.0004)**	−0.36 (0.013)	−0.34 (0.173)	−0.49 (0.217)
Number of relapses in the 2 previous years	−0.04 (0.724)	−0.06 (0.700)	0.11 (0.658)	0.00 (1.000)
Number of Gd-enhancing lesions	0.06 (0.652)	−0.00 (0.994)	0.08 (0.744)	0.51 (0.247)
T2LL	−0.25 (0.034)	−0.19 (0.210)	−0.32 (0.200)	−0.07 (0.879)
TLR2				
Duration of disease	−0.01 (0.906)	−0.12 (0.440)	0.17 (0.513)	0.24 (0.560)
EDSS	−0.21 (0.072)	−0.26 (0.077)	0.12 (0.623)	−0.15 (0.728)
Number of relapses in the 2 previous years	−0.12 (0.303)	−0.01 (0.929)	−0.35 (0.154)	−0.22 (0.604)
Number of Gd-enhancing lesions	0.01 (0.921)	0.04 (0.775)	−0.16 (0.530)	0.24 (0.599)
T2LL	−0.21 (0.085)	−0.24 (0.115)	−0.10 (0.693)	−0.14 (0.760)
IFNAR1				
Duration of disease	0.12 (0.323)	0.12 (0.429)	0.12 (0.628)	0.17 (0.686)
EDSS	0.17 (0.168)	0.17 (0.278)	−0.07 (0.778)	−0.76 (0.028)
Number of relapses in the 2 previous years	−0.01 (0.951)	0.06 (0.701)	−0.26 (0.296)	−0.11 (0.797)
Number of Gd-enhancing lesions	−0.02 (0.862)	−0.04 (0.787)	0.06 (0.823)	0.11 (0.811)
T2LL	0.21 (0.088)	0.22 (0.144)	0.28 (0.261)	−0.43 (0.337)

Data are expressed as Spearman correlation coefficients: r (p values). Bold values indicate statistically significant correlations following Bonferroni correction.
EDSS = Expanded Disability Status Scale; IFN = interferon; IR = intermediate responders to IFNβ; MS = whole multiple sclerosis (MS) group; NR = nonresponders to IFNβ; R = responders to IFNβ; T2LL = T2 lesion load.

Fig 3C). For CXCL10, trends toward increased expression levels were observed in nonresponders and intermediate responders when compared with responders ($p = 0.075$ and $p = 0.090$, respectively; see Fig 3C). No significant differences between groups were observed for *TNF*.

IFNAR1 Expression in Monocytes Is Elevated at Baseline in MS Patients and Nonresponders to Treatment

We next explored the implication of the type I IFN signaling pathway in the disease and the response to IFNβ by measuring IFNAR1 expression at baseline in monocytes from patients and controls. As depicted in Figure 4A, IFNAR1 expression in monocytes was significantly higher in the whole MS group compared with controls ($p = 0.039$). Comparison of IFNAR1 expression in MS patients further stratified based on their response to IFNβ treatment revealed significantly higher IFNAR1 expression in nonresponders when compared with responders and controls ($p = 0.003$ and $p = 0.002$, respectively; see Fig 4A). Of note, patients classified as intermediate responders were also characterized by increased IFNAR1 expression in monocytes, and differences were statistically significant when compared with controls ($p = 0.028$). A trend toward increased IFNAR1 expression was also observed in intermediate responders compared with responders ($p = 0.086$).

No significant correlations were observed between baseline clinical and radiological variables and IFNAR1 expression in the whole MS group or in patients stratified by the IFNβ response phenotype (see Table 2).

IFNβ Induction Is Altered in Nonresponders to Treatment

We next aimed to investigate the effect of exogenous IFNβ on the type I IFN pathway of responders and

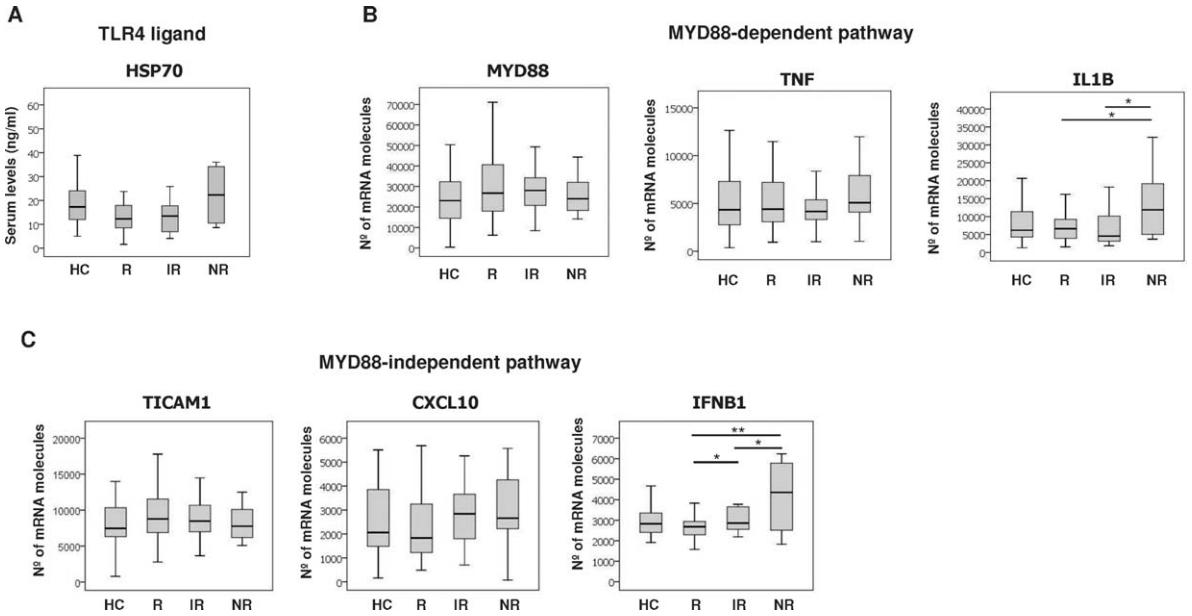


FIGURE 3: Baseline levels of HSP70 and TLR4 downstream genes in MS patients with different response phenotypes and healthy controls. Box plots showing (A) serum levels of the TLR4 ligand HSP70 and (B) expression levels of MYD88-dependent and (C) MYD88-independent TLR4 downstream genes. As described in Patients and Methods, serum levels of HSP70 were determined by ELISA and represented as ng/ml. Expression levels of TLR4 downstream genes were determined by real-time PCR absolute quantification and results expressed as number of mRNA molecules. *IL1B* expression is increased in nonresponders compared with responders (*$p = 0.019$) and intermediate responders (*$p = 0.014$). *IFNB1* expression is elevated in nonresponders compared with responders ($p = 0.005$) and intermediate responders (*$p = 0.033$), and in intermediate responders compared with responders (*$p = 0.033$). ELISA = enzyme-linked immunosorbent assay; HSP70 = heat shock protein 70; *IFNB1* = interferon, beta 1, fibroblast; *IL1B* = interleukin 1 beta; IR = intermediate responders to IFNβ (n = 18); mRNA = messenger RNA; MYD88 = myeloid differentiation primary response gene 88; NR = nonresponders to IFNβ (n = 18); HC = healthy controls (n = 32); PCR = polymerase chain reaction; R = responders to IFNβ (n = 49); TLR = Toll-like receptor.**

nonresponders to treatment by evaluating both the changes in IFNAR1 expression induced by IFNβ after 12 months of treatment and the ability of IFNβ to induce its own expression. As depicted in Figure 4B, ex vivo determination of IFNAR1 expression in monocytes before and after 12 months of IFNβ treatment did not reveal significant differences in the whole MS group or in patients stratified by the response to treatment. However, a lack of significant induction of IFNβ was observed in baseline PBMC from nonresponders after in vitro culture with IFNβ for 24 hours (magnitude of median increase [Δ] = 1.9; $p = 0.172$; see Fig 4C). In responders, IFNβ resulted in a significant induction of its own expression ($\Delta = 5.4$; $p = 0.024$), whereas in controls the addition of IFNβ was associated with induction ratios that were in between responders and nonresponders ($\Delta = 3.4$; $p = 0.084$) (see Fig 4C).

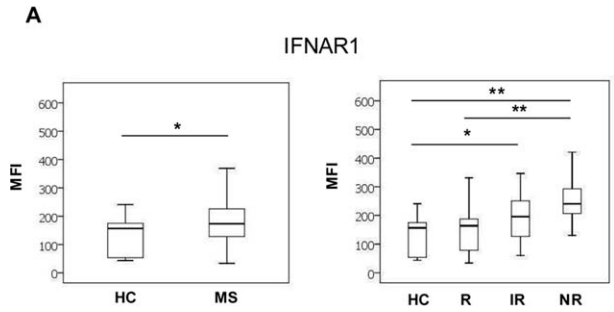
Expression Levels of IRAK3, a Negative Regulator of TLR4 Signaling, Are Decreased in IFNβ Responders

As a next step, the findings of an increased expression of IFNAR1 and TLR4 downstream genes such as *IFNB1*, *IL1B*, and *CXCL10* in nonresponders prompted us to investigate the expression levels of genes involved in the regulation of the TLR4 and the type I IFN pathways. Figure 5 depicts the Ct values obtained for these genes, which are inversely related to quantity. Expression levels of *IRAK3* in responders were lower than in nonresponders and controls, and differences reached statistical significance when compared with nonresponders ($p = 0.002$), and a trend was observed when compared with controls ($p = 0.011$). Based on this finding, IRAK3 expression was also determined in a group of intermediate responders (n = 16), and levels were found to be similar to the responder group (data not shown).

No statistically significant differences were observed between groups in the expression levels of *TYK2*, *PTPN6, PTPN11*, *SARM1*, *SIKE*, *SIGIRR*, and *RIPK3* (Fig 5).

Discussion

In a previous study by our group we reported that PBMC, most likely monocytes, from a subgroup of IFNβ nonresponders were characterized by overexpression of type I IFN responsive genes at baseline.[5] However, it was unknown whether this lack of response was due to alterations in the pathways through which IFNβ



B Changes in IFNAR1 expression

Groups	IFNAR1		
	BL	IFN	p-values
MS	177.3 (89.2)	173.8 (92.3)	0.579
R	162.9 (85.3)	163.3 (86.1)	0.929
IR	182.6 (80.8)	154.6 (83.2)	0.199
NR	258.4 (101.8)	286.6 (86.1)	0.111

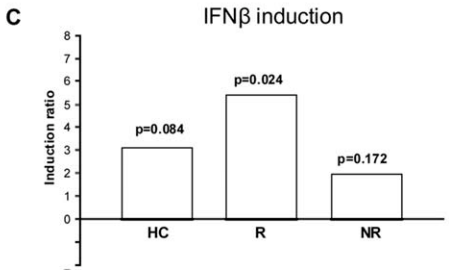


FIGURE 4: Comparison of baseline and IFNβ-induced IFNAR1 expression in monocytes and IFNB1 expression levels after in vitro exposure to IFNβ in MS patients stratified by their response to treatment and healthy controls. (A) Box plots showing IFNAR1 expression in CD14+ monocytes from MS patients and healthy controls. Determination of IFNAR1 expression was performed by flow cytometry, as described in Patients and Methods. IFNAR1 expression is increased in the whole MS group compared with controls (*p = 0.039; left). In patients classified by their response to IFNβ (right), IFNAR1 expression is elevated in nonresponders vs responders and controls (p = 0.003 and p = 0.002, respectively), and in intermediate responders compared with controls (*p = 0.028). Results are expressed as MFI. Analysis was also performed with the percentage of CD14+ cells expressing IFNAR1 and resulted in similar differences (data not shown). HC (n = 32); IR (n = 18); MS group (n = 71); R (n = 45); NR (n = 8). (B) Table showing changes in IFNAR1 expression induced by IFNβ after 12 months of treatment. Data are expressed as mean values (standard deviation) of MFI levels. IR (n = 15); MS (n = 60); NR (n = 6); R (n = 39). (C) Bars represent induction ratios of *IFNB1* expression in PBMC after in vitro addition of IFNβ-1b (100IU/ml) for 24 hours. Induction ratios were calculated as the quotient of median number of *IFNB1* mRNA molecules obtained in PBMC cultured in the presence (numerator) or absence (denominator) of IFNβ-1b. *IFNB1* expression was determined by real-time PCR absolute quantification. HC (n = 4); R: responders (n = 5); NR (n = 5). BL = baseline; HC = healthy controls; IFN = 1-year interferon β treatment; IFNAR1 = IFN receptor 1; *IFNB1* = interferon, beta 1, fibroblast; IR = intermediate responders to IFNβ; MFI = median fluorescence intensity; mRNA = messenger RNA; MS = multiple sclerosis; NR = nonresponders to IFNβ; PBMC = peripheral blood mononuclear cells; PCR = polymerase chain reaction; R = responders to IFNβ.**

exerts its effects, such as the type I IFN pathway or related pathways, or was a consequence of higher disease activity in nonresponders. In the present study, we aimed to explore the implication of the TLR4 signaling pathway, which is in close relationship with the type I IFN pathway,[11] in MS disease activity and the response to IFNβ.

Despite the finding of lower TLR4 expression in monocytes from MS patients at baseline compared with controls and the significant negative correlation observed between TLR4 expression and EDSS scores, its lack of association with other clinical and radiological variables of disease activity together with the presence of similar EAE disease courses in wild-type and TLR4-deficient mice do not further support a role of TLR4 in MS disease activity. Previous EAE studies in TLR4-deficient mice have resulted in discordant results.[14,15]

A major finding that emerged from the investigation of the TLR4 pathway in the response to IFNβ was the presence of an increased baseline expression of endogenous IFNβ in patients who will show a lack of response to treatment. This finding is in agreement with a previous study by our group,[5] in which type I IFN bioactivity measured in baseline serum samples was found to be elevated in nonresponders compared with responders, and may explain both the overexpression of type I IFN responsive genes reported in nonresponders in the previous study and the increased expression levels of other TLR4 downstream genes such as *IL1B* and *CXCL10* observed for nonresponders in the present study. This finding is also in agreement with a recent publication by Axtell and colleagues[16] in which the authors reported the presence of increased serum IFNβ levels in a subgroup of IFNβ nonresponders.

It is worth mentioning that the increase observed in endogenous IFNβ expression levels and monocytic IFNAR1 expression, while more prominent, was not restricted to the subgroup of patients classified as IFNβ nonresponders based on stringent clinical criteria that

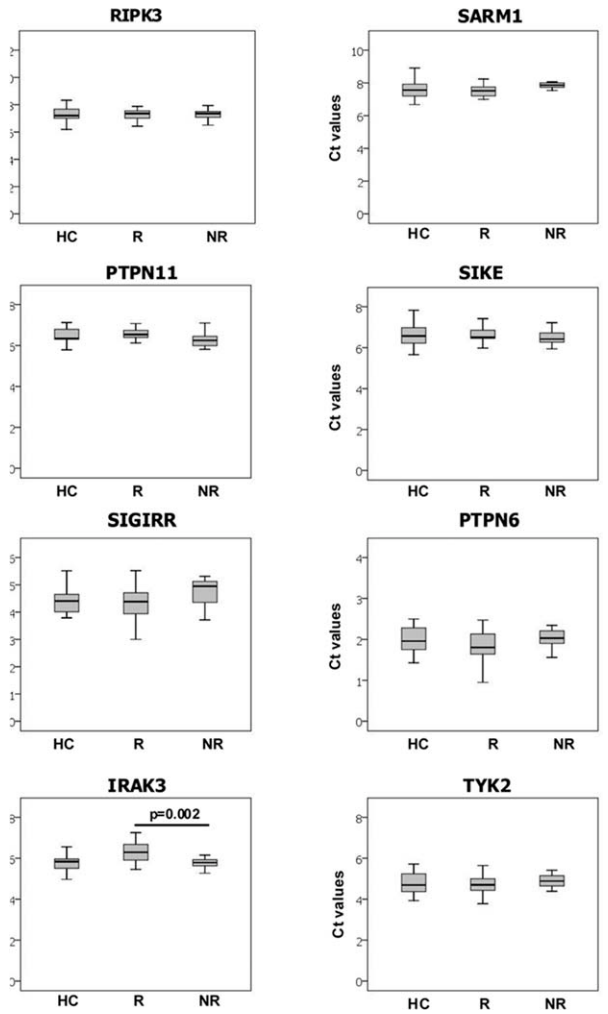


FIGURE 5: Comparison of baseline gene expression levels of key regulators of TLR4 and type I IFN pathways in MS patients stratified based on the response to IFNβ treatment and healthy controls. mRNA expression levels for each gene were determined by real-time RT-PCR relative quantification, as described in Patients and Methods. *IRAK3* expression is deficient in R compared with NR ($p = 0.002$). The y-axis represents the Ct values obtained for each individual after *GAPDH* subtraction (ΔCt values). Ct is inversely related to quantity, and higher Ct values are indicative of lower mRNA expression levels. HC (n = 18); NR (n = 18); R (n = 18). Ct = threshold cycle; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; HC = healthy controls; IFN = interferon; *IRAK3* = interleukin-1 receptor-associated kinase 3; mRNA = messenger RNA; MS = multiple sclerosis; NR = nonresponders to IFNβ; R = responders to IFNβ; RT-PCR = reverse transcription polymerase chain reaction; TLR = Toll-like receptor.

required both the presence of relapses and progression on neurological disability. In this regard, increased IFNβ and IFNAR1 expression was also a feature of patients labeled as IFNβ intermediate responders according to less stringent criteria that included either the presence of relapses or progression on neurological disability. Insomuch as a large proportion of patients showing lack of response to IFNβ fall into this intermediate response phenotype, findings such as those mentioned above in these patients may have significant relevance to clinical practice. However, it should be emphasized that no consensus on the definition of lack of response to IFNβ treatment has been reached yet, and the definitions that were used in the present study to classify patients into responders, nonresponders, and intermediate responders were based on data from previous studies by our group using response criteria that were validated by a long-term follow-up.[4,12]

In the present study, the finding of high endogenous IFNβ expression levels in nonresponders may have different origins and do not suggest per se an involvement of the TLR4 pathway in the response to IFNβ. Supporting this notion, expression levels of TLR4 upstream molecules such as *MYD88* and *TICAM1*, expression of the membrane receptor itself, and serum levels of HSP70, a TLR4 ligand, were comparable between the different IFNβ response phenotypes and healthy controls. However, investigation of the expression levels of key regulators of the TLR4 signaling pathway revealed an interesting finding in relation to *IRAK3* that points to an implication of this pathway in the response to treatment. *IRAK3* was found to be significantly differentially expressed between IFNβ responders and nonresponders. Much to our surprise, the defect in *IRAK3* expression appeared to be present in IFNβ responders, as *IRAK3* expression levels were similar between nonresponders and healthy controls and they clearly differed from the levels found in responders. IRAK3, also known as IRAKM, is primarily expressed in monocytes and macrophages[17] and functions as a negative regulator of TLR signaling.[18]

IRAK3 expression is induced by lipopolysaccharide (LPS) and inhibits the dissociation of IRAK1 and IRAK4 from MYD88 and thus the formation of IRAK1-TRAF6 (TNF-receptor-associated factor 6) complexes, which are necessary for downstream signaling and nuclear factor (NF)-κB activation.[18] Based on our expression findings, the inhibitory effect of IRAK3 on the TLR4 MYD88-dependent pathway should be diminished and, as a result, the production of proinflammatory cytokines upon TLR4 stimulation increased in responders compared with nonresponders. Interestingly, it has been shown that IRAK1 is required for the induction of proinflammatory cytokines, but is someway inhibitory for the induction of type I IFNs.[19] These observations stem from studies with SHP1 (*PTPN6*), another negative regulator of TLR signaling acting on IRAK1, in which SHP1 inhibits NF-κB activation but in addition boosts the production of type I IFNs.[19,20]

Of note, the restricted expression of *IRAK3* to the monocytes/macrophage lineage is in line with previous observations from our group pointing to a selective alteration of the type I IFN pathway in monocytes from IFNβ nonresponders.[5] One of the major findings derived from the investigation of the type I IFN signaling pathway in the response to IFNβ treatment was the increase expression of IFNAR1 observed in monocytes from nonresponders at baseline, which was already reported by our group and somehow validates our previous findings,[5] since the present study was conducted in an independent cohort of IFNβ-treated patients. Interestingly, similar to the results in IFNβ expression the elevated IFNAR1 expression was also observed in MS patients with intermediate response phenotypes, suggesting that the molecular mechanisms underlying IFNβ treatment failure are the same for all MS patients showing a lack of response to IFNβ regardless of the stringency of the criteria used to classify patients.

While most of the determinations were performed in PBMC from responders and nonresponders collected at baseline, similar ex vivo studies in patients following 12 months of treatment revealed either strong inductions of TLR4 and TLR2 expression or a lack of significant induction of IFNAR1 expression by IFNβ. However, all MS patients responded similarly to IFNβ in terms of changes in TLR4, TLR2, and IFNAR1 expression induced by treatment regardless of their response phenotypes. Of note, when PBMC from MS patients were challenged to induce IFNβ by the in vitro exposure to IFNβ, the capacity of IFNβ to induce its own expression was clearly deficient in cells from nonresponders compared with responders, which suggests that in patients receiving exogenous IFNβ the presence of high baseline endogenous IFNβ levels may result in an attenuation of the intracellular pathways amplifying IFNβ signaling and, consequently, in a decrease induction of IFNβ-responsive genes by exogenous IFNβ.

One matter that deserves further discussion is the "behavior" of healthy controls as comparison group for the findings observed in responders and nonresponders. For some determinations, findings in healthy controls appear to be closer to nonresponders than responders. One such example is *IRAK3* expression in healthy controls, which was found to be similar to nonresponders. However, contrary to nonresponders, PBMC from controls were not characterized by elevated expression levels of TLR4 downstream genes or IFNAR1 expression in monocytes. While IRAK3 findings may be playing a role in the response to IFNβ, other molecular mechanisms must be operating to explain the increased production of type I IFNs and altered monocytic type I IFN pathway observed in nonresponders vs responders but not vs healthy controls.

To summarize, PBMC from MS patients who will show a lack of response to IFNβ are characterized by an increased endogenous IFNβ expression at baseline compared with responders, which may explain the type I IFN signature reported in a previous study in a subgroup of IFNβ nonresponders. Furthermore, the differences in the expression levels observed for *IRAK3* and IFNAR1 between responders and nonresponders provide compelling evidence of the involvement of the TLR4 and type I IFN signaling pathways in the response to IFNβ, and point to monocytes as the main players in determining the response outcome of MS patients who will receive treatment with IFNβ.

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Potential Conflict of Interest

M.C. has consulted for Bayer Schering Pharma, Biogen Idec, Merck Serono, and Teva Pharmaceuticals; and has received payment for lectures including service on speakers bureaus for Bayer Schering Pharma, Merck Serono, Teva Pharmaceuticals, Novartis. X.M. received support for travel to meetings for the study or other purposes from NMSS; has consulted for Bayer Schering Pharma, Biogen Idec, EMD Merck Serono, Genentech, Genzyme, Novartis, Sanofi-Aventis, Teva Pharmaceuticals and Almirall; has grants/grants pending from Bayer Schering Pharma, Biogen Idec, EMD Merck Serono, Genentech, Genzyme, Novartis, Sanofi-Aventis, Teva Pharmaceuticals and Almirall; and has received payment for lectures including service on speakers bureaus from Bayer Schering Pharma, Biogen Idec, EMD Merck Serono, Genentech, Genzyme, Novartis, Sanofi-Aventis, Teva Pharmaceuticals and Almirall.

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