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The Role of Endogenous IFN-β in the Regulation of Th17 Responses in Patients with Relapsing-Remitting Multiple Sclerosis

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IFN-β has been used as a first-line therapy for relapsing-remitting multiple sclerosis (RRMS). Because only a few studies have addressed the role of endogenous IFN-β in the pathogenesis of the disease, our objective was to characterize its role in the transcriptional regulation of pathogenic Th17 cytokines in patients with RRMS. In vitro studies have demonstrated that IFN-β inhibits IL-17A, IL-17F, IL-21, IL-22, and IFN-γ secretion in CD4+ lymphocytes through the induction of suppressor of cytokine secretion 1 and suppressor of cytokine secretion 3. We found that patients with RRMS have increased serum and cerebrospinal fluid Th17 (IL-17A and IL-17F) cytokine levels in comparison with the control subjects, suggesting that deficient endogenous IFN-β secretion or signaling can contribute to the dysregulation of those pathogenic cytokines in CD4+ cells. We identified that the endogenous IFN-β from serum of RRMS patients induced a significantly lower IFN-inducible gene expression in comparison with healthy controls. In addition, in vitro studies have revealed deficient endogenous and exogenous IFN-β signaling in the CD4+ cells derived from patients with MS. Interestingly, upon inhibition of the endogenous IFN-β signaling by silencing IFN regulatory factor (IRF) 7 gene expression, the resting CD4+ T cells secreted significantly higher level of IL-17A, IL-17F, IL-21, IL-22, and IL-9, suggesting that endogenous IFN-β suppresses the secretion of these pathogenic cytokines. In vivo recombinant IFN-β-1a treatment induced IFNAR1 and its downstream signaling molecules’ gene expression, suggesting that treatment reconstitutes a deficient endogenous IFN-β regulation of the CD4+ T cells’ pathogenic cytokine production in patients with MS. The Journal of Immunology, 2014, 192: 5610–5617.

Only a few studies have addressed the role of endogenous IFN-β in the pathogenesis of multiple sclerosis (MS) (1, 2). The aim of this study was to characterize the role of endogenous IFN-β in the transcriptional regulation of pathogenic Th17 cytokines in patients with RRMS. The online version of this article contains supplemental material. Abbreviations used in this article: BDCA2, blood DC Ag 2; CIS, clinically isolated syndrome suggestive of MS; CSF, cerebrospinal fluid; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; HC, healthy control; IRF, IFN regulatory factor; MRI, magnetic resonance imaging; MS, multiple sclerosis; Mx1, mixovirus resistance 1; pDC, plasmacytoid dendritic cell; PRKR, protein kinase RNA regulated protein; qRT-PCR, quantitative real-time PCR; ROI, region of interest; ROR, retinoic acid-related orphan nuclear hormone receptor; RRMS, relapsing-remitting MS; siRNA, small interfering RNA; SN, supernatant; SOCS, suppressor of cytokine secretion.

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The gene expression data presented in this article have been submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53716) under accession number GSE53716.

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Materials and Methods

Patients

One hundred twenty-six RRMS patients, six CIS patients, 21 control subjects with no evidence of the inflammatory disease, and 74 HCs were enrolled in the study upon signing an institutional review board–approved form. The inclusion criteria consisted of an RRMS diagnosis (17), age of 18–65 years, and absence of disability status score of 1.5–5.5. The exclusion criteria consisted of an RRMS diagnosis (17), age of 18–65 years, and absence of disability status score of 1.5–5.5. The exclusion criterion was treatment with immunomodulatory or immunosuppressive therapy at the time of blood sample collection. The treatment-free period was at least 1 month after methylprednisolone, and 3 mo after IFN-β, and glatiramer acetate treatment. Patients treated previously with immunosuppressive therapies were not enrolled in the study. Control subjects who donated CSF samples collected at the time of their diagnostic workup did not have evidence of inflammatory disease. They had the following diagnoses: chronic pain (6), headache (5), cerebrovascular disease (4), altered mental status, Bell’s palsy, hereditary spastic paraplegia, peripheral neuropathy, fatigue, and metabolic encephalopathy (one patient for each diagnosis). Six CIS patients were included in the study; their first clinical presentation occurred within 1 year of the blood sample collection, and brain magnetic resonance imaging (MRI) revealing at least two CNS demyelinating lesions.

Flow cytometry

Fresh PBMCs derived from 16 patients with RRMS and 16 HCs were incubated in serum-free medium and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma) for 2 h, and brefeldin A (1:1000 dilution; eBioscience) for an additional 3 h for the intracellular cytokine staining (18). Where indicated, PBMCs (1 × 10⁶ cells/ml) derived from an independent cohort of 10 RMMS patients were cultured in serum-free medium in the absence or presence of IFN-β–1a (1000 IU/ml; EMD Serono) for 24 h, followed by PMA and ionomycin stimulation for the intracellular cytokine staining. Intracellular staining for pSTAT, RORc, and FOXP3 was demonstrated baseline IRF7-mediated inhibition of Th17 and Th9 cytokine secretion in CD4+ cells. IFN-β–1a treatment of patients with clinically isolated syndrome suggestive of MS (CIS) induced IFNAR1 and its downstream signaling molecules’ gene expression. These findings suggest that high-dose recombinant IFN-β–1a therapy may reconstitute a deficient endogenous IFN-β regulation of the CD4+ T cells’ pathogenic cytokine production in MS.

Affymetrix gene arrays

Differential gene expression induced in vivo IFN-β–1a treatment was determined using Affymetrix Human Genome U133 (HG-U133) arrays (Affymetrix) that contain 45,000 probe sets representing 39,000 transcripts derived from ∼33,000 human genes. PBMCs (6 × 10⁷) were separated from 6 CIS patients at baseline and after IFN-β–1a in vivo treatment (month 12). The total RNA was extracted and hybridized for the gene expression measurements. The statistical analysis was performed as reported previously (19); p < 0.05 was considered significant.
Statistics

Statistical analyses of the comparisons between two groups were performed using nonparametric Wilcoxon ranked sum test for the paired samples (Figs. 1A, 1B, 1D–F, 3B) and Mann–Whitney U test for unpaired samples (Figs. 2A–C, 3A). For the data with a normal distribution confirmed using a Shapiro-Wilk test (R software), we performed paired Student t test (Fig. 3C).

Power calculations were performed to ensure that sample size was sufficient to test the hypothesis in each dataset by using R software. We used the standardized Wilcoxon ranked sum test and Mann–Whitney U test, which approximately follow standard normal distributions under the null hypothesis. To calculate the power, we set the significance level of the test at 0.05 and determined the acceptance region of the standard normal distribution. In each case, the power was calculated as the probability that a random variable following normal distribution with mean T and variance 1 falls outside the acceptance region of the standard normal distribution.

Multiple comparison control was performed using Bonferroni test. Repeated-measures ANOVA was used for the comparisons between multiple groups (Figs. 4, 5). Linear correlation was performed using GraphPad InStat software (GraphPad Software) (Fig. 2E); p < 0.05 was considered significant.

Results

IFN-β–1a inhibits Th17 cytokine secretion

Following our previous studies reporting on the IFN-β–mediated inhibition of Th17 cell differentiation via its effect on the Th17 polarizing cytokines secreted by DCs (19) and B cells (15), this in vitro study was performed to characterize the mechanisms of the IFN-β’s direct regulation of Th17 cell responses. IFN-β–1a in vitro treatment of PBMCs derived from 10 RRMS patients significantly decreased the percentages of IL-17A, IL-17F, IL-21, IL-22, and IFN-γ–producing CD4+ T cells, and increased percentage of IL-4–positive cells (Fig. 1A). IFN-β–1a in vitro treatment of CD4+ cells separated from the same RMMS patients increased the percentage of CD4+ pSTAT1+ cells (10.3-fold) and decreased percentage of the cells expressing the Th17 transcription factor RORc (−2.8-fold; Fig. 1B). The percentage of FOXP3+ CD4+ cells did not change (data not shown). Western blotting studies of CD4+ cells separated from three additional RMMS patients (Fig. 1C) revealed an increased pSTAT1 and IRF7 expression (9.8-fold and 8.5-fold, respectively) and decreased RORc expression (−2.6-fold; all p < 0.05) in IFN-β–treated cells. Furthermore, IFN-β–1a decreased the secretion of IL-17A, IL-17F, IL-22, IL-9, and IFN-γ, and it increased the secretion of the immunomodulatory cytokines IL-4 and TGF-β1 by the CD4+ T cells separated from 10 RMMS patients (Fig. 1D).

Studies of the molecular mechanisms of the IFN-β cytokine transcriptional regulation in the same cell cultures revealed that it induced a significant increase in suppressor of cytokine secretion (SOCS)1 and SOCS3 gene expression (Fig. 1E).

FIGURE 1. IFN-β–1a inhibits Th17 cytokine secretion. (A) PBMCs (1 × 10^6/ml) derived from 10 RRMS patients were cultured in serum-free medium in the absence or presence of IFN-β–1a (1000 U/ml) for 24 h, followed by PMA and ionomycin stimulation for the intracellular cytokine staining. The cells were stained with fluorescein-conjugated Abs and αCD4 mAb for gating. (B) The same PBMCs were stained with the indicated fluorescein-conjugated Abs. (C) Magnetic bead-separated CD4+ T cells (1 × 10^6/ml) from an additional three RMMS patients were cultured in the absence or presence of IFN-β–1a for 24 h prior to the protein extraction. The Western blotting results represent one of three similar experiments. (D) Resting CD4+ T cells (1 × 10^6/ml) from an independent group of 10 RRMS patients were cultured in the absence or presence of IFN-β–1a 72 h prior to SN collection. The secretion of the indicated cytokines was measured using ELISA. (E and F) CD4+ cells derived from the same 10 RMMS patients as in Fig. 1D were used for gene expression studies using qRT-PCR. Statistical analysis was performed using a Wilcoxon ranked sum test.
qRT-PCR measurements of the gene expression in the above cultures from 10 RMMS patients revealed a significant induction of IRF7, TLR7, and MyD88 and decreased expression of IL-1R1 (Fig. 1F), whose signaling plays a critical role in Th17 cell differentiation (21).

Th17 cell cytokine production is increased in RMMS patients

We next measured the concentrations of prototypical Th17 (IL-17A and IL-17F), Th1 (IFN-γ), and Th2 (IL-4) cytokines in the sera and CSF samples from the RMMS patients (23 and 21 patients, respectively) and from the control subjects (22 and 21 donors, respectively), whose CSF samples were obtained during their diagnostic workup. Patients’ and control subjects’ demographic and disease activity characteristics are provided in Supplemental Table I. Control subjects did not have evidence of inflammatory diseases. The RMMS patients exhibited a significantly higher production of IL-17A and IL-17F in sera (Fig. 2A) and CSF (Fig. 2B), and increased IFN-γ serum levels in comparison with the control subjects. The percentages of ex vivo–detected, IL-17A–producing CD4+ T lymphocytes were significantly increased, and the percentages of IL-4–producing cells were decreased in the blood samples from 16 RMM patients in comparison with the 16 HCs (Fig. 2C, 2D). We next measured the volume of brain MRI T2 lesions, which represent the total lesion burden accumulated over the duration of the disease, and hypointense T1 lesions, which reflect a more advanced lesions characterized by axonal loss (22), in 15 RMMS patients whose MRIs were obtained at the

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** RRMS patients exhibit activated Th17 cell responses. (A) Serum samples from 23 RRMS patients and 22 HCs and (B) CSF from 21 RRMS patients and 21 control subjects were collected, and the production of IL-17A, IL-17F, IFN-γ, and IL-4 was measured using ELISA. (C) Fresh PBMCs isolated from an independent group of 16 RMMS patients and 16 HCs were stained with the fluorescein-conjugated Abs and αCD4 mAb for gating. Isotype controls were used to determine the background. The percentages of the cells expressing each molecule in gated CD4+ T cells were determined by flow cytometry. Statistical analysis was performed using Mann–Whitney U test. (D) The figure presents representative PBMC staining from one RRMS patient and one HC. (E) Correlation analysis was used to examine the relationship between serum IL-17A and IL-17F cytokine levels and brain MRI T2 and T1 lesion volumes in 15 patients for whom an MRI scan was obtained at the time of the serum collection.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** RRMS patients have a deficient endogenous IFN-β production. (A) IFN-β–reactive WISH cells were cultured in the absence or presence of serum samples from 20 RRMS patients and 20 HCs for 2 h prior to RNA extraction. The expression of IFN-β–inducible Ms1 and PRKR genes was measured by qRT-PCR. Statistical analysis was performed using Mann–Whitney U test. (B) PBMCs derived from 13 RMMS patients and 13 matched HCs were stained for the pDC marker BDCA2, and the percentage of pDCs was determined in the gated live cells. Statistical analysis was performed using Wilcoxon ranked sum test.
time of the serum collection. Correlation analysis revealed that serum IL-17F levels in RRMS patients positively correlated with their brain MRI T2 and T1 lesion volumes, whereas serum IL-17A levels were positively correlated with T1 lesion volumes (Fig. 2E).

Deficient endogenous IFN-β production in RRMS patients

Because of the technical difficulties associated with direct measurement of endogenous IFN-β in complex biological fluids, such as serum (23), recent studies measuring the endogenous IFN-β bioactivity were performed using cell-based bioassays (24). To measure endogenous IFN-β serum production in the context of the identified cytokine changes in RRMS patients, we have performed a sensitive in vitro assay validated in a large number of patients (20, 24). Human epithelial IFNAR+ WISH cells were cultured with medium, or with 50% diluted serum samples from 20 RRMS patients and 20 HCs for 2 h. The level of detection of serum IFN-β that induced the expression of IFN-inducible genes was 0.1 pg/ml. The expression of IFN-β-induced Mx1 and PRKR genes was significantly decreased in the RRMS serum-treated WISH cells, which suggests a deficient endogenous IFN-β secretion in RRMS patients in comparison with HCs (Fig. 3A).

Consistent with those results, our studies of pDCs, the main cell source of IFN-β, have identified a significantly decreased percentage of BDCA2+ pDCs in the PBMCs derived from 13 RRMS patients in comparison with the same number of age- and sex-matched HCs (Fig. 3B).

IFN-β induces a deficient signaling in CD4+ cells derived from RRMS patients

Western blotting experiments on the CD4+ T cells derived from three RRMS patients revealed decreased baseline pSTAT1, pSTAT3, TLR7, and MyD88 expression (2.72-, 2.67-, 2.60-, and 2.14-fold, respectively) in comparison with matched HCs, which may reflect decreased endogenous IFN-β secretion. However, in vitro treatment with the exogenous IFN-β–1a induced pSTAT1, IRF7, and MyD88 protein expression that were less prominent (2.16-, 2.79-, and 2.23-fold) in RRMS patients in comparison with HCs (Fig. 4).

**FIGURE 4.** IFN-β–1a induces deficient IFNAR1 signaling in CD4+ cells derived from MS patients in comparison with HCs. Magnetic bead-separated CD4+ T cells (1×10^6/ml) from three RRMS patients and three matched HCs were cultured in the absence or presence of IFN-β–1a for 24 h prior to the protein extraction. The expression of the indicated proteins was measured by Western blotting. The results present one of three similar experiments.

**FIGURE 5.** Endogenous IFN-β suppresses the basal Th17 cytokine secretion. (A and B) CD4+ cells (3×10^6 per condition) derived from four RRMS patients were transfected with siRNA IRF7 or control siRNA. Cells were cultured in serum-free medium in the absence or presence of IFN-β–1a for 24 h prior to the protein extraction and for 72 h prior to SN collection. (A) The expression of the indicated proteins was measured using Western blotting. The results represent one of four similar experiments. (B) The secretion of the indicated cytokines was measured in the same cultures using ELISA. Statistical analysis was performed using a repeated-measures ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001. **(C) IFNβ, SOCS1, and SOCS3 gene expression was measured using RT-PCR in CD4+ T cells derived from three additional RRMS patients transfected with siRNA IRF7 or control siRNA. The results are presented as the relative gene expression normalized against 18S mRNA. Statistical analysis was performed using paired t tests.
In summary, in vitro results have characterized the mechanisms of the direct Th17 cell suppression by IFN-β, which may be deficient in RRMS patients because of decreased endogenous IFN-β secretion and signaling.

**IRF7 mediates the immunomodulatory effects of IFN-β on CD4+ T cell cytokine production**

IRF7 is the key downstream molecule in the IFN-β signaling pathway and the main positive-feedback regulator of IFNAR signaling. To address whether baseline IFN-β production inhibits Th17 cytokine secretion, we silenced IRF7 gene expression in CD4+ T cells from four RRMS patients using siRNA (protein expression decreased by 87%; Fig. 5A). As expected, IRF7 silencing did not affect the expression of proximal IFNAR, pSTAT1, and pSTAT3 (Fig. 5A); however, it abolished the induction of TLR7 and MyD88 expression by IFN-β–1a, confirming its critical role in IFN-β signaling (19).

Most importantly, upon IRF7 silencing, the resting CD4+ T cells secreted significantly higher levels of IL-17A, IL-17F, IL-21, IL-22, and IL-9 secretion, and the induction of IL-10 secretion (Fig. 5A). In resting CD4+ T cells separated from an additional three RRMS patients, IRF7 silencing inhibited endogenous IFN-β and SOCS1 and SOCS3 gene expression (Fig. 5C), suggesting a role for endogenous IFN-β/IRF7 signaling in sustaining basal IFN-β, SOCS1, and SOCS3 expression.

**IFN-β–1a treatment of RRMS patients induced IFNAR1 signaling and inhibited Th17 cell responses**

To characterize the immunomodulatory effects of IFN-β–1a therapy (Rebiif), we studied the effect of a standard dose (44 μg injected s.c. three times per week) in six patients with clinically isolated syndrome suggestive of MS (25). The Affymetrix gene arrays were performed on PBMCs obtained before and after 12 mo of treatment. Patients’ demographic and disease activity information is provided in Supplemental Table II. IFN-β–1a treatment significantly changed the expression of 6396 genes (p < 0.05), including 190 immune response genes. We found a significantly increased gene expression for IFNAR1 and its signaling molecules JAK1, JAK2, JAK3, STAT1, STAT2, STAT3, STAT5B, and STAT6; the TLRs’ signaling molecules TRAF6, MyD88, and IRAK4; and SOCS1. In contrast, after 12 mo of treatment, there was a decreased gene expression for IL-17C, IL-23R, IL-22RA1, IL-9R, and IL-11, which regulate Th17 cell responses (Fig. 6).

**Discussion**

Only a few studies have addressed the role of endogenous IFN-β in the pathogenesis of the autoimmune response. Independent work from three groups has identified IFNAR-induced IL-27 secretion in myeloid cells as a key mechanism of IFN-β regulation of the Th17 cell–mediated autoimmune response (1, 26). Guo et al. (1)
have reported that IFNAR1-deficient mice had higher susceptibility to EAE, implying that IFNAR1 signaling in the macrophages and neutrophils is essential for the negative regulation of the Th17 cellular response. Prinz et al. (2) have confirmed that a conditional IFNAR1 deletion in myeloid cells led to severe disease. Shinohara et al. (26) have further demonstrated that the IFNAR-dependent inhibition of intracellular osteopontin de-repressed IL-27 secretion and prevented Th17 responses and EAE. They concluded that a constitutive autocrine IFNAR signaling in DCs is sufficient to suppress Th17 cellular responses.

Several studies have provided indirect evidence for a deficient type I IFN production in RRMS (27, 28), but direct serum IFN-β measurements using ELISA have been challenging because of the presence of heterophilic serum proteins that nonspecifically bind to the capture and detection Abs in the assay (29, 30). Therefore, studies of the endogenous IFN-β in patients with RRMS have focused on the functional assays measuring IFN-β-inducible gene expression, primarily MxA, whose low levels are associated with relapses in RRMS patients (31). Although these studies imply that low-endogenous IFN-β levels may be associated with the disease activity, no studies of cytokine regulation by endogenous IFN-β have been reported in patients with MS.

We report a significantly decreased endogenous IFN-β biologic activity in serum of RRMS patients in comparison with HCs, based on the significantly decreased IFN-induced gene expression in bioassay using a IFN-β-responsive cell line.

pDCs are the main IFN-β producers, secreting ~1000-fold more type I IFNs than other cell subsets. Our study has identified a significantly decreased percentage of BDCA2+pDCs in PBMCs of RRMS patients in comparison with HCs, consistent with a previous report (32). In addition to decreased numbers in the peripheral circulation, the pDCs from MS patients have an impaired maturation, characterized by the lower ex vivo expression of CD86 and 4-1BBL (33). MS patients have an altered ratio of pDC subsets, with lower numbers of IFN-α-productionpDC1s and higher numbers of mature IL-6-producing pDC2s, in comparison with HCs (34). pDC depletion during the active disease exacerbated EAE (35), which was characterized by increased numbers of Th1 and Th17 cells within the CNS. Subsequent studies (28, 36) have identified a deficient IFN-α secretion in response to CpG, and decreased expression of maturation markers on the MS patients’ pDCs, which may be associated with their impaired function. In contrast to pDCs, which represent only 0.1% of PBMCs, the regulation of endogenous IFN-β secretion by other cell subsets has not yet been characterized.

The mechanisms of IFN-β regulation of CD4+ T cell cytokine secretion was characterized in the in vitro experiments, revealing that IFN-β directly inhibited CD4+ cell secretion of IL-17A, IL-17F, IL-21, IL-22, and IFN-γ and induced the secretion of immunoregulatory cytokines IL-4, and TGF-β. Our previous studies on DCs and B cells (15, 19) and current experiments on CD4+ T cells have revealed that the IFN-β in vitro treatment induces SOCS3 and SOCS5 expression.

Cytokine secretion profiling of the serum and CSF samples in our study revealed increased IL-17A and IL-17F levels in the RRMS patients in comparison with the control subjects. Notably, IL-17F serum levels were positively correlated with patients’ brain MRI total T2 and destructive T1 brain MRI lesion volumes, whereas IL-17A serum levels were positively correlated with T1 lesion volumes, reflecting the pathogenic role of Th17 cytokines in the formation of the CNS lesions (9, 10).

IRF7 silencing experiments in CD4+ cells have demonstrated that the constitutive endogenous and exogenous IFN-β-mediated inhibition of IL-17A, IL-17F, IL-21, IL-22, and IL-9 suppression is directly regulated by IRF7, as their secretion was disinhibited upon IRF7 silencing. Endogenous IFN-β may thus contribute to the maintenance of peripheral immune tolerance via tonic suppression of the proinflammatory cytokine secretion.

Western blotting studies revealed decreased baseline pSTAT1, pSTAT3, IRF7, and MyD88 expression in the CD45 cells derived from MS patients in comparison with HCs, reflecting deficient endogenous IFN-β signaling. In addition, in vitro IFN-β–1a treatment of CD4+ cells revealed decreased induction of pSTAT1, pSTAT3, IRF7, and MyD88 in the MS patients in comparison with the HCs, indicating deficient exogenous IFN-β signaling in MS.

Ex vivo gene expression profiling at baseline and following 1 year of IFN-β–1a therapy in CIS patients revealed decreased Th17 gene expression in the context of activated IFNAR1 signaling. Our study has detected a significant inhibition of IL-17C expression, which was recently reported to induce STAT3 expression, and in cooperation with RORγt and RORα induces Th17 cell differentiation and enhances EAE disease activity (37). Multiple studies have reported on the IFN-β treatment-induced gene expression changes in RRMS patients (38–40), however our study reports on the Th17 gene expression changes in CIS patients following IFN-β–1a therapy. The findings are consistent with the results of Durelli et al. (11), who reported that Th17 cells have a higher IFNAR1 expression than Th1 cells and are selectively susceptible to inhibition by IFN-β–1a.

Based on the above studies, we propose that the endogenous IFN-β plays an active role in the suppression of the inflammatory responses and in the maintenance of peripheral immune tolerance in the healthy state. Its deficient secretion and signaling in RRMS patients might contribute to de-repression of the Th17-mediated autoimmune response, which is normalized upon chronic therapeutic administration of high-dose recombinant IFN-β–1a. Moreover, sensitive bioassays of the serum endogenous IFN-β bioactivity may be used to identify RRMS patients with low endogenous IFN-β levels that will likely optimally respond to IFN-β treatment (41), whereas RRMS patients with higher endogenous IFN-β levels may be stratified for other currently available immunomodulatory therapies.

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Disclosures
The authors have no financial conflicts of interest.

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