Search for Specific Biomarkers of IFNβ Bioactivity in Patients with Multiple Sclerosis

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Abstract

Myxovirus A (MxA), a protein encoded by the MX1 gene with antiviral activity, has proven to be a sensitive measure of IFNβ bioactivity in multiple sclerosis (MS). However, the use of MxA as a biomarker of IFNβ bioactivity has been criticized for the lack of evidence of its role on disease pathogenesis and the clinical response to IFNβ. Here, we aimed to identify specific biomarkers of IFNβ bioactivity in order to compare their gene expression induction by type I IFNs with the MxA, and to investigate their potential role in MS pathogenesis. Gene expression microarrays were performed in PBMC from MS patients who developed neutralizing antibodies (NAB) to IFNβ at 12 and/or 24 months of treatment and patients who remained NAB negative. Nine genes followed patterns in gene expression over time similar to the MX1, which was considered the gold standard gene, and were selected for further experiments: IFI6, IFI27, IFI44L1, IFIT1, HERC5, LYZ, RSAD2, SIGLEC1, and USP18. In vitro experiments in PBMC from healthy controls revealed specific induction of selected biomarkers by IFNβ but not IFNγ, and several markers, in particular USP18 and HERC5, were shown to be significantly induced at lower IFNβ concentrations and more selective than the MX1 as biomarkers of IFNβ bioactivity. In addition, USP18 expression was deficient in MS patients compared with healthy controls (p = 0.0004). We propose specific biomarkers that may be considered in addition to the MxA to evaluate IFNβ bioactivity, and to further explore their implication in MS pathogenesis.

Introduction

In 1993, IFNβ was the first FDA-approved drug for the treatment of relapsing-remitting MS (RRMS), and since then it has widely been used in clinical practice. IFNβ has demonstrated beneficial effects on decreasing the number of clinical relapses and disease activity measured by magnetic resonance imaging [1–3]. The mechanisms of action by which IFNβ produces its therapeutic effects in MS are not yet fully understood, however, IFNβ beneficial effects are most likely associated with its immunomodulatory properties.

IFNβ is a type I IFN that binds a heterodimeric cell surface receptor composed of the IFN receptor 1 (IFNAR1) and 2 (IFNAR2) subunits and activates the JAK-STAT signaling pathway. As a result, IFN-stimulated gene factor 3 (ISGF3) complexes are formed and translocated to the nucleus where they bind to IFN-stimulated response elements (ISREs) and initiate the transcription of type I IFN-responsive genes [4]. Among the different type I IFN-responsive genes, myxovirus resistance protein A (MxA), a GTPase protein encoded by the MX1 gene with potent antiviral activity [5], has proven to be one of the most sensitive and specific markers of IFNβ bioactivity [6,7]. MxA expression is significantly reduced during the development of neutralizing antibodies (NABs) [8–10], and its measurement has provided the basis for in vitro and in vivo assays to determine the presence of NABs [11,12]. However, there is a lack of clear roles of MxA as a biomarker on disease pathogenesis or in the therapeutic response to IFNβ.

In the present study, we aimed to identify new biomarkers of IFNβ bioactivity in order to compare their specificities as genes induced by type I IFNs with the MxA, and evaluate their potential implication in MS pathogenesis.

Results

Microarray studies identify biomarkers of IFNβ bioactivity with similar gene expression patterns to the MX1

We first performed gene expression microarrays in PBMC collected at different time points from IFNβ-treated patients. Supplementary Tables S1 and S2 show the top canonical
pathways that were identified in up- and down-regulated genes respectively during IFNβ treatment compared to the baseline condition. As expected, the type I IFN signaling pathway was one of the most significant pathways identified among up-regulated genes.

In order to identify new markers of IFNβ bioactivity, we stratified patients based on the presence and absence of NABs at 12 and/or 24 months of IFNβ treatment. Nine genes fulfilled the conditions described in the Methods section and followed patterns of gene expression over time similar to the MX1, the gold standard gene, and were chosen for further experiments (Table 1). As shown in Figure 1A, selected genes were significantly induced by IFNβ treatment after 3 months of treatment and their expression levels were reduced by the presence of NABs and reversed in NAB negative conditions.

Further analysis of potential transcription factors binding to the promoter region of selected genes revealed that seven of them (MX1, IFI27, IFT11, RSAD2, USP18, IFI44L, and HERC5) had ISRE responding elements (STAT1 transcription factor binding sites) in upstream regions very close to the annotated transcription initiation sites, findings that support the specificity of selected biomarkers as type I IFN induced genes.

We next performed real time RT-PCR of selected genes in order to validate microarray findings. As depicted in Figure 1B, mRNA expression levels measured by PCR over time in NAB positive and negative patients mirrored those obtained with gene expression microarrays.

Selected IFNβ bioactivity markers are specifically induced by type I IFNs

As a next step, we performed in vitro experiments to characterize the specific induction of selected biomarkers by type I (IFNβ) but not type II (IFNγ) IFNs. First, we cultured PBMC from healthy controls for 8 hours in the presence or absence of different concentrations of Avonex, Rebi, Betaferon, and IFNγ. As shown in Figure 2, all genes were selectively induced by IFNβ, as indicated by the differences in gene expression observed for IFNβ and IFNγ. The different types of IFNβ resulted in similar levels of gene expression and were considered together for calculations. Four genes had a lower limit of quantification (LLOQ) of 0.1 IU/ml: HERC5 (p = 0.007), USP18 (p = 0.01), IFI27 (p = 0.02), and IFI6 (p = 0.03) (Figure 2, arrows). The remaining genes, including MX1, reached statistical significance in their gene expression inductions at higher IFNβ concentrations (LLOQ: 1 IU/ml). Except for RSAD2, all the selected biomarkers were shown to be more selective than the MX1 gene, as indicated by the p-values associated with the area under the curve (AUC) of the difference between IFNβ and IFNγ. USP18 had the lowest p-value (p = 2.3 × 10⁻¹⁷) and was considered to be the most selective IFNβ biomarker. Four genes (IFI27, IFT11, RSAD2, and USP18) had stronger inductions in gene expression by IFNβ compared with the MX1, whereas IFI6, IFI44L, HERC5 and SIGLEC1 showed gene expression levels comparable to the MX1. Finally, Ly6E was up-regulated at lower levels (Figure 2).

From these dose-dependent experiments, a concentration of 100 IU/ml was considered optimal for gene expression induction and selected for further experiments.

Next, we cultured PBMC from healthy controls at different time points with 100 IU/ml of IFNβ and IFNγ. As depicted in Figure 3, comparisons of the AUC obtained for gene expression at the different time points revealed HERC5 (p = 2.4 × 10⁻¹⁹) and USP18 (p = 2.6 × 10⁻¹⁵) as the genes showing the highest differences in their expression levels between IFNβ and IFNγ. The remaining genes showed lower selectivity values compared with the MX1 (p = 2.2 × 10⁻¹⁵). Similar to the dose-dependent induction, IFI27, IFT11, RSAD2, and USP18 were more up-regulated at the different time points by IFNβ than the MX1. On the other hand, IFI6, IFI44L, HERC5, and SIGLEC1 showed comparable levels of gene expression induction to the MX1, whereas Ly6E was the least induced gene at all time points (Figure 3).

For most of the biomarkers, peak levels of gene expression occurred after 8 hours of cell culture and this time point was chosen for further experiments.

These data indicate that, although all the selected genes are specifically induced by type I but not type II IFNs, several biomarkers appear to be induced at lower IFNβ concentrations and more selective than the MX1.

Gene expression of selected biomarkers is gradually inhibited by increasing NAB titres

We next evaluated the capacity of high and low NAB titres to inhibit the expression of selected IFNβ bioactivity biomarkers. As depicted in Supplementary Figure S1, all biomarkers showed similar profiles of gene expression inhibition by different NAB dilutions, and gene expression was greatly reduced by high NAB titres (undiluted serum and serum dilutions ranging from 1:5 to 1:27). At lower NAB titres (1:81 serum dilutions), except for SIGLEC1 gene expression of selected biomarkers was reduced by more than 50% of the expression levels obtained for the positive control. At 1:243 serum dilutions, except for SIGLEC1, IFI44L, and Ly6E gene expression of the remaining biomarkers was reduced by greater than 25% of the positive control expression levels. Interestingly, RSAD2 showed the highest degree of inhibition in gene expression by low NAB titres, and was the only IFNβ bioactivity biomarker whose expression was reduced by more than 50% of the positive control condition at the highest serum dilutions (1:729), and greater than 50% after 1:243 dilutions (Supplementary Figure S1).

Despite similar levels of NAB-induced gene expression inhibition observed for selected biomarkers, these results point to RSAD2 as the most sensitive biomarker to capture the blocking effect of low NAB titres.

Abrogation of gene expression of selected biomarkers following cell activation

To evaluate whether selected biomarkers could be indirectly induced via the production of cytokines other than IFNβ, PBMC from healthy controls were non-specifically activated with LPS plus PHA in the presence or absence of a high-titre NAB positive serum. As shown in Figure 4, IFNβ accounted for the majority of gene expression induction by non-specific cell activation, as IFNβ blocking was associated with a more than 80% reduction in the expression levels for MX1, IFI44L, HERC5, and Ly6E, and greater than 90% reduction for IFI6, IFI27, IFT11, RSAD2, SIGLEC1, and USP18. As expected from dose- and time-dependent experiments, IFNγ contributed little to cell activation-induced gene expression, and IFNγ blocking only resulted in a small additional decrease in gene expression that ranged from 1.5% for SIGLEC1 to 7.1% for IFI44L (Figure 4).

These findings indicate that cell activation-induced up-regulation of selected biomarkers is mostly mediated by the effects of IFNβ, and other cytokines included IFNγ appear to contribute little to their expression.

USP18 expression is deficient in MS patients

We finally aimed to evaluate the potential implication of selected biomarkers in MS pathogenesis. To achieve this, expression levels
Table 1. Selected markers of IFNβ bioactivity from gene expression microarrays.

| Affymetrix probe set | Symbol | Description | Other aliases and designations | Chromosome location |
|----------------------|--------|-------------|-------------------------------|---------------------|
| 202086_at           | MX1*   | myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) | MxA                 | 21q22.3             |
| 204415_at           | IFI6   | interferon, alpha-inducible protein 6 | IFI6-16, G1P3       | 1p35                |
| 202411_at           | IFI27  | interferon, alpha-inducible protein 27 | ISG12              | 14q32               |
| 204439_at           | IFI44L | interferon-induced protein 44-like | C1orf29            | 1p31.1              |
| 203153_at           | IFIT1  | interferon-induced protein with tetratricopeptide repeats 1 | IFI56, ISG56       | 10q25-q26           |
| 219863_at           | HERC5  | hectar domain and RLD 5 | CEB1, CEBP1         | 4q22.1              |
| 202145_at           | LY6E   | lymphocyte antigen 6 complex, locus E | RIGE                | 8q24.3              |
| 213797_at/242625_at | RSAD2  | radical S-adenosyl methionine domain containing 2 | viperin            | 2p25.2              |
| 219519_s_at         | SIGLEC1| sialic acid binding Ig-like lectin 1, sialoadhesin | CD169              | 20p13               |
| 219211_at           | USP18  | ubiquitin specific peptidase 18 | ISG43              | 22q11.21            |

*MX1 was used as the gold standard gene.
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for these biomarkers were compared between untreated RRMS patients and healthy controls. Interestingly, only USP18 survived correction for multiple testing, and expression levels for this gene were significantly lower in MS patients compared with controls (p = 0.0004; Figure 5). Trends towards lower expression in MS patients were also observed for HERC5 (p = 0.018) and Ly6E (p = 0.012), although differences did not reach the threshold for statistical significance after Bonferroni correction (alpha = 0.005). Expression levels for the remaining genes were similar between MS patients and healthy controls (Figure 5).

No significant correlations were observed between USP18 expression levels and variables such as gender, age at onset, EDSS scores at the time of blood collection, number of relapses in the 2 previous years, and disease duration (p>0.05).

Discussion

MxA is specifically induced by type I IFNs and has demonstrated to be a reliable and sensitive measure of the biological response to IFNβ [6,7]. However, it has no confirmed roles in MS pathogenesis or in the clinical response to IFNβ. By applying gene expression microarrays to PBMC from patients who developed NABs to IFNβ and patients that remained NAB negative, we identified 9 biomarkers that followed changes in gene expression over time similar to the MX1, the gold standard gene. While some of these biomarkers have been published in previous studies to evaluate the biological response to IFNβ [13,14] (Supplementary Table S3), others have not been tested yet. In the present study, we compared the potential for selected biomarkers to evaluate IFNβ bioactivity. Interestingly, although MX1 induction was highly selective for type I IFNs, dose- and time-dependent induction experiments revealed several biomarkers of IFNβ bioactivity that were more selective, and significantly induced by lower IFNβ concentrations and at higher levels than the MX1. The finding of similar profiles of gene expression inhibition by different NAB dilutions for all selected biomarkers supports their use to measure the in vivo effects of NABs on IFNβ bioactivity. Finally, the gene expression abrogation experiments following non-specific stimulation indicate that cytokines other than IFNβ contribute little to the expression of selected biomarkers and reinforce their specificity by type I IFNs. Although not proven in the present study, the low gene expression levels that remained after inhibiting the effects of both IFNβ and IFNγ were most likely due to the action of IFNα, another type I IFN.

USP18 was one of the most selective biomarkers of IFNβ bioactivity, and was significantly induced at the lowest IFNβ concentration and up-regulated to a greater degree by type I IFNs compared to the MX1 gene. Furthermore, it was the only biomarker found to be differentially expressed between MS patients and controls, which suggests that USP18 may play a role in the pathogenesis of MS. USP18 codes for a type I IFN-inducible cysteine protease that deconjugates ISG15, a ubiquitin-like protein, from target proteins [15]. Interestingly, USP18 has been shown to negatively regulate the type I IFN signalling pathway, and its deficiency results in enhanced and prolonged STAT1 phosphorylation [15–17]. This action appears to be independent of its protease activity and mediated by the specific binding of USP18 to IFNAR2, which then blocks the interaction between JAK1 and the IFN receptor and results in inhibition of the downstream phosphorylation cascade [18]. Although further studies are needed, it is tempting to speculate that a deficient expression of USP18 in MS patients may lead to overactivation of the type I IFN pathway and have implications in the therapeutic response to IFNβ. In fact, overexpression of type I IFN-responsive genes has been associated with a decrease biological and clinical response to IFNβ in MS patients [19,20]. Whether or not responders and non-responders to IFNβ differ in their allelic frequencies for USP18 is an open question.

Together with USP18, HERC5 was highly selective as IFNβ bioactivity biomarker, significantly induced at the lowest IFNβ concentration, and showed induction levels comparable to the MX1. HERC5 codes for a protein ligase that is involved in the ISG15 conjugation process (ISGylation) upon stimulation with type I IFNs [21].

Similar to the MX1, RSAD2 was significantly induced at a concentration of IFNβ of 1 IU/ml, but showed stronger induction in gene expression by type I IFNs although with lower selectivity. RSAD2 (also known as viperin) encodes an antiviral protein that is involved in innate immunity against the infection of many DNA and RNA viruses. Of note, RSAD2 showed the highest degree of inhibition in gene expression by high dilutions of serum from a NAB positive patient. The inhibiting effect of high and low NAB titres on RSAD2 expression was also evaluated in a recent study [14]. These findings suggest that
RSAD2 measurement may be considered in the design of new and more sensitive assays to determine NABs.

Similar to the MX1, SIGLEC1 and Ly6E had a LLOQ of 1 IU/ml. In dose-dependent induction experiments, they were shown to be more selective than the MX1 as IFNb bioactivity biomarkers. However, Ly6E induction levels were the lowest following stimulation with IFNb, and SIGLEC1 was the least sensitive biomarker to capture the blocking effect of low NAB titres. SIGLEC1 (also known as CD169) codes for a macrophage-restricted sialic acid receptor, which mediates adhesive interactions with lymphoid and myeloid cells [22]. Although little is known on the function of Ly6E, Ly6 proteins may be playing roles in cell signalling and cell adhesion processes [23,24]. Interestingly, SIGLEC1 and Ly6E were found to be up-regulated in peripheral blood cells, mainly monocytes, from patients with other autoimmune disorders such as systemic sclerosis and systemic lupus erythematosus compared with healthy controls [25–28], and mRNA and protein levels were shown to correlate with disease activity in lupus patients [25,27,28]. Studies correlating SIGLEC1 and Ly6E levels with disease activity or the response to IFNb have not been performed in MS.

In dose-dependent induction experiments, IFI6 and IFI27 were significantly induced at lower IFNb concentrations and more

Figure 1. (A) Changes in gene expression observed with microarrays for selected IFNb bioactivity markers at baseline (T = 0), and after 3, 12, and 24 months of treatment. Four patients developed NABs at 12 and/or 24 months (Patients 1–4) and 4 patients remained NAB negative at these time points (Patients 5–8). (B) Validation of microarray findings by real time RT-PCR in representative patients belonging to the different categories (Patients 1, 2, 3, and 5). Given the much stronger induction in gene expression observed for IFI27, graphs corresponding to its expression were depicted separately for the sake of clarity. Open squares: Ly6E; open circles: IFIT1; open triangles: IFI6; open inverted triangles: USP18; open diamonds: HERC3; asterisks: IFI44L; solid squares: MX1; solid circles: SIGLEC1; solid inverted triangles: IFI27; solid diamonds: RSAD2. + refers to induction in gene expression after 3 months of treatment. NAB positive determination. -: NAB negative determination.

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In dose-dependent induction experiments, IFI6 and IFI27 were significantly induced at lower IFNb concentrations and more
While IFI6 showed comparable induction levels to the MX1, IFI27 was by far the most up-regulated gene following stimulation with type I IFNs. Of note, IFI27 was proposed as a sensitive marker of IFNβ bioactivity in a recent study [13], and in a one-year time course transcriptomic study IFI6 was found among the genes consistently up-regulated by IFNβ [29]. IFI6 and IFI27 belong to the FAM14 family of proteins and have roles in the regulation of apoptosis. IFI6 encodes an anti-apoptotic protein that inhibits depolarization of mitochondrial membrane potential, cytochrome c release, and caspase-3 activity [30]. Interestingly, IFI6 has also been shown to antagonize the effects of TRAIL (TNF-related apoptosis-inducing ligand) by inhibiting the intrinsic apoptotic pathway through mitochondrial stabilization [31]. The protein encoded by IFI27 associates with or inserts into the mitochondrial membrane, and its up-regulation has been reported to lead to decreased viable cell numbers and enhanced sensitivity to DNA-damage induced apoptosis [32].

Given the important role that apoptosis plays in the pathogenesis of MS, further studies to explore the implication of IFI6 and IFI27 in disease pathogenesis are warranted.

Finally, LLOQ of IFIT1 and IFI44L was the same as the MX1 (1 IU/ml). Whereas in the dose-dependent experiments IFI44L showed similar selectivity and induction levels to the MX1, IFIT1 appeared to be more selective and induced to a higher degree compared to the MX1. IFIT1 encodes a protein that is rapidly induced in response not only to viral infections but also non-viral stimuli such as LPS, IL-1 and TNFα [33,34], and may be involved in cell apoptosis via interaction with the eukaryotic elongation factor-1A (eEF1A) [35]. Little evidence exists in the literature regarding the function of the protein encoded by IFI44L. However, it is important to mention that a related gene, IFI44, and IFIT1 were found to be among the genes that best predicted the response to IFNβ treatment in MS patients [19].

In summary, we propose specific biomarkers that may be considered in addition to the MxA to measure the biological response to IFNβ and the in vivo effects of NABs. Although more
studies are needed, findings from the present study suggest that some of these selected biomarkers may also be playing roles in the pathogenesis of MS and/or the therapeutic response to IFNβ.

**Materials and Methods**

**Ethics Statement**

The study was approved by the Hospital Universitari Vall d’Hebron Ethics Committee [PR/AG/33/2008] and all patients gave written informed consent to be included in the study.

**Gene expression microarrays**

PBMC from RRMS patients were collected before and during IFNβ treatment and stored in liquid nitrogen until used. Gene expression microarrays (Affymetrix Human Genome U133 Plus 2.0) were performed in PBMC from 8 RRMS patients at baseline and after 3, 12 and 24 months of IFNβ treatment. All patients were females and the mean age (SD) was 43.1 years (8.8). Four patients were treated with subcutaneous IFNβ-1b (Betaferon), and the remaining received subcutaneous IFNβ-1a (Rebif). Four patients were negative for NABs at 12 and 24 months and 4 patients developed NABs at 12 and/or 24 months (one patient was NAB positive at 12 and 24 months, another patient was negative at 12 and positive at 24 months, and 2 patients were positive at 12 and negative at 24 months).

Quality control, preprocessing and analysis of microarray data were performed as previously described [19]. We aimed to identify genes that followed temporal expression patterns similar to the

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**Figure 4. Abrogation of gene expression of selected biomarkers following non-specific cell activation (A–J).** PBMC from 3 healthy controls were cultured for 8 hours in preincubated medium with PHA plus LPS in the presence or absence of undiluted high-titre NAB positive serum with and without anti-IFNγ antibodies, as described in Methods. Results are expressed as fold change in gene expression relative to a condition of unstimulated cells and with a value of 1 after normalization (not shown in the graphs for the sake of clarity). PBMC cultured with 100 IU/ml of Betaferon in the presence or absence of high-titre NAB positive serum were used as positive controls (graphs on the left). Bars represent SEM. Arrows indicate the difference in gene expression observed after the addition of anti-IFNγ antibodies. NAB: neutralizing antibodies to IFNβ.

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**Validation of selected IFNβ bioactivity markers by real time RT-PCR**

In 4 patients, expression levels of selected genes were also determined by real time RT-PCR in order to validate microarray findings. Total RNA was taken from the same samples that had been used for the microarrays. cDNA was synthesized from total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, U.S.A.). Amplifications were performed in duplicate using Taqman probes specific for the genes selected from microarray studies (Applied Biosystems). The housekeeping gene GAPDH was used as an endogenous control. The threshold cycle (C_T) value for each reaction, and the relative level of gene expression for each sample were calculated using the 2^−ΔΔC_T method [37]. In brief, GAPDH 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 ΔC_T value. The differences (ΔΔC_T) between the ΔC_T values obtained for the untreated baseline samples (calibrators) and the ΔC_T values for the IFNβ-treated samples (at 3, 12 and 24 months) were determined. The relative quantitative value for the treated samples was then expressed as 2^−ΔΔC_T, representing the fold change in gene expression normalized to the endogenous control and relative to the calibrators.

**Dose- and time-dependent induction of selected IFNβ bioactivity markers**

For dose-dependent experiments, fresh PBMC from 6 healthy controls [3 females/3 males; mean age: 27.5 years (7.1)] were isolated by Ficoll-Isopaque density gradient centrifugation (Gibco BRL, Life Technologies LTD, UK), washed twice and resuspended in culture medium (RPMI medium 1640 supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM HEPES buffer, 50 units/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL)). PBMC (2 x 10⁶ cells/ml) were cultured for 24 hours with intramuscular IFNβ-1a (Avonex), Rebif, Betaferon, and human recombinant IFNγ at different concentrations: 0.1, 10, 100, and 1000 IU/ml. After cell culture, mRNA expression levels of selected IFNβ bioactivity markers were determined by real time RT-PCR, as previously described. Changes in gene expression were always compared with cells cultured in the absence of IFNβ (referred to as 0 IU/ml; calibrators).

For time-dependent experiments, freshly isolated PBMC from 7 healthy controls [3 females/4 males; mean age: 27.5 years (5.7)] were cultured as previously described in the presence or absence of 100 IU/ml of Avonex, Rebif, Betaferon, and human recombinant IFNγ for 2, 4, 6, 8, and 24 hours. After cell culture, gene expression levels for selected markers were determined by real time RT-PCR, as described above. Changes in gene expression were always referred to a baseline uncultured condition (0 h; calibrators). Previously, gene expression levels obtained for the
different biomarkers in untreated cells cultured for the same time points were subtracted from the values obtained after treatment with IFNβ and IFNγ.

NAB-induced gene expression inhibition

Undiluted and serially diluted serum (1:3, 1:9, 1:27, 1:81, 1:243, 1:729) collected from a patient treated with Betaferon who developed NABs at high titres (>1280) was preincubated for 1 hour in the presence or absence of 100 IU/ml of Betaferon. Subsequently, freshly isolated PBMC from 3 healthy controls (2 females/1 male; mean age: 24.7 years [21]) were cultured for 8 hours with preincubated medium. After cell culture, mRNA expression levels of selected IFNβ bioactivity markers were determined by real time RT-PCR, as described above. IFNβ-induced expression levels were compared with those obtained from cells cultured without IFNβ in the presence of serum from a NAB negative patient (calibrators). PBMC cultured with 100 IU/ml of Betaferon was used as positive control.

Abrogation of gene expression induced by non-specific cell activation

Freshly isolated PBMC from 3 healthy controls (2 females/1 male; mean age: 29.3 years [4.9]) were cultured for 8 hours in preincubated medium with PHA (3 μg/ml) plus LPS (1 μg/ml) in the presence or absence of undiluted high-titre NAB positive serum (>1280) with and without anti-IFNβ antibodies (100 μg/ml) at 37°C for 1 hour. After cell culture, gene expression of selected biomarkers was determined by real time RT-PCR, as previously described. Cell activation-induced expression levels were compared with those obtained from unstimulated cells cultured in the presence of serum from a NAB negative patient (calibrators). PBMC cultured with 100 IU/ml of Betaferon in the presence or absence of high-titre NAB positive serum were used as positive controls of NAB-induced inhibition.

Gene expression levels for selected bioactivity markers in MS patients and controls

Fresh PBMC were isolated from 14 untreated RRMS patients (64.3% females; mean age (standard deviation): 42.1 years (9.6); mean number of relapses in the previous 2 years: 0.9 (0.9); mean disease duration: 12.4 years (7.1); median EDSS at the time of blood collection (interquartile range): 2.0 (1.5–3.0)]. A group of 15 healthy controls [53.3% females; mean age (standard deviation): 42.1 years (9.6); mean number of relapses in the previous 2 years: 0.9 (0.9); mean disease duration: 12.4 years (7.1); median EDSS at the time of blood collection (interquartile range): 2.0 (1.5–3.0)] was also included in the study.

After RNA extraction, mRNA expression levels for selected biomarkers were determined by real time RT-PCR, as described above. Gene expression values obtained for MS patients were referred to the expression levels observed in controls (calibrators).

Statistical analysis

For dose-dependent experiments, the following parameters were considered: (i) Sensitivity was evaluated by the LLOQ and defined as the minimum IFNβ concentration that induced a statistically significant increase in gene expression when compared with the untreated condition, and it was calculated by paired t-tests adjusting for multiple testing using the Bonferroni approach. (ii) Selectivity was defined, for each gene, as the difference observed in gene expression between different concentrations of type I and type II IFNs, and it was calculated by comparing the AUC obtained for IFNβ and IFNγ. The p-value associated with the AUC of the difference was calculated by means of a t-type statistic that uses the critical value from a t-distribution with Satterthwaite’s approximation [30] to the degrees of freedom for calculation of confidence intervals.

Similarly, for time-dependent selectivity was defined as the difference observed in gene expression between type I and type II IFNs at the different time points of in vitro cell culture, and it was analyzed by computing the AUC of the difference, as described above.

NAB-induced gene expression inhibition was evaluated by comparing the NAB-positive serum dilutions that were associated with reductions in gene expression of selected biomarkers greater than 25% and 50% of the expression levels obtained for the positive control condition.

A Mann-Whitneys test was used to test for significant differences in gene expression levels between MS patients and controls. Insomuch as 10 different genes were evaluated, Bonferroni correction was used to correct the alpha level for multiple comparisons (alpha = 0.005).

Statistical calculations were performed with R language and the SPSS 11.5 package (SPSS Inc, Chicago, IL) for MS-Windows.

Supporting Information

Figure S1 NAB-induced gene expression inhibition of selected biomarkers. Undiluted and increasingly diluted serum from an IFNβ-treated patient who developed high NAB titres were preincubated for 1 hour in the presence or absence of 100 IU/ml of Betaferon, and then added to PBMC from 3 healthy controls for 8 hours, as described in Methods. Results are expressed as fold change in gene expression relative to a condition of cells cultured without IFNβ and with a value of 1 after normalization (not shown in the graphs for the sake of clarity). Bars represent SEM. Dotted lines indicate the expression levels that correspond to 25% and 50% reductions in gene expression of the positive control condition. US: undiluted serum. PC: positive control. NAB: neutralizing antibodies to IFNβ.

Table S1 Top canonical pathways up-regulated during treatment with IFNβ.

Table S2 Top canonical pathways down-regulated during treatment with IFNβ.

Table S3 Summary of studies related with selected IFNβ bioactivity markers.

Methods S1

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Author Contributions

Conceived and designed the experiments: SM MFB MC. Performed the experiments: SM MFB FD NF. Analyzed the data: MCRdV EV LN RNN. Contributed reagents/materials/analysis tools: FM JR XM. Wrote the paper: SM MC.

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