Activation of the JAK-STAT Signaling Pathway after In Vitro Stimulation with IFNβ in Multiple Sclerosis Patients According to the Therapeutic Response to IFNβ

Isaac Hurtado-Guerrero, Maria Jesús Pinto-Medel, Patricia Urbaneja, Jose Luis Rodríguez-Bada, Antonio León, Miguel Guerrero, Óscar Fernández, Laura Leyva*, Begoña Oliver-Martos**

Unidad de Gestión Clínica de Neurociencias, Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Regional Universitario de Málaga, Universidad de Málaga, Málaga, Spain

* These authors contributed equally to this work.
* begoliver@gmail.com (BOM); leyvafer@gmail.com (LL)

Abstract

Interferon beta (IFNβ) is a common treatment used for multiple sclerosis (MS) which acts through the activation of the JAK-STAT pathway. However, this therapy is not always effective and currently there are no reliable biomarkers to predict therapeutic response. We postulate that the heterogeneity in the response to IFNβ therapy could be related to differential activation patterns of the JAK-STAT signaling pathway. Our aim was to evaluate the basal levels and the short term activation of this pathway after IFNβ stimulation in untreated and IFNβ treated patients, as well as according to therapeutic response. Therefore, cell surface levels of IFNAR subunits (IFNAR1 and IFNAR2) and the activated forms of STAT1 and STAT2 were assessed in peripheral blood mononuclear cells from MS patients by flow cytometry. Basal levels of each of the markers strongly correlated with the expression of the others in untreated patients, but many of these correlations lost significance in treated patients and after short term activation with IFNβ. Patients who had undergone IFNβ treatment showed higher basal levels of IFNAR1 and pSTAT1, but a reduced response to in vitro exposure to IFNβ. Conversely, untreated patients, with lower basal levels, showed a greater ability of short term activation of this pathway. Monocytes from responder patients had lower IFNAR1 levels (p = 0.039) and higher IFNAR2 levels (p = 0.035) than non-responders just after IFNβ stimulation. A cluster analysis showed that levels of IFNAR1, IFNAR2 and pSTAT1-2 in monocytes grouped 13 out of 19 responder patients with a similar expression pattern, showing an association of this pattern with the phenotype of good response to IFNβ (p = 0.013). Our findings suggest that an activation pattern of the IFNβ signaling pathway in monocytes could be associated with a clinical phenotype of good response to IFNβ treatment and that a differential modulation of the IFNAR subunits in monocytes could be related with treatment effectiveness.
Introduction

Multiple sclerosis (MS) is a chronic, inflammatory, demyelinating and presumably autoimmune disease of the central nervous system. It is characterized by the presence of multifocal lesions with variable axonal degeneration that result in progressive neurological disability [1]. Interferon beta (IFNβ) is a common treatment used for patients with relapsing-onset MS [2]; however there is a large percentage of patients in which the IFNβ therapy fails to reduce the relapse rate and the disability progression [3]. Clinicians should identify these non responder patients as early as possible, in order to offer other alternative therapies that are currently available. However, although several efforts have been made, at the moment there is no reliable biomarker to predict the response to IFNβ treatment and only neutralizing antibodies (NABs) are considered to be a biomarker of IFNβ bioactivity that might affect clinical decision making [4].

IFNβ exerts its biological activity through interaction with a heterodimeric type I interferon receptor (IFNAR), composed of the IFNAR1 and IFNAR2 subunits [5] and through activation of the JAK-STAT signaling pathway [6]. This interaction results in the phosphorylation of IFNAR1 and IFNAR2 in tyrosine residues by Janus kinases, Tyk2 and JAK1, that subsequently result in the phosphorylation of critical residues of the transcriptional factors of the STAT family, mainly STAT1 (tyrosine 701) and STAT2 (tyrosine 689), leading to the formation of a heterodimer. This heterodimer associates with a third subunit, interferon regulatory factor 9 (IRF9/p48), to form the transcriptional complex IFN-stimulated gene factor 3 (ISGF3), which in turn, translocates to the nucleus to activate genes containing the IFN-stimulated response elements (ISRE) [7–10].

The JAK-STAT pathway is utilized by many other cytokines for signaling, and its activation is critical for the orchestration of immune responses [11]. The dysregulation of this pathway has pathological implications in autoimmune diseases such as rheumatoid arthritis, lupus erythematosus and psoriasis [12–15]. Accordingly, in the last years, therapies targeting the JAK-STAT signaling pathway have emerged for rheumatoid arthritis and other inflammatory diseases [16].

In MS patients, alterations in the JAK-STAT signaling pathway have also been described. The different leukocyte subsets showed a differential activation of STATs in response to systemic injection of IFNβ1a or in vitro stimulation [17]. Furthermore, the activation of this pathway in MS has been related with different clinical issues. Phosphorylated STAT1 (pSTAT1) has been proposed as a marker of disease activity since an up-regulation of pSTAT1 in peripheral blood mononuclear cells (PBMC) was observed in the active phase of the disease [18]. Moreover, those patients who subsequently became non-responders showed a greater activation in the JAK-STAT signaling pathway before the onset of IFNβ therapy, with an elevation of IFNAR1 and pSTAT1 levels in monocytes [19].

The aim of the present study was to assess the levels of some proteins of the JAK-STAT signaling pathway at baseline and after short term activation with IFNβ, in untreated and IFNβ-treated patients and to determine whether the activation patterns of those proteins discriminated responders from non-responders to IFNβ therapy.

Materials and Methods

Subjects

This cross-sectional study enrolled forty eight patients with clinically definite relapsing remitting MS [20,21] from the Multiple Sclerosis Unit at Regional University Hospital in Málaga (Spain). Among them, 17 patients were treatment-naïve for at least 6 months, and 31 patients were treated with IFNβ1a or 1b, for 12–14 months. None of them had received corticosteroids.
in the three months prior to blood sampling. All the patients were sampled during remissions and, in the case of treated patients after 12–14 months of IFNβ therapy, within 10–12 hours post-injection.

The presence of neutralizing antibodies (NABs) against IFNβ in serum samples of treated patients was tested by the cytopathic effect test following the WHO recommendations, as previously described [22]. The NABs were tested in the serum samples collected at the same time point as the blood used for isolating PBMC to avoid any possible influence on the activation of the Jak-STAT signaling pathway. Only those patients who were NABs negative were included in the study.

After 12 months of IFNβ therapy, patients were classified according to their clinical and MRI activity [23]. Each patient could be positive for relapses, progression or MRI activity. Those patients exhibiting at least one relapse during the first year of therapy were considered positive for relapses; those who showed a progression of disability in the Expanded Disability Status Scale (EDSS) score of at least one point during the first year of therapy (confirmed at 6 months) were considered positive for progression; finally, those developing three or more active lesions (either new or enlarging T2 lesions compared with baseline MRI scan or gadolinium-enhancing lesions) on the MRI performed after 1 year of therapy were classified as positive for MRI activity.

Patients were considered non-responders according to the occurrence of two or three positive variables during the first year of therapy, otherwise they were considered responders.

The samples were provided by the Biobank of the Andalusian Public Health System. All patients participating in the study gave their written informed consent and protocols were approved by institutional ethical committees (CEI Málaga Nordeste).

The demographic and clinical characteristics of the subjects are summarized in Table 1.

**Sample collection**

Fresh lithium heparinised blood was obtained by venipuncture from clinically stable MS patients. PBMC were isolated using a ficoll-hypaque gradient, as described in the supplier’s

| Table 1. Demographic and clinical characteristics of multiple sclerosis patients. |
|---------------------------------|---------|---------|---------|---------|---------|---------|
|                                  | Untreated | IFNβ Treated | p-Value (a) | Responders | Non-responders | p-Value (b) |
| Number of subjects              | 17       | 31       |          | 19       | 12      |          |
| Female/male                     | 10/7     | 19/12    | n.s.     | 12/7     | 6/6     | n.s.     |
| Age (years)                     | 37 ± 11  | 36 ± 10  | n.s.     | 36 ± 10  | 35 ± 11 | n.s.     |
| MS duration (years)             | 6.5 ± 5.4| 6.9 ± 5.4| n.s.     | 6.4 ± 5.6| 7.7 ± 5.2| n.s.     |
| EDSS at baseline                | 1 (0–3.75)| 1 (0–1.5) | n.s.     | 0 (0–1)  | 1 (0–2.5)| n.s.     |
| EDSS after a year of therapy    | -        | 1 (0–1.5)|          | 0 (0–1)  | 1.75 (1–2.875)| 0.003    |
| Number of relapses before treatment onset | 1 (0–1.5) | 1 (0–1)  | n.s.     | 1 (1–1)  | 1 (0–2) | n.s.     |
| Number of relapses in the first year of therapy | - | 0 (0–1)  |          | 0 (0–0)  | 1 (1–1) | 9 x10⁻⁶ |
| Number of patients with MRI activity before interferon onset | - | - |          | 6/19     | 10/12   | <0.005   |
| Number of patients with MRI activity after 1 year of IFNβ therapy | - | - |          | 1/19     | 9/12    | <0.00005 |

Quantitative data are presented as mean ± standard deviation (age and MS duration) or as median (inter-quartile range) for EDSS and number of relapses.

(a) P-values obtained between untreated and treated patients by chi-square test (gender), T-test (age, duration) or Mann-Whitney test (EDSS and number of relapses).

(b) P-values obtained between responder and non responder patients by chi-square test (gender, MRI activity), T-test (age, duration) or Mann-Whitney test (EDSS and number of relapses).

n.s non significant, MS multiple sclerosis, EDSS expanded disability status scale, IFNβ interferon beta.

doi:10.1371/journal.pone.0170031.t001
protocol (ICN Biomedicals Inc., OH, USA). After that, cells were cryopreserved in RPMI-1640 medium (BioWhittaker), 40% heat-inactivated fetal calf serum (FBS) (BioWhittaker) and 10% DMSO (Sigma), until use.

**IFNβ stimulation and flow cytometry**

A panel of four markers was selected including the two subunits of IFNAR (IFNAR1 and IFNAR2) and the activated forms of STAT (pSTAT1 and pSTAT2), to cluster patients based on signaling profiles.

PBMC from untreated and treated MS patients were thawed and suspended in pre-warmed RPMI-1640 medium (BioWhittaker), supplemented with 2 mM L-glutamine (ICN Biomedicals), 20% FBS (BioWhittaker) and 0.032 mg/ml gentamicin (Normon). PBMC were washed by centrifugation, re-suspended in the same medium without FBS (1 million /ml) and incubated at 37˚C for 90 min, in order to obtain the lowest level of activation.

We first analyzed early kinetics of STAT phosphorylation induced by a single dose of 1000 IU/ml IFNβ1a (Avonex, Biogen, Inc.) in Jurkat cells. Then, the preliminary experiments with PBMC from MS patients showed that within 30 min of stimulation, phosphorylation of STAT1 and STAT2 was close to maximum levels, so we chose this time and dose for cell stimulation in the study (data not shown).

Cells from each subject were stimulated either with 1000 IU/ml IFNβ1a to allow the signal transduction and the phosphorylation of the proteins or with RPMI-1640 medium without FBS during 30 minutes at 37˚C (unstimulated cells considered as basal levels of activation).

Following stimulation, the cells were fixed with Cytofix (BD Biosciences) at 37˚C for 10 min, washed twice with Perm/Wash Buffer (BD Biosciences) and permeabilized with Perm-Buffer III (BD Biosciences) at 4˚C for 20 min. After two additional washes, the cells were stained for 30 min in the dark, at room temperature with fluorescein isothiocyanate, phycoerythrin, phycoerythrin-cyanine, peridin chlorophyll protein, Alexa Fluor-488 and allophycocyanin labelled specific monoclonal antibodies (MAb) for the following molecules: IFNAR1 (R&D, FAB245F), IFNAR2 (PBL 21385–3), phospho-STAT1 (Y701) (BD Biosciences 612596), phospho-STAT2 (Y689) (R&D IC2890F), CD3 (BD Biosciences 345766), CD8 (BD Biosciences 560917) and CD14 (BD Biosciences 555399) S1 Table. Previously, the antibodies were titrated for optimal separation and staining. Four or five colour stainings with different combinations of MAb were performed in order to evaluate the expression of the IFNAR subunits and the activation of STAT's in the different cell populations. Isotype-matched controls were used to verify the staining specificity of the antibodies.

Cells were washed and acquired in a FACSCanto II™ flow cytometer (BD Biosciences) using the FACSDiva software (BD Biosciences). At least 50,000 events were acquired from each sample. The gating strategy is shown in Fig 1. The expression of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 was determined in unstimulated and IFNβ-stimulated CD4⁺, CD8⁺ T cells and monocytes, and the data were analyzed as geometric mean fluorescent intensities (MFI) for each marker within each cell subpopulation.

**Statistical analysis**

Expression of each variable was tested for distribution using the Kolmogorov–Smirnov test. Distribution of the quantitative variables following a normal distribution (age and MS duration) in the patient subgroups were analyzed by the T test. In the case of variables that did not follow a normal distribution (EDSS and number of relapses), non-parametric tests were applied.
Basal levels of expression in unstimulated cells were analyzed as MFI, and the Mann-Whitney U test was used for the comparisons between two groups. Changes in IFNAR1, IFNAR2, pSTAT1 and pSTAT2 after in vitro stimulation with IFNß were analyzed as the following ratio: MFI of stimulated cells / MFI of unstimulated cells (MFI\textsubscript{s}/MFI\textsubscript{us}) using the Mann-Whitney U test. For the heat map representation, log\textsubscript{2} was applied to this ratio \[24\]. Statistical significance was set at p < 0.05. The correlation between the expression of the different markers in MS patients was assessed using the Pearson correlation coefficient.

Fig 1. Gating strategy to determine IFNAR1, IFNAR2, pSTAT1 and pSTAT2 expression on different lymphocyte subsets. First, based on the combination of FSC (size) and SSC (granularity) attributes of the acquired events, lymphocyte and monocyte gates were selected (a). T lymphocytes were identified by gating on CD3+ cells (b), then they were transferred to a new dot plot and were analyzed by a specific antibody against CD8. T CD8+ lymphocytes were identified as CD3+ and CD8+ cells and T CD4+ lymphocytes as CD3+ and CD8- cells (c). Monocytes were identified as CD14+ and CD3- (d). Overlay histograms from a representative patient of each study group that depict the MFI of unstimulated (open histograms) and IFNß-stimulated (1000UI/mL, 30 min) (filled histograms) cells expressing IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in CD4+, CD8+ T lymphocytes and monocytes. The numbers next to each of the histograms indicate the MFI values for unstimulated and stimulated cells (in brackets) (e). FSC: forward scatter, SSC: side scatter, FITC: fluorescein isothiocyanate, PE: phycoerythrin, PE-Cy7: phycoerythrin-cyanine, PerCP: peridinin chlorophyll protein, Alexa488: Alexa Fluor-488, APC: allophycocyanin.

doi:10.1371/journal.pone.0170031.g001
Four parameters (IFNAR1, IFNAR2, pSTAT1 and pSTAT2) were included as variables in an unsupervised average linkage hierarchical clustering, executed with Genesis software [25] to search for a specific pattern related to therapeutic response to IFNβ. After that, the variable "presence or absence of the pattern identified by the clustering" was associated with the response to IFNβ therapy by the chi-square test.

**Results**

**Correlations of protein levels**

**Baseline correlation.** Untreated patients showed a strong positive correlation between the expression levels of each of the markers with the levels of the other three markers in the three unstimulated cell subsets (Fig 2A). However, in IFNβ treated patients, IFNAR1 levels did not correlate with IFNAR2 levels in any of the cell subsets. Additionally, IFNAR1 levels did not correlate with STAT1 or STAT2 levels in monocytes, and IFNAR2 levels did not correlate with STAT1 in CD8+ T cells or with STAT2 levels in CD8+ T cells and monocytes, as observed in Fig 2B.

**Correlation after short-term in vitro stimulation with IFNβ.** Correlations between the expression levels of each of the markers with the levels of the other three markers in the three cell subsets were lost after in vitro activation with IFNβ. In untreated patients, only IFNAR1 expression correlated with IFNAR2 expression in the 3 cell subsets. This correlation was replicated in the treated patients in the T cell subsets, but was lost in the stimulated monocytes, in which the only correlations observed were between IFNAR1-STAT2 levels and IFNAR2-STAT1 levels (Fig 3).

**JAK-STAT signaling pathway in unstimulated cells and after short term IFNβ stimulation. Comparisons between untreated and treated patients**

**Baseline expression.** Before evaluating the effects of IFNβ stimulation, the expression of the steady-state levels of each of the markers of the JAK-STAT signaling pathway was assessed in three cellular subsets (CD4+ T cells, CD8+ T cells, and CD14+ monocytes) in untreated and treated MS patients. Among all the subsets, the monocytes showed the highest MFI for all the markers in unstimulated conditions, as shown in Table 2.

The differences in the basal levels between untreated and treated patients were assessed by comparing MFI in unstimulated cells from both groups of patients. IFNβ treated patients showed significantly higher MFI\textsubscript{us} of IFNAR1 and pSTAT1 in the CD4+ T cells ($p = 0.019$; $p = 0.020$), CD8+ T cells ($p = 0.001$; $p = 0.026$), and monocytes ($p < 0.0001$; $p = 0.008$) than untreated patients, as observed in Table 2 and Fig 4.

To evaluate the different profiles of the JAK-STAT signaling pathway in unstimulated cells, data of each study group were represented in a heat map. To normalize the data, the average per group of the MFI in unstimulated cells (MFI\textsubscript{us mean group}) was divided by the average of the MFI from all the patients (MFI\textsubscript{us mean all}) for a specific marker, in each of the unstimulated cell subsets, and then, the ratios (MFI\textsubscript{us mean group} / MFI\textsubscript{us mean all}) for IFNAR1, IFNAR2, pSTAT1, pSTAT2 were log\textsubscript{2} transformed.

These values were represented graphically by colouring log ratios of 0 in black (unchanged proteins), increasingly positive log ratios with yellows of increasing intensity and increasingly negative log ratios with violets of increasing intensity. Long term IFNβ-treated patients showed a higher activation pattern in unstimulated conditions than untreated patients, as can be observed in the heat map (Fig 5a).
Expression after short-term in vitro stimulation with IFNβ. The differences of the in vitro stimulation with IFNβ between untreated and IFNβ treated patients were analyzed as the ratio (MFI_{un}/MFI_{us}) for IFNAR1, IFNAR2, pSTAT1, pSTAT2 in each cell subset. This ratio provided us with information about the activation state of the cells with short term stimulation.
Fig 3. Correlations between levels of expression of the different markers in the three stimulated cell subsets (CD4+ T cells, CD8+ T cells, CD14+ monocytes) in untreated and IFNß-treated MS patients. The conditions of IFNß stimulation were 1000U/mL, 30 min. The correlation between the log2 [MFIstimulated/MFIunstimulated] of the different markers was assessed using the Pearson correlation coefficient. MFIstimulated: mean fluorescence intensity in IFNß-stimulated cells; MFIunstimulated: mean fluorescence intensity in unstimulated cells.

doi:10.1371/journal.pone.0170031.g003
with IFNβ and, therefore, indirectly, about the functionality of the IFNβ signaling pathway. With IFNβ stimulation, monocytes from untreated patients showed lower IFNAR1 expression (p = 0.012) and higher STAT2 expression (p = 0.027) than those from treated patients, but showed no differences in IFNAR2 and pSTAT1 levels between both groups. Conversely, in both T cell subsets, in vitro stimulation with IFNβ slightly increased IFNAR1 levels to a higher extent in untreated patients than in those patients previously treated with IFNβ (p = 0.044 and p = 0.040, respectively in CD4+ and CD8+ T cells), as shown in Fig 6.

To look for different profiles of activation of the JAK-STAT signaling pathway after in vitro IFNβ stimulation, the averages of MFIus for IFNAR1, IFNAR2, pSTAT1, pSTAT2 of each study group were log₂ transformed and represented in a heat map, as explained before. Overall, the heat map revealed that untreated patients showed a higher activation pattern after stimulation with IFNβ than patients previously treated with prolonged systemic IFNβ therapy (Fig 5b).

### JAK-STAT signaling pathway in responder and sub-optimal responder patients

#### Baseline expression. Basal levels of the JAK-STAT signaling pathway markers in treated patients according to the therapeutic response to IFNβ were assessed in unstimulated cells. No significant differences in the MFIus of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in the three cell subsets were observed between responder and sub-optimal responder patients, as shown in Table 3. As mentioned before, the highest MFI for all the markers were observed in the monocyte subset.

At baseline, levels of IFNAR1 correlated with levels of STAT1 and STAT2 in both T cell subsets, both in responders and non-responders. Additionally, STAT1 expression correlated with STAT2 expression in the three cell subsets in both groups, as shown in Table 4.

To construct the heat map in unstimulated cells, the data of each marker were normalized as follows: log₂ (MFIus mean group / MFIus mean all). Although some differences can be seen between both groups, they did not reach statistical significance (Fig 5c).

|                | Non-treated | Treated      | p   |
|----------------|-------------|-------------|-----|
| CD4 IFNAR1     | 247.65 ± 33.52 | 274.9 ± 37.79 | 0.019 |
| IFNAR2         | 243.82 ± 43.61 | 254.9 ± 25.10 | NS   |
| pSTAT1         | 243.12 ± 28.74 | 266.52 ± 33.81 | 0.020 |
| pSTAT2         | 259.88 ± 35.28 | 270.71 ± 38.20 | NS   |
| CD8 IFNAR1     | 262.23 ± 42.52 | 315.97 ± 54.96 | 0.001 |
| IFNAR2         | 270.41 ± 53.31 | 277.16 ± 45.08 | NS   |
| pSTAT1         | 245.76 ± 37.75 | 273.58 ± 35.16 | 0.026 |
| pSTAT2         | 267.88 ± 40.08 | 288.16 ± 41.94 | NS   |
| CD14 IFNAR1    | 735.36 ± 136.79 | 1143.81 ± 290.34 | <0.0001 |
| IFNAR2         | 556.59 ± 130.45 | 583.29 ± 100.18 | NS   |
| pSTAT1         | 648.23 ± 173.80 | 794.48 ± 176.49 | 0.008 |
| pSTAT2         | 1061.18 ± 298.78 | 1090.29 ± 232.95 | NS   |

Data are expressed as mean values ± standard deviation

doi:10.1371/journal.pone.0170031.t002
Expression after short-term in vitro stimulation with IFNβ. The differences in the activation of the signaling cascade after stimulation with IFNβ between responders and suboptimal responders were analyzed as the ratio (MFI\textsubscript{ms}/MFI\textsubscript{us}) for each of the four markers, S2 Table. Monocytes from responder patients showed slightly lower IFNAR1 levels (p = 0.039) and higher IFNAR2 levels (p = 0.035) than monocytes from non-responders (Fig 7). However, differences between responders and non-responders in the ability to activate STAT proteins (assessed by tyrosine phosphorylation) after IFNβ stimulation were subtle and did not reach statistical significance.

After short term in vitro activation with IFNβ, the changes in the levels of IFNAR1 and IFNAR2 correlated significantly in CD4+ (r = 0.583; p = 0.009) and CD8+ T cells from responders exclusively (r = 0.726; p = 0.00043), a correlation that was not found in monocytes (Fig 8).

Fig 4. Differences in the expression of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in unstimulated cells between untreated and treated patients. MFI\textsubscript{us} of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in CD4+, CD8+ and CD14+ subsets from untreated and treated patients. The Mann-Whitney U test was used for the comparison between two groups.

doi:10.1371/journal.pone.0170031.g004
Different patterns of JAK-STAT signaling pathway after in vitro stimulation according to therapeutic response. As in the other conditions, the average of log₂ (MFI_us/MFI_us) of responders and non-responders was represented in a heat map (Fig 5d), where some differences could be observed. In order to identify the different functional patterns in the IFNβ signaling pathway related to the therapeutic response to IFNβ, the log₂ (MFI_us/MFI_us) of each patient was further represented in a heat map (Fig 9). The unsupervised average linkage hierarchical clustering showed that the levels of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in monocytes were able to group 13 out of 19 (68%) responders with a similar expression pattern. This "responder" pattern in monocytes was characterized by a decrease in IFNAR1 levels, and a simultaneous increase in IFNAR2, pSTAT1 and pSTAT2 after in vitro stimulation with IFNβ.
The chi-square test showed an association between the presence of this pattern and the clinical phenotype of good therapeutic response to IFNß (p = 0.013). However, this pattern related with the therapeutic response to IFNß could not be identified in CD4⁺ or CD8⁺ T cell sub-populations.

**Discussion**

The mechanism of IFNß therapy in relapsing-remitting multiple sclerosis is not completely understood, however it is well known that its action starts with the interaction with IFNAR and the activation of the JAK and STAT proteins[26]. The evaluation of the cell surface receptor and the phosphoproteins just after IFNß1a stimulation allows us to characterise the short term activation of the JAK-STAT signaling pathway. Our hypothesis was that a differential
activation of this pathway could help to explain differences in responsiveness to IFNβ therapy and that patients could be grouped based on signaling profiles.

First of all, the signaling pathway was characterized in untreated and IFNβ-treated patients, to evaluate the effect of the prolonged systemic IFNβ therapy. Physiological expression levels of IFNAR1 and IFNAR2 are very low [27] and, as other low copy-number proteins, IFNAR1 and IFNAR2 show a large variance in the receptor concentrations of individual cells [28]. Strikingly, in our cohort, basal levels of each of the markers correlated strongly with the expression of the others in untreated patients, but many of these correlations lost significance in previously treated patients, so systemic therapy with IFNβ is influencing the basal expression of these markers. Accordingly, it has been described that type I IFN binding to their receptor induces IFNAR1 internalization and degradation via lysosomal receptor proteolysis [29–33], while IFNAR2 expression displays a considerably slower basal turnover [33].

Under unstimulated conditions, levels of IFNAR1 and pSTAT1 were significantly higher in IFNβ-treated patients than in untreated patients in the three sub-populations. However, after short term stimulation with IFNβ, monocytes from untreated patients had a more prominent response to the stimulus than those from treated patients, showing a higher decrease in the

| Table 3. Mean Fluorescence Intensity of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in unstimulated cells (MFIun). Comparison between responders and sub-optimal responders to IFNβ therapy. |
| MFI in UNSTIMULATED CELLS |
|--------------------------|
|                          | Responders | Non responders | p   |
| CD4                      |            |               |     |
| IFNAR1                   | 271.32 ± 41.39 | 280.58 ± 32.15 | N.S. |
| IFNAR2                   | 257.84 ± 25.64 | 250.25 ± 24.58 | N.S. |
| pSTAT1                   | 264.95 ± 36.31 | 269 ± 30.80   | N.S. |
| pSTAT2                   | 270.68 ± 43.61 | 270.75 ± 29.46 | N.S. |
| CD8                      |            |               |     |
| IFNAR1                   | 314.21 ± 61.94 | 318.75 ± 44.10 | N.S. |
| IFNAR2                   | 285.05 ± 51.87 | 264.67 ± 29.37 | N.S. |
| pSTAT1                   | 269 ± 39.80  | 280.83 ± 26.18 | N.S. |
| pSTAT2                   | 287.11 ± 48.55 | 289.83 ± 30.58 | N.S. |
| CD14                     |            |               |     |
| IFNAR1                   | 1136.58 ± 281.91 | 1155.25 ± 315.64 | N.S. |
| IFNAR2                   | 597.37 ± 71.61  | 561 ± 134.52   | N.S. |
| pSTAT1                   | 784.79 ± 159.39 | 809.83 ± 207.25 | N.S. |
| pSTAT2                   | 1113.26 ± 229.07 | 1053.92 ± 244.51 | N.S. |

Data are expressed as mean values ± standard deviation

doi:10.1371/journal.pone.0170031.t003

Table 4. Significant correlations between Mean Fluorescence Intensities of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in unstimulated cells (MFIun). Comparison between responders and sub-optimal responders to IFNβ therapy.

| Correlations | Response to IFNβ therapy | CD4+ |               | CD8+ |               | CD14+ |
|--------------|--------------------------|------|--------------|------|--------------|-------|
|              | r           | p    | r           | p    | r           | p     |
| IFNAR1-STAT1 | R           | 0.850| 4 x 10^-8   | 0.828| 1.2 x 10^-5 |       |
|              | NR          | 0.662| 0.019       | 0.772| 0.003       |       |
| IFNAR1-pSTAT2| R           | 0.926| 1.2 x 10^-8 | 0.893| 2.7 x 10^-7 |       |
|              | NR          | 0.752| 0.005       | 0.765| 0.004       |       |
| pSTAT1-pSTAT2| R           | 0.933| 5.5 x 10^-9 | 0.960| 7.1 x 10^-11| 0.652| 0.002 |
|              | NR          | 0.757| 0.004       | 0.838| 0.001       | 0.873| 0.0002|

R: responders; NR: non-responders; r: Pearson correlation coefficient

doi:10.1371/journal.pone.0170031.t004
expression of IFNAR1 and a higher activation of pSTAT2. Accordingly, monocytes and T cells from patients with prolonged IFNβ treatment showed higher basal levels of IFNAR1 and pSTAT1 but showed a reduced response to *in vitro* exposure to IFNβ. Conversely, untreated patients, with a lower activation state under unstimulated conditions, showed a higher ability of short term activation of the pathway after *in vitro* stimulus with IFNβ. These results suggest a de-sensitization to the IFNβ stimulus in previously treated patients as a consequence of the continued exposure to IFNβ treatment [34, 35]. Otherwise, STAT-1, and -2 were not fully constitutively activated in T cells and monocytes, as *in vitro* stimulation with IFNβ increased their activation in both untreated and treated patients, although the ability to respond to this cytokine by activating pSTATs was slightly reduced in previously treated patients. Continuous systemic therapy with IFNβ may diminish the capacity of these cells to respond to this cytokine through these transduction factors.

**Fig 7.** Differences in the expression of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 after short term IFNβ stimulation between responders and suboptimal responders. Expression is represented as the ratio of the mean fluorescence intensity in stimulated cells (MFI) between the MFI of unstimulated cells (MFIus) of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 in CD4+, CD8+ and CD14+ subsets from responder and non-responder patients. The conditions of IFNβ stimulation were 1000UI/mL, 30 min. The Mann-Whitney U test was used for the comparison between the two groups.

doi:10.1371/journal.pone.0170031.g007
Among all the analyzed cell subsets, monocytes showed a higher response to in vitro stimulation with IFNß than CD4\(^+\) and CD8\(^+\) T cells, so IFNß stimulation seems to trigger cell type specific responses, as has been previously described in MS [17].

Regarding the therapeutic response to IFNß, it has been previously described that before the onset of IFNß therapy, those patients who subsequently became non responders showed higher IFNAR1 and pSTAT1 levels in monocytes than those who responded satisfactorily to this therapy [19], but there is a lack of JAK-STAT pathway data concerning patients undergoing IFNß treatment, as in this study.

We have shown that responders and non-responders showed no differences in the expression of any of the markers under steady-state conditions. However, responders are the group that better reflect the modulation of biological responsiveness to in vitro IFNß stimulation. Monocytes from responder patients underwent a rapid down-modulation of the cell surface IFNAR1 and an up-regulation of the IFNAR2 subunit. This observation suggests that, in non-responders a more important de-sensitization of the JAK-STAT pathway could occur as a consequence of the prolonged systemic treatment [34]. This could be a cause that justifies the lack of response with an important biological rationale, that should be investigated in a greater cohort of patients.

We thought that it would be interesting to address whether a differential modulation of the subunits occurs according to the therapeutic response, as the modulation of IFNAR1 and IFNAR2 levels is one of the main regulatory mechanisms of the duration and potency of cell responsiveness to IFNß. Several studies have demonstrated that IFNß induces endocytosis and degradation of IFNAR1 to regulate the cell signaling [36–38] en just 30 minutes after IFNß stimulation, a down-regulation of IFNAR1 has been described in some cell lines, while IFNAR2 expression displayed a considerably slower basal turnover and the extent of its down-regulation diminished as IFNAR1 expression decreased in cells [33]. Additionally, although IFNAR2 is the binding subunit, IFNAR1 can also interact with IFNß and the complex is able
to transmit signals to induce genes independently of IFNAR2 [39], a relevant fact that could explain differences in the response to IFNβ treatment.

In our study, after in vitro stimulation with IFNβ, the cell surface expression of IFNAR1 decreased while that of IFNAR2 increased in monocytes from responders. These changes suggest that, in responders, a differential regulation of the two receptor subunits is possible and the increase in IFNAR2 surface expression may compensate the well known IFNAR1 down-regulation induced by continuous exposure to IFNβ, in an attempt to maintain the activation of the JAK-STAT signaling pathway that will result in transcriptional activation or repression of interferon-regulated genes.

Fig 9. Individual activation pattern of JAK-STAT signaling pathway after IFNβ in vitro stimulation related with the responsiveness to IFNβ therapy. The log2 (MFI_stim/MFI_unstim) of IFNAR1, IFNAR2, pSTAT1 and pSTAT2 of each treated patient were represented in a heat map for each of the cellular sub-populations. Unchanged proteins are displayed in black, over-expressed proteins are displayed in yellow, while down-regulated proteins are shown in violet. In monocytes, a non-supervised average linkage hierarchical clustering grouped 13 out of 19 of responder patients with a similar expression pattern (blue square). MFI_stim mean fluorescence intensity of stimulated cells, MFI_unstim mean fluorescence intensity of unstimulated cells, NR non-responders to IFNβ therapy, R responders to IFNβ therapy.

doi:10.1371/journal.pone.0170031.g009
The changes of the cell surface receptor and the phospho-proteins just after IFNβ in vitro stimulation were used to address whether the patients could be grouped based on the activation of the JAK-STAT signaling pathway, using an unsupervised linkage hierarchical clustering. It was possible to identify a pattern in monocytes that was present in 68.4% of responder patients based on the similarity of their cell surface receptor levels and the expression of phospho-STAT proteins. This pattern, significantly associated with the phenotype of good responsiveness to IFNβ treatment, was characterised by a decrease in IFNAR1 levels and an increase in IFNAR2, pSTAT1 and pSTAT2 levels upon stimulation with IFNβ. Only two non-responders were also grouped within this pattern, so that the lack of response in these patients seems not to be directly related with the JAK-STAT signaling pathway, nor with the presence of NABs, as all the patients included in the study were NABs negative. Other factors that have not be considered in the study, such as the lack of compliance could explain this lack of response.

As JAK-STAT signaling alone is not enough to explain all the biological effects of type I IFNs, and the molecular mechanisms of the therapeutic response to IFNβ are not yet completely understood, it would be interesting to study other alternative kinases or transcription factors, as well as the inclusion of other downstream markers in the IFNβ signalling pathway that could explain why some responder patients did not cluster within the profile of good response and that would have strengthened the study.

Due to the complexity of MS pathology and the heterogeneity in the response to therapy, it might be very difficult to establish a single response biomarker. The approach in this study has allowed us to associate a clinical phenotype of good responsiveness to IFNβ treatment with a functional pattern of the IFNβ signaling pathway in monocytes, but further investigation is required to elucidate completely the role of the JAK-STAT signaling pathway in the responsiveness to IFNβ therapy.

Supporting Information
S1 Table. Detailed information of the antibodies used in flow cytometry.
(DOC)
S2 Table. Data set responder and non-responder patients.
(XLS)

Acknowledgments
We sincerely thank all the patients that participated in this study.

Author Contributions
Conceptualization: LL BOM.
Formal analysis: LL BOM MJPM.
Funding acquisition: LL.
Investigation: IHG MJPM JLRB.
Methodology: LL BOM.
Project administration: OF.
Resources: PU MG AL.
Validation: BOM.
Writing – original draft: LL BOM.
Writing – review & editing: LL BOM OF.

References
1. McFarland HF, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. Nat Immunol. 2007. 8: 913–9. doi: 10.1038/ni1507 PMID: 17712344
2. Kieseier BC. The mechanism of action of interferon-beta in relapsing multiple sclerosis. CNS Drugs. 2011. 25: 491–502. doi: 10.2165/11591110-000000000-00000 PMID: 21649449
3. Rio J, Nos C, Tintore M, Tellez N, Galan I, Pelayo R, et al. Defining the response to interferon-beta in relapsing-remitting multiple sclerosis patients. Ann Neurol. 2006. 59: 344–52. doi: 10.1002/ana.20740 PMID: 16437558
4. Comabella M, Montalban X. Body fluid biomarkers in multiple sclerosis. Lancet Neurol. 2014. 13: 113–126. doi: 10.1016/S1474-4422(13)70233-3 PMID: 24331797
5. Lutfalla G, Gardiner K, Proudhon D, Vielh E, Uze G. The structure of the human interferon alpha/beta receptor gene. J Biol Chem. 1992. 267: 2802–9. PMID: 1370838
6. Stark GR, Darnell JE Jr. The JAK-STAT pathway at twenty. Immunity. 2012. 36: 503–514. doi: 10.1016/j.immuni.2012.03.013 PMID: 22520844
7. Velazquez L, Fellous M, Stark GR, Pellegri S. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. Cell. 1992. 70: 313–22. PMID: 1386289
8. Croze E, Russell-Harde D, Wagner TC, Pu H, Pfeffer LM, Perez HD. The human type I interferon receptor. Identification of the interferon beta-specific receptor-associated phosphoprotein. J Biol Chem. 1996. 271: 33165–33166. PMID: 8969169
9. van Boxel-Dezaire AH, Rani MR, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity. 2006. 25: 361–372. doi: 10.1016/j.immuni.2006.08.014 PMID: 16979568
10. de Weerd NA, Nguyen T. The interferons and their receptors—distribution and regulation. Immunol Cell Biol. 2012. 90: 489–491. doi: 10.1038/icb.2012.9 PMID: 22410872
11. O’Shea JJ, Plenge R. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. Immunity. 2012. 36: 542–550. doi: 10.1016/j.immuni.2012.03.014 PMID: 22520847
12. Axtell RC, Raman C. Janus-like effects of type I interferon in autoimmune diseases. Immunol Rev. 2012. 248: 23–35. doi: 10.1111/j.1600-065X.2012.01131.x PMID: 22725952
13. Kasperkovicz PV, Verbeet NL, Smeets TJ, van Rietscho ten JG, Kraan MC, van der Pouw Kraan TC, et al. Ann Rheum Dis. 2004. 63: 233–239. doi: 10.1136/ard.2003.013276 PMID: 14962955
14. Ramirez-Velez G, Medina F, Ramirez-Montano L, Zarazua-Lozada A, Hernandez R, Llorente L, et al. Constitutive phosphorylation of interferon receptor A-associated signaling proteins in systemic lupus erythematosus. PLoS One. 2012. 7: e41441. doi: 10.1371/journal.pone.0041414 PMID: 22859983
15. Hald A, Andreás RM, Salskov-Iversen ML, Kjellerup RB, Iversen L, Johansen C. STAT1 expression and activation is increased in lesional psoriatic skin. Br J Dermatol. 2013. 168: 302–310. doi: 10.1111/bjd.12049 PMID: 23013371
16. Schwartz DM, Bonelli M, Gadina M, O’Shea JJ. Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases. Nat Rev Rheumatol. 2016. 12: 25–36. doi: 10.1038/nrrheum.2015.167 PMID: 26633291
17. Zula JA, Green HC, Ransohoff RM, Rudick RA, Stark GR, van Boxel-Dezaire AH. The role of cell-type-specific responses in IFN-β therapy of multiple sclerosis. Proc Natl Acad Sci U S A. 2011. 108: 19689–19694. doi: 10.1073/pnas.1117347108 PMID: 22106296
18. Frisullo G, Angelucci F, Caggiula M, Nociti V, Iorio R, Patanella AK, et al. pSTAT1, pSTAT3, and T-bet expression in peripheral blood mononuclear cells from relapsing-remitting multiple sclerosis patients correlates with disease activity. J Neurosci Res. 2006. 84: 1027–1036. doi: 10.1002/jnr.20995 PMID: 16865709
19. Comabella M, Lunemann JD, Rio J, Sanchez A, Lopez C, Julia E, et al. A type I interferon signature in monocytes is associated with poor response to interferon-beta in multiple sclerosis. Brain. 2009. 132: 3353–65. doi: 10.1093/brain/awp226 PMID: 19741051
20. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann. Neurol. 2001. 50: 121–127. PMID: 11456302
21. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann. Neurol. 2011. 69: 292–302. doi: 10.1002/ana.22366 PMID: 21387374

22. Oliver B, Orpez T, Mayorga C, Pinto-Medel MJ, Leyva L, López-Gómez C, Marín C, Luque G, Ortega-Pinazo J, Fernández O. Neutralizing antibodies against IFN beta in patients with multiple sclerosis: a comparative study of two cytopathic effect tests (CPE) for their detection. J Immunol Methods. 2009 Dec 31; 351(1–2):41–5. doi: 10.1016/j.jim.2009.09.005 PMID: 19786034

23. Rio J, Castillo J, Rovira A, Tintore M, Sastre-Garriga J, Horga A, et al. Measures in the first year of therapy predict the response to interferon beta in MS. Mult Scler. 2009. 15: 848–53. doi: 10.1177/1352458509104591 PMID: 19542263

24. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud Ø, Gjertsen BT, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell. 2004. 118: 217–228. doi: 10.1016/j.cell.2004.06.028 PMID: 15260991

25. Sturn A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics. 2002. 18: 207–208. PMID: 11836235

26. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat. Rev. Immunol. 2005. 5: 375–386. doi: 10.1038/nr1604 PMID: 15864272

27. Cohen B et al. Ligand-induced association of the type I interferon receptor components. Mol Cell Biol 1995; 15: 4208–4214 PMID: 7623815

28. Cai L, Friedman N, and Xie XS. Stochastic protein expression in individual cells at the single molecule level, Nature 2006; 440:358–362. doi: 10.1038/nature04599 PMID: 16541077

29. Constantinescu S. N., Croze E., Wang C., Murti A., Basu L., Mullersman J. E., and Pfeffer L. M.. Role of interferon α/β receptor chain 1 in the structure and transmembrane signaling of the interferon α/β receptor complex. Proc. Natl. Acad. Sci. USA 1994; 91:9602; PMID: 7524081

30. Fantuzzi L, Eid P, Malorni W, Rainald G, Gauzzi MC, Pellegrini S, Belardelli F, Gessani S. Post-translational up-regulation of the cell surface-associated α-component of the human type I interferon receptor during differentiation of peripheral blood monocytes: role in the biological response to type I interferon. Eur J Immunol. 1997; 27(5):1075–81; doi: 10.1002/eji.1830270506 PMID: 9174595

31. Liu J, Plotnikov A, Banerjee A, Suresh Kumar KG, Ragimbeau J, Marijanovic Z, Baker DP, Pellegrini S, Fuchs SY. Ligand-independent pathway that controls stability of interferon alpha receptor. Biochem Biophys Res Commun. 2008 Mar 7; 367(2):388–93. doi: 10.1016/j.bbrc.2007.12.137 PMID: 18166147

32. Gauzzi MG, Canini I, Eid P, Belardelli F, Gessani S. Loss of type I IFN receptors and impaired IFN responsiveness during terminal maturation of monocyte-derived human dendritic cells. J Immunol 2002; 169:3038–3045 PMID: 12218119

33. Marijanovic Z, Ragimbeau J, van der Heyden J, Uzé G, Pellegrini S. Comparable potency of IFNalpha2 and IFNbeta on immediate JAK/STAT activation but differential down-regulation of IFNAR2. Biochem J. 2007 Oct 1; 407(1):141–51. doi: 10.1042/BJ20070605 PMID: 17627610

34. François-Newton V, Magno de Freitas Almeida G, Payelle-Brogard B, Monneron D, Pichard-Garcia L, Piehler J, et al. USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon alpha receptor. PLoS One. 2011. 6: e22200. doi: 10.1371/journal.pone.0022200 PMID: 21779393

35. Sandler NG, Bosinger SE, Estes JD, Zhu RT, Tharp GK, Boritz E, et al. Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. Nature. 2014. 511: 601–605. doi: 10.1038/nature13554 PMID: 25043006

36. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nat Rev Immunol. 2014. 14: 36–49. doi: 10.1038/nri3581 PMID: 24362405

37. Kumar KG, Krolewski JJ, Fuchs SY. Phosphorylation and specific ubiquitin acceptor sites are required for ubiquitination and degradation of the IFNAR1 subunit of type I interferon receptor. J Biol Chem. 2004. 279: 46614–46620. doi: 10.1074/jbc.M407082200 PMID: 15337770

38. Kumar KG, Barriere H, Carbone CJ, Liu J, Swaninathan G, Xu P, et al. Site-specific ubiquitination exposes a linear motif to promote interferon-alpha receptor endocytosis. J Cell Biol. 2007. 179: 935–50. doi: 10.1083/jcb.200706034 PMID: 18056411

39. de Weerd NA, Vivian JP, Nguyen TK, Mangan NE, Gould JA, Braniff SJ, et al. Structural basis of a unique interferon-β signaling axis mediated via the receptor IFNAR1. Nat Immunol. 2013. 14: 901–907. doi: 10.1038/ni.2667 PMID: 23872679