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Title:

Decreased Interferon-β induced STAT-4 activation in immune cells and clinical outcome in MS

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Running title:

Interferon-β signalling in patients with MS

Abstract

Objectives. Interferon-β (IFN-β) is used in the treatment of multiple sclerosis (MS). IFN-β activation of signal transduction and activation of transcription (STAT)-4 is linked to its immunomodulatory effects. Previous studies suggest a type I IFN deficit in immune cells of MS patients, but data on interferon-α/β receptor (IFNAR) expression and the relationship with treatment response are conflicting. Here we compare IFN-β-mediated STAT4 activation in immune cells of untreated MS patients and controls.

Materials & methods. Peripheral blood mononuclear cells (PBMC) from 27 untreated patients with relapsing MS, obtained before the initiation of IFN-β treatment, and 12 matched controls were treated *in vitro* with IFN-β. Total and phosphorylated STAT4 (pSTAT4) and IFNAR were measured by flow cytometry and quantitative PCR. The patients were followed-up for 5 years.

Results. pSTAT4 induction by IFN-β was lower in MS patients than in controls, as was expression of IFNAR. pSTAT4 expression did not correlate with the clinical outcome at five years, measured by EDSS change. There was a negative correlation between the baseline IFNAR1 mRNA levels and relapse rate.

Conclusions. The results suggest decreased IFN-β responsiveness in MS patients, associated with reduced STAT4 activation and reduced IFNAR expression. This reduced responsiveness does not appear to affect the long term clinical outcome of IFN-β treatment.

Key words: multiple sclerosis, interferon beta, immunomodulation

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Introduction

The type I interferon, Interferon-β (IFN-β) has been used in the treatment of multiple sclerosis (MS) for many years (1, 2) and remains a mainstay disease modifying treatment for the relapsing forms of the disease. IFN-β, together with IFN-α utilises a shared heterodimeric receptor, IFNAR, composed of two chains, IFNAR1 and IFNAR2, and signals via Janus kinases Tyk2 and Jak1, resulting in the formation of heterodimers of signal transducer and activator of transcription 1 (STAT1) and STAT2, which on translocation to the nucleus associate with IFN regulatory factor 9 (IRF9) forming the heterotrimeric complex IFN stimulated gene factor 3 (ISGF3). This complex activates IFN-dependent genes. After Janus kinase activation, IFN-β also activates STAT1 homodimers, and, primarily in T and NK cells, through docking of STAT2 to STAT4, can induce phosphorylation of STAT4 and activation of gene expression via STAT4. The effect of IFN-β in MS is often partial and variable. Moreover, it is cell-type specific, related to differential activation of STATs, sometimes with opposite effects in different cell types (3).

We have shown that IFN-β in T cells leads to the induction of anti-inflammatory cytokine IL-10 in a STAT4 activation-dependent manner, explaining in part the immunomodulatory effects of IFN-β (4). This IL-10 induction was required for the suppression by IFN-β of IFN-γ induction by IL-12 (4).

Production of type I IFN is deficient in patients with MS, and several studies showed a decreased responsiveness of MS patients to IFN type I (5, 6), reflected, for example, in reduced expression of IFN-stimulated genes and decreased phosphorylation of STAT1 (7).

The aim of this current study was to determine the effects of IFN-β on the phosphorylation of STAT4 in MS patients compared to controls as an indication of response to IFN-β, and to determine its relationship with the expression of interferon type 1 receptor (IFNAR). We also

aimed to determine if the effects of IFN-β on phosphorylation of STAT4 in MS patients correlate with their 5-year clinical course as measured by the change in the expanded disability scale (EDSS) score and clinical relapses.

Materials and Methods

The Nottingham Research Ethics Committee (UK) approved this study.

Subjects included in the study

27 relapsing-remitting MS patients (17 women, 10 men; age range 29-60, mean age 43; mean Expanded disability status scale, EDSS 3.6, range 1-6) and 12 healthy controls (7 women and 5 men; mean age 42) were included in the study. The patients with MS had not previously received disease modifying drugs, were clinically stable at baseline (not in relapse) and had had 2 relapses in the last 2 years. Participants gave written informed consent prior to participation in the study.

In all MS subjects, treatment with IFN-β was commenced as part of their clinical care. 24 patients were treated (13 with IFN-β 1b and 11 with IFN-β 1a) and all patients were followed up in clinic for 5 years. EDSS at 5 years of follow-up was compared with initial EDSS score.

Cytokines and antibodies

The following reagents were obtained: IFN-β (Rebif, a gift from Merck Serono, UK); polyclonal rabbit anti-STAT4 antibody, polyclonal rabbit antiphospho-STAT4 antibody (Zymed Laboratories, Inc.,San Francisco, CA, USA); anti-human IFN-α receptor-1 (IFNAR-1) mAb and mouse IgG isotype control were purchased from R&D Systems (Oxford, UK).

Cell preparation

PBMC from healthy donors and MS patients were isolated by standard gradient centrifugation with Histopaque 1077 (Sigma-Aldrich, Dorset, UK). The mononuclear cells were prepared at $1x10^6$ cells/ml in media consisting of RPMI 1640, 2 mM glutamine, 20 mM Hepes, 0.1 mg/ml penicillin and streptomycin, and 10% FCS (Sigma-Aldrich).

Cell stimulation

PBMCs were left unstimulated or incubated with 10 ng/ml IFN-β (2.72 U/ml of Rebif®) for 30 min at 37°C. Varying IFN-β concentrations were analysed for their effect on STAT4 phosphorylation; concentrations used here were those that produced the peak pSTAT4. Optimal duration of stimulation for pSTAT4 results shown here was 30 min.

Intracellular staining

Following incubation, $1x10^6$ cells/ml were fixed in 1ml ice-cold 70% ethanol and incubated on ice for 20 min. The cells were then washed by centrifugation once in PBA (PBS, 0.5% bovine serum albumin and 1% sodium azide [SigmaAldrich]), once in saponin buffer (PBA + 0.1% saponin [Sigma-Aldrich]) and once in 10% foetal calf serum (FCS) in saponin buffer at 300 g for 5 min. 0.5 µg of rabbit polyclonal anti-phosphorylated STAT4 pTyr693 or rabbit polyclonal STAT4 (Zymed, San Francisco, CA, USA) [adapted from (8)] were added and incubated at room temperature for 30 min. Following incubation the cells were washed by centrifugation with saponin buffer. 1 µg of PE conjugated goat anti-rabbit IgG was added for 30 min at room temperature. Cells were washed with saponin buffer and resuspended in 0.5% formaldehyde.

Surface staining

Following incubation with IFN-β, 1x10^6cells/ml were washed by centrifugation in 2% FCS RPMI, and 10µl IFNAR-1 antibody were added for 30 min. After washing, cells were incubated with 5 µl goat anti-mouse FITC or goat anti-rat-PE, for 30 min. then washed and resuspended in 0.5% formaldehyde.

Quantitative real-time polymerase chain reaction (PCR)

RNA was extracted using RNeasy miniprep kit (Qiagen, Valencia, CA, USA) following manufacturers' instructions. First strand cDNA synthesis was initiated from 0.5 µg total RNA, using random hexamers (Promega, Madison, WI, USA), and avian myeloblastosis virus reverse transcriptase (Promega) using conditions as described by the manufacturer, in a final volume of 25 µl. Oligonucleotide primers designed for the Genbank sequences (NM 000629; NM 207585) were: IFNAR1 forward 5' TGCTGCGAAAGTCTTCTTGA 3'; reverse 5' TGCTTTCAACTTCTGAGGAACA 3' IFNAR2 forward 5' CACCAT AGTGACACTG AAATG 3'; reverse 5' TTGGAAGCCATGGATATGGT 3'; β2microglobulin forward 5' CTCCGTGGCCTT AGCTGTG 3' reverse 5' ATGTGTCTGGGTTT CATCCATC 3'. Real-time PCR used SYBRgreen fluorescence method with SYBRgreen master mix (Stratagene, La Jolla, CA, USA) as specified by the manufacturer. PCR reactions were performed in triplicate on a MX4000® Multiplex System (Stratagene) using standard default thermal cycling conditions. Non-template controls were loaded in triplicate. Quantification of transcripts was carried out using the relative standard curve method as per Applied Biosystems (Foster City, CA, USA; 1997) (9). An equal aliquot of undiluted cDNA from each sample was pooled together. This pool was serially diluted (neat, 1:2, 1:5, 1:10, 1:20) to produce standards, from which the Ct value was converted to ng total RNA equivalent used for first-strand synthesis. mRNA expression for each gene is normalized to internal standard (β2 microglobulin expression).

Measurements

PBMCs were evaluated on Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the results were analysed using software WINMDI 2.8. Statistical analysis employed the paired T-test. R^2 was used to assess correlation between IFNAR expression and STAT4 activation. Spearman's rank correlation was used to correlate EDSS change and pre-treatment IFNAR expression. $p \leq 0.05$ was considered significant.

Results

Activation of STAT4 by IFN-β

We first confirmed previous results showing that IFN-β increases pSTAT4. IFN-β stimulation increased pSTAT4 compared to unstimulated cells for MS and controls (Figure 1). No difference in total STAT4 was observed (not shown).

We then compared STAT4 activation in patients and controls. pSTAT4 induction was significantly higher in controls (mean fluorescence intensity, MFI 125±10) than in MS patients (MFI 98±5) ($p = 0.031$) (Figure 1).

IFNAR expression in patients and controls

We next investigated upstream of STAT4, at the receptor level. IFNAR positive cells, by flow cytometry with IFNAR1 antibody, were 15% ±2% for MS v. 36% ±7% for controls. IFNAR expression was lower ($p=0.034$) in MS patients compared to controls (Figure 2).

We then assessed IFNAR1 and IFNAR2 mRNA expression in PBMCs. MS patients and controls had similar IFNAR2 expression; however, IFNAR1 expression was significantly lower in MS patients when compared to controls (P =0.028) (Figure 3).

We also found a positive correlation between IFNAR expression and IFN-β induced pSTAT4 in MS patients (p=0.03) (Figure 4).

Clinical outcome at 5 years and IFNAR expression

EDSS scores at baseline and after 5 years of clinical follow-up were compared. Overall mean EDSS change was 1.1 points. 17 patients had an EDSS worsening at 5 years. The change in EDSS did not correlate with total pre-treatment IFNAR expression. This lack of correlation was observed in all patient subgroups (with or without change in EDSS; treated with IFN-β1a or IFN-β1b), and both IFNAR subunits. In the 22 patients with reliable documentation of relapses, there was a negative correlation between the baseline IFNAR1 mRNA levels and relapse rate (p=0.05).

Discussion

Here we show that untreated MS patients have reduced IFN responses as reflected by reduced STAT4 activation due to reduced IFNAR expression.

In our treatment-naive MS patients, IFNAR1 expression was significantly lower than in controls. This confirms previous reports on IFNAR subtypes mRNA pointing to lower IFNAR1 mRNA levels in untreated MS patients than in healthy controls (10, 11). The mechanism may involve down-regulation of IFNAR by inflammatory cytokines, as we showed (4). High levels of gene expression of IFNAR correlate with greater biological response to IFN-β as measured by cumulative induction of Myxovirus protein-A (MxA), a

marker of interferon-β bioactivity (12). Other authors have shown that long-term treatment with IFN-β could increase the levels of IFNAR1 mRNA in subjects in whom MxA has been induced (10). Low levels of expression of the IFNAR subunits can be one of the causes of low-responsiveness to IFN-β in MS.

STAT4 signalling has been associated with Th1 development, inflammation, and autoimmunity; however its roles are not completely understood. STAT4 Th1 signalling suppresses Foxp3 positive T regulatory cells (Treg) and favours autoimmune disease (13). The transition of a subset of Th17 precursors to Th1-like cells is contingent upon STAT4 expression (14).

STAT4 represents a core signalling hub in MS (4). Mice deficient in STAT4 are resistant to inflammation induction in the animal model of MS (15). STAT4 promotes IFN-γ expression in humans. IFN-γ induces inflammation and relapses in MS (16), although its role in MS and its experimental models is incompletely elucidated (17). IL-12 - induced expansion of Th1 cells plays an important role in MS. Responses to IL-12 are mediated mainly through STAT4 (18). The ability of both IL-12 and IFN-β to activate STAT4 in T cells is relevant to the actions of those cytokines in MS. IL-12 and IFN-β are considered to have biologically opposing effects. IL-12 is increased (19) and considered detrimental in MS (20) but IFN-β treatment does not appear to potentiate IL-12 proinflammatory effects. We have shown that prior exposure to IFN-β enhances STAT4 activation by IL-12, but the net effect is not proinflammatory because of parallel induction of IL-10 by IFN-β (4). In progressive stages of MS IL-10 loses its ability to suppress IFN-γ and IL-12, while IFN-β treatment loses efficacy considerably in this phase of the disease. Therefore, the beneficial immunomodulatory effects of IFN-β may in part be a result of its ability to suppress IL-12 (4).

The type I IFN pathway of STAT4 activation is more important in human immune responses than in mouse (21). Type I IFN-induced STAT4 activation requires interactions of STAT4 with STAT2, and there is genetic dissimilarity between murine and human STAT2 (22). Here we show a decreased IFN-β STAT4 signalling and IFNAR expression in whole, unseparated PBMCs from people with MS. There are major differences in the responses of primary human leukocyte subsets to IFN-β (23). Although the activation of STAT4 by type I IFN was thought initially to occur only in T and NK cells, STAT4 activation is also shown in endothelial cells, activated monocytes, and dendritic cells (DCs) (24). IFN-β induces distinct gene expression response patterns in human monocytes versus T cells (25). Differential activation of a variety of signalling proteins including STATs by IFN-β can explain the significant patient-to-patient variation in gene expression observed after treatment with IFN-β in the PBMCs from MS patients (26). Immune priming can change, positively or negatively, the quantity and quality of signal transduction by type I IFNs (24). The decreased STAT4 signalling in response to IFN-β found in the PBMC in the group of untreated MS patients reported here could therefore reflect the baseline MS immunological milieu in the absence of therapeutic immunomodulation.

Because of the role of STAT4 in mediating inflammation in MS and, at the same time, in mediating IFN-β anti-inflammatory effects, we planned to look at long term clinical correlates of STAT4 activation in untreated MS patients just prior to initiating disease modifying treatment with IFN-β. The patient group was then followed up for 5 years. Disability progression at 5 years is often used as a measure of long-term response to treatments in MS (27, 28). Of note, the patients in this study were early participants in the UK Multiple Sclerosis Risk Sharing Scheme (29). This large observational clinical cohort study recently showed that treatment with IFN-β reduces disability progression measured by EDSS

scores over 6 years of treatment (29). Early inclusions in the Risk Sharing Scheme (and access to treatment with IFN) consisted predominantly of patients with longer disease duration and thus more advanced disease, more disability and higher EDSS scores. This explains the relatively high EDSS scores at baseline in our patient group (mean EDSS 3.5). Generally, MS patients who reach EDSS 3 may already enter secondary progression (30). This may explain why this group showed evidence of disability progression over 5 years; this progression is difficult to quantify and compare to the overall Risk Sharing Scheme results, or what would be expected from the natural history of MS. However, in this cohort, clinical progression was not predicted by the pre-treatment in vitro responsiveness to IFN-β. Whether the disease modifying treatment altered the responsiveness and the expression of STAT4 and IFNAR in the short or medium term is unknown.

The lack of correlation of pSTAT4 expression before treatment with IFN-β to disability status at five years suggests the lack of a specific defect in STAT4 signalling that would link with the progression of disability. Of note, it was shown that, although the baseline expression of genes involved in IFN-β related pathways can differ between different untreated MS patients, and treatment with IFN-β can differentially induce gene expression (strong induction in those patients with low endogenous IFN-like activity, but only weak induction in patients with high endogenous IFN-like activity before treatment), patients with high and low endogenous IFN-like activity at baseline show similar clinical long-term courses of disease (31).

We found a negative, albeit marginal, correlation of relapses with IFNAR1 mRNA expression ($r= -0.4$; $p=0.05$). This may suggest that IFNAR expression can be a predictor of relapses under treatment. Nevertheless, a reliable documentation of relapses was possible only in the 22 of patients followed (81%), and under-reporting of MS relapses cannot be

excluded. In a multi-centre, retrospective study including 103 patients with MS from 7 UK centres, 46% of patients did not report at least one relapse in the past and 28% of most recent relapses were unreported (32). Several reasons may contribute to this, including rare review schedules (32).

The occurrence of relapses remains the principal factor for starting disease modifying treatments (DMT) under current MS treatment guidance (33). The risk of severe MS relapses is lower in patients treated with DMTs (34). While cost-effectiveness analyses in MS tend to focus on long term disability, individual relapses have a major impact on patients′ working and family lives, which is often overlooked (32). This highlights relapse reduction as a worthy treatment goal in MS.

Regular MRI follow-up data were not available for the group of patients in this study. MRI after the first year of treatment with IFN-β can be a good predictor of the outcome at 4-years (35), and the occurrence of 2 or more new T2 lesions or new gadolinium-enhancing lesions in patients with variable responses to IFN-β associate a significantly increased risk of future relapses and progression (36).

In this study, we intended to determine whether pre-treatment activation of pSTAT4 predicts longer term outcome. Therefore, we did not assess the medium or long term effects of IFN-β treatment on STAT4 signalling and IFNAR expression. Subnormal responses to type I IFN in MS can be corrected by long term IFN-β therapy which is able to prime the subnormal IFN response and elevate STAT signalling levels (37). Gene expression profiling studies show that the IFN-β deficiency exhibited by MS patients can be corrected over time by repeated IFN-β administration (38) however IFN signalling and gene activation pathways are partially resistant to IFN therapy in MS (2). Of note, the decreased IFN-β response in MS patients can

be improved by combination therapy with other treatments with synergistic effects. We have previously shown that prior exposure of PBMC of healthy controls and patients with MS to dexamethasone results in increased sensitivity of the cells to IFN-β, to an increase in pSTAT4 generation and in upregulation of its receptor expression (39). We showed similar enhancement of IFN-β signalling in cells from healthy controls and people with MS by curcumin (40).

In conclusion, here we show for the first time that IFN-β induced STAT4 signalling is reduced in untreated people with MS as compared to healthy subjects. The reduced pSTAT4 induction by IFN-β in MS patients compared to controls indicates a decreased ability to activate IFN-β signalling pathway via STAT4. This, combined with the reduction of IFNAR expression, suggests that untreated MS patients have decreased IFN-β responsiveness. Therapeutic strategies to improve this responsiveness, for example by synergistic combinations may be beneficial in the treatment for MS in the future.

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Conflict of Interest

CS Constantinescu has received research support, travel support for meetings and consultancy fees from Biogen Idec, Bayer-Schering, Genzyme, Merck Serono, Morphosys, Novartis, Roche, Sanofi-Pasteur MSD and Teva. R Tanasescu has received travel support for meetings from Biogen-Idec, Novartis and Teva. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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