RESEARCH PAPER

TRAIL and TRAIL receptors splice variants during long-term interferon β treatment of patients with multiple sclerosis: evaluation as biomarkers for therapeutic response

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ABSTRACT

Objective We aimed to assess the effects of interferon β (IFNβ) treatment on the expression of the splice variants of the Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) and its receptors in different cell subpopulations (CD14+, CD4+ and CD8+) from patients with multiple sclerosis (MS), and to determine whether this expression discriminated responders from non-responders to IFNβ therapy.

Methods We examined mRNA expression of the TRAIL and TRAIL receptors variants in patients with MS, at baseline and after one year of IFNβ therapy, according to responsiveness to this drug.

Results Long-term therapy with IFNβ increased the expression of TRAIL-α in T cell subsets exclusively from responders and decreased the expression of the isoform 2 of TRAILR-2 in monocytes from responders as well as non-responders. Lower expression of TRAIL-α, and higher expression of TRAIL-β in monocytes and T cell, was found before the onset of IFNβ therapy in patients who will subsequently become responders. Baseline expression of TRAILR-1 was also significantly higher in monocytes and CD4+ T cells from responders.

Conclusions The present study shows that long-term IFNβ treatment has a direct influence on TRAIL-α and TRAILR-2 isoform 2 expression. Besides, receiver operating characteristic analysis revealed that the baseline expression of TRAIL-α in monocytes and T cells, and that of TRAILR-1 in monocytes and CD4+ T cells, showed a predictive value of the clinical response to IFNβ therapy, pointing to a role of TRAIL system in the mechanism of action of IFNβ in MS that will need further investigation.

INTRODUCTION

Recombinant interferon β (IFNβ) is one of the most widely used first line therapies in multiple sclerosis (MS), but up to 50% of patients under treatment continue to experience relapses and progression of the disease and are considered suboptimal responders.1 It is, therefore, strongly desirable to count with biomarkers that allow prediction of the therapeutic response to IFNβ.

The Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL)/TRAIL Receptor System has been shown to be implicated in MS pathogenesis2-4 as well as in the mechanisms of action of IFNβ.5,6 Expression of TRAIL and its four surface receptors in different cell types during IFNβ treatment has been studied,7,8 and TRAIL mRNA expression in leucocytes has been proposed as a biomarker for the therapeutic response to IFNβ in patients with MS.9 Nevertheless, none of these studies has taken into account the different splicing variants of each gene, which could lead to the expression of non-functional proteins. Three different splice variants have been discovered for TRAIL: TRAIL-α, the functional isoform; TRAIL-β, which lacks exon 3; and TRAIL-γ, which lacks exons 2 and 3. The loss of exon 3 leads to the lack of the extracellular domain and, therefore, to the absence of apoptotic potential.5

TRAILR-2 has two different apoptotic variants: TRAILR-2 isoform 1 (TRICK2b), which has an 87 nucleotides insertion and TRAILR-2 isoform 2 (TRICK2a), which is homologous to TRAILR-1.10 Finally, there are two different splice variants for TRAILR-4: TRAILR-4 α, the long isoform, and TRAILR-4 β, which lacks exon 3.11 The loss of this exon leads to the truncation of the first cysteine rich domain 1, involved in the ligand-receptor complex, which may alter its ability to bind TRAIL.

In this study, we monitored the expression kinetics of TRAIL and its receptors after induction with IFNβ. Although kinetics of TRAIL expression have previously been reported,12 it remains unknown whether TRAIL receptors follow the same pattern.

The aim of the present work was to assess the effects of IFNβ treatment on the expression of the splice variants of TRAIL and its receptors in different cell subpopulations from patients with MS, and to determine whether these expressions discriminated responders from non-responders to IFNβ therapy.

METHODS

Subjects
Forty-two Spanish patients with MS prone to start IFNβ treatment were recruited from the Multiple Sclerosis Unit at Malaga Regional University Hospital (Malaga, Spain) and followed for a minimum of 2 years of treatment.

Study inclusion criteria included definite MS according to the McDonald criteria,13 and treatment-naive at baseline for at least 6 months.


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Once they started therapy with IFNβ, patients had to be on a stable dose for at least 24 months. Blood samples were obtained during remissions before the first IFNβ administration (baseline) and again after 12–14 months (serum and peripheral blood mononuclear cells (PBMC)) and 24 months (serum) on that therapy.

As controls (HC), twenty age-matched healthy unrelated subjects were selected.

Samples were processed following standard procedures and frozen immediately after they were received by the Málaga Regional Hospital Biobank, as part of Andalusian Public Health System Biobank. All individuals participating in the study gave their informed consent and protocols were approved by an institutional ethical committee (Comisión de Ética y de Investigación del Hospital Regional Universitario Carlos Haya).

Two patients initially taking part in the study were excluded due to the presence of high and permanent titres of neutralising antibodies (NABs) against IFNβ. The demographic and clinical variables of the remaining 40 patients with MS and HC are listed in table 1.

**Definition of response to IFNβ therapy**

Patients were classified as optimal responders if they had neither relapses within the previous year nor progression of the disease over the same follow-up period, and as non-responders if they experienced one or more relapses or an increase of at least one point in the Expanded Disability Status Scale (EDSS) that persisted for a minimum of two consecutive visits separated by a 6-month interval.14

**Detection of Neutralising Antibodies NABs**

The presence of NABs in serum was checked after 12 and 24 months of treatment by the IFN-induced inhibition of virus cytopathic effect on human cells in culture, following the WHO test (clinical course and type of IFN). The neutralisation titre of serum samples was calculated according to Kawade et al.16 Titres ≥20 TRU/mL were considered as positive.

**Determination of expression kinetics in the Jurkat T-cell leukaemia cell line**

Owing to the limited yield of PBMC obtained from each patient, gene expression kinetics were carried out in an immortalised human T lymphoblastoid cell line. Jurkat cells (Clone E.6, American Type Culture Collection) were cultured in 12-well plates, at $1.5 \times 10^6$ cells/mL, in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1.3% Hepes, 50 μg/mL gentamicin and 2 mM L-glutamine. After incubation at 37°C and 5% CO₂ for 24 h, cells were washed and incubated for 2 h with the former media without FBS. Cells for pre-stimulation condition (3 replicates) were collected in 1 mL of Tripure Isolation Reagent (Roche Diagnostic GmbH) and the remaining cells were stimulated with 200 UI/mL of IFNβ-1a (Avonex, Biogen Idec) and collected in the same way at 4, 8, 12 and 24 h after IFNβ induction.

**Gene expression in patients with MS**

Blood samples were taken before the first IFNβ administration and after 1 year of treatment. PBMC were isolated by a Ficoll-Hypaque density gradient. Monocytes (CD14+), CD4+ T cells and CD8+ T cells were isolated using immunomagnetic microbeads and LS MACS columns (Miltenyi Biotec GmbH), following the manufacturer’s protocol. Purity of each cellular subset (95–97%) was assessed by flow cytometry. After isolation, the cellular subsets were cultured in 96 well plates with medium without FBS. Cells were induced with 200 UI/mL of IFNβ-1a and collected in Tripure at 4 and 24 h after induction.

**RNA isolation, primer design and quantitative reverse transcription PCR**

Total RNA was isolated from Jurkat cells and PBMC using a modification of the phenol–chloroform method. Total RNA yield and quality of product was assessed with a Nanodrop 2000 Spectrophotometer.

For complementary DNA (cDNA) synthesis, 1 μg of total RNA was reverse transcribed with the M-MLV reverse transcriptase as described elsewhere.18 The cDNA was stored at −80°C until use.

Primers for all amplified products are shown in table 2. Primers for TRAILR-1 and TRAILR-3 were designed using Primer 3 software.19 Primers for TRAIL, TRAILR-2 and TRAILR-4 were custom designed to make them complementary to the specific exon boundaries of each splice variant.

Conventional PCR with temperatures ranging from 57°C to 63°C were performed to ensure specificity of primer pairs and to determine the optimal annealing temperature. Annealing at

### Table 1 Demographic and clinical characteristics of the participants at baseline

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy controls</th>
<th>Responders</th>
<th>Non-responders</th>
<th>p Values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=20</td>
<td>n=24</td>
<td>n=16</td>
<td></td>
</tr>
<tr>
<td>Female/male ratio (% women)</td>
<td>1/6 (70)</td>
<td>17/7 (70.8)</td>
<td>12/4 (75)</td>
<td>0.867</td>
</tr>
<tr>
<td>Age at baseline (years)</td>
<td>34.0 (31.7–43.5)</td>
<td>34.5 (28.5–45.7)</td>
<td>34.0 (29.2–46.0)</td>
<td>0.950</td>
</tr>
<tr>
<td>Duration of disease</td>
<td>4.0 (2.0–7.7)</td>
<td>5.0 (3.2–6.7)</td>
<td>0.774</td>
<td></td>
</tr>
<tr>
<td>RR/SP ratio (% RR)</td>
<td>2/22 (91.7)</td>
<td>15/1 (93.8)</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>EDSS at baseline</td>
<td>1.0 (1.0–1.37)</td>
<td>1.25 (1.0–2.87)</td>
<td>0.101</td>
<td></td>
</tr>
<tr>
<td>Number of relapses in the 2 previous years</td>
<td>1.0 (1.0–2.0)</td>
<td>2.0 (1.0–2.0)</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td>Type of IFNβ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intramuscular IFNβ-1a (%)</td>
<td>4 (16.7)</td>
<td>1 (6.2)</td>
<td>0.422</td>
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<tr>
<td>Subcutaneous IFNβ-1a (%)</td>
<td>19 (79.2)</td>
<td>13 (81.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous IFNβ-1b (%)</td>
<td>1 (4.1)</td>
<td>2 (12.5)</td>
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<td></td>
</tr>
</tbody>
</table>

*Quantitative data are presented as median (IQR).

*p Values: Refers to p values obtained following comparisons between healthy controls, responders and non-responders by means of a Kruskal-Wallis test (age) and χ² test (gender), as well as p values obtained following comparisons between responders and non-responders by means of a Mann-Whitney U test (duration, EDSS and number of relapses) and χ² or Fisher test (clinical course and type of IFNβ).

EDSS, Expanded Disability Status Scale; IFN, interferon; RR, relapsing–remitting; SP, secondary progressive; TRAIL, Tumour necrosis factor-Related Apoptosis Inducing Ligand.
Table 2 Primers used for amplification of the splice variants of TRAIL and TRAIL receptors

<table>
<thead>
<tr>
<th>Product name</th>
<th>Primer</th>
<th>Sequence 5’→3’</th>
<th>Location</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL-α</td>
<td>TRAIL-fwd1</td>
<td>TITCAGTTCAGAAGAAGGC</td>
<td>Exons 3–4 joint</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>TRAIL-rev1</td>
<td>GCCAGAGGTTCTTATCTC</td>
<td>Exons 4–5 joint</td>
<td>195</td>
</tr>
<tr>
<td>TRAIL-β</td>
<td>TRAIL-fwd2</td>
<td>AGCTGCTGAAAGAAGAGG</td>
<td>Exons 2–4 joint</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>TRAIL-rev2</td>
<td>GGTCCTGCCAGGCTGCAA</td>
<td>Exon</td>
<td>145</td>
</tr>
<tr>
<td>TRAIL-γ</td>
<td>TRAIL-fwd3</td>
<td>ACGGTTGGAAGCAGAAAAGC</td>
<td>Exons 1–4 joint</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>TRAIL-rev1</td>
<td>GCAGACGACCTTCTTCTATCT</td>
<td>Exons 4–5 joint</td>
<td>145</td>
</tr>
<tr>
<td>TRAILR-1</td>
<td>TRAILR-fwd1</td>
<td>ACCAGGAAACACAGGATGTC</td>
<td>Exon</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>TRAILR-rev1</td>
<td>GCAGACGACCTTCTTCTATCT</td>
<td>Exons 4–5 joint</td>
<td>145</td>
</tr>
<tr>
<td>TRAILR-2 isoform 1</td>
<td>TRAILR-fwd2</td>
<td>CAGTGGCAAGAAGGGCACTT</td>
<td>Exon 4</td>
<td>145</td>
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<tr>
<td></td>
<td>TRAILR2-rev1</td>
<td>CCCCAGTTCTTGTGACCT</td>
<td>Exons 5</td>
<td>108</td>
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<td>TRAILR-2 isoform 2</td>
<td>TRAILR-fwd2</td>
<td>CAGTGGCAAGAAGGGCACTT</td>
<td>Exon 4</td>
<td>145</td>
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<td></td>
<td>TRAILR2-rev2</td>
<td>GGATGCTGATCTTTTG</td>
<td>Exon</td>
<td>108</td>
</tr>
<tr>
<td>TRAILR-3</td>
<td>TRAILR3-fwd</td>
<td>TGCAGACGAGGGTGTTGATTA</td>
<td>Exon 3</td>
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</tr>
<tr>
<td></td>
<td>TRAILR3-rev</td>
<td>GTGCTAGTGTTGAGAAGTCTT</td>
<td>Exon 3</td>
<td>108</td>
</tr>
<tr>
<td>TRAILR-4 α</td>
<td>TRAILR4-fwd1</td>
<td>CAATTTGCTCTTCCTGTGC</td>
<td>Exons 3</td>
<td>164</td>
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<td></td>
<td>TRAILR4-rev</td>
<td>CTCGAGAAGCTTGCTTCTA</td>
<td>Exons 4–5 joint</td>
<td>145</td>
</tr>
<tr>
<td>TRAILR-4 β</td>
<td>TRAILR4-fwd2</td>
<td>GAGGTGTCAGCGGTCAA</td>
<td>Exons 2–4 joint</td>
<td>137</td>
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<tr>
<td></td>
<td>TRAILR4-rev</td>
<td>CTCGAGAAGCTTGCTTCTA</td>
<td>Exons 4–5 joint</td>
<td>145</td>
</tr>
<tr>
<td>GADPH</td>
<td>GADPH-fwd</td>
<td>GAAGATTTGAAGGGATTC</td>
<td>Exon</td>
<td>225</td>
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<tr>
<td></td>
<td>GADPH-rev</td>
<td>GAAGATTTGAAGGGATTC</td>
<td>Exon</td>
<td>225</td>
</tr>
</tbody>
</table>

TRAIL, Tumour necrosis factor-Related Apoptosis Inducing Ligand.

57°C was optimal for TRAILR-1 and TRAIL variants, at 60°C for GAPDH and at 58°C for the remaining genes.

Quantitative PCR was performed in duplicate in a Rotor Gene Q Thermocycler (Qiagen GmbH) in a 20 μl reaction mix containing DEPC-treated water, 20 mM primers (forward and reverse), Quantitect SYBR Green PCR Master Mix (Qiagen) and cDNA. The programme consisted of a step of 15 min at 95°C, followed by 40 cycles of 95°C for 30 s, 57°C or 60°C for 30 s and 72°C for 30 s. A melting step from 65°C to 95°C was run, increasing 0.5°C every 5 s.

The relative TRAIL and TRAIL receptor variants messenger RNA (mRNA) expression levels were calculated according to the 2−ΔΔCt method, first by normalising to GAPDH and then to a calibrator sample.

Statistical analysis

Comparisons of demographic characteristics at baseline between HC, responder and non-respondent patients with MS were performed by Kruskal-Wallis test (age) and χ² test (gender). Comparisons of clinical characteristics at baseline between responders and non-responders were performed by means of a Mann-Whitney U test (duration, EDSS and number of relapses) and χ² or Fisher test (clinical course and type of IFNβ).

A Wilcoxon test was used to compare relative expression between pretreatment samples and samples from patients after 1 year of treatment with IFNβ.

A Mann-Whitney U test was used to compare relative expression of TRAIL and its receptors between responders and suboptimal responders to IFNβ therapy.

Receiver operating characteristic (ROC) analyses were performed to evaluate the predictive value of gene expression before treatment onset on therapeutic response to IFNβ.

RESULTS

Gene expression kinetics of TRAIL and TRAIL receptors in Jurkat cells

The expression kinetics of each gene after in vitro induction with IFNβ was determined in Jurkat cells (figure 1). The three

TRAIL variants showed a similar expression pattern: gene expression was upregulated during 1–4 h after IFNβ stimulation (peak at 4 h), and declined thereafter, followed by a second increment after 24 h, in agreement with previous data.12

TRAILR-1 and TRAILR-2 shared the same expression pattern, characterised by a decreased expression in the first hours, and a peak 24 h after IFNβ induction. The decoy receptors also began to be induced at 8–12 h post-stimulation with IFNβ, showing a progressive increment with a marked peak at 24 h. Thus, in PBMC, the expression of TRAIL and its receptors was assessed at 4 and 24 h after in vitro stimulation with IFNβ, respectively.

Gene expression of TRAIL and TRAIL receptors in untreated patients with MS and HC

On in vitro stimulation with IFNβ, mRNA expression of TRAIL-α was significantly increased in HC compared to untreated patients with MS in the three cellular subsets (p=0.023 in monocytes, p=0.00004 in CD4+ T cells and p= 0.021 in CD8+ T cells), as observed in figure 2. We detected no other significant differences in the expression of TRAIL and receptor variants at the RNA level.

Basal gene expression of TRAIL and TRAIL receptors in patients with MS who will subsequently become responders or non-responders to IFNβ therapy

Within the patient group, prior to IFNβ treatment onset, we found higher mRNA expression of TRAIL-α in monocytes, CD4+ and CD8+ T cells from patients who will subsequently be classified as non-responders than from responders (p=0.008, p=0.00006 and 0.003, respectively), as shown in figure 3. Conversely, the mRNA expression of the TRAIL-β isoform was significantly higher in CD4+ and CD8+ T cells from patients who will become responders (p=0.03 and p=0.025), and showed the same trend of expression in monocytes (p=0.052). Additionally, expression of TRAILR-1 was also significantly higher in monocytes and CD4+ T cells from patients who will become responders.
In the search for biomarkers to predict the therapeutic response to IFNβ, ROC analyses were performed to evaluate the predictive discriminating value of TRAIL-α, TRAIL-β and TRAILR-1 expression on in vitro induction with IFNβ before treatment onset, on different cell subsets from patients with MS.

Values of areas under the curve (AUC), sensitivity, specificity, positive predictive value and negative predictive value are shown in table 3. Expression of TRAIL-α in the three cellular subsets better predicted those patients who will subsequently become non-responders to treatment, with AUC >0.7 and sensitivities greater than 80% in the three cases. Conversely, expression of TRAILR-1 on monocytes and CD4+ T cells better predicted those patients who will subsequently become responders to treatment, with AUC >0.8, sensitivities greater than 90% and specificities greater than 75%. TRAIL-β expression did not seem a good biomarker to predict therapeutic response to IFNβ, as AUCs were under 0.7.

Effects of long-term IFNβ therapy on TRAIL and TRAIL receptors expression in PBMC from patients with MS

Relative expression of TRAIL and TRAIL receptors was significantly modified along the first year of therapy only for two genes. Expression of the isoform 2 of the death receptor TRAILR-2 was significantly reduced in monocytes from first-year responder as well as non-responder patients (p=0.004 and 0.016, respectively). Expression of TRAIL-α was significantly increased in both T cell subsets but exclusively in patients who showed a clinical response to systemic IFNβ treatment (p=0.004 in CD4+ cells and p=0.002 in CD8+ cells from responders). Induction of TRAIL-α mRNA expression in monocytes by IFNβ therapy was achieved in 13 of 24 responders and, therefore, overall expression in this subset was not significantly increased (figure 4).

DISCUSSION

TRAIL expression in PBMC has been proposed as a biomarker for therapeutic response to IFNβ in MS,5 but different splice variants were not taken into account. Several diseases have been related to alternative splice variant or aberrant splicing.20 We hypothesise that alternative splice variants could be implicated in response to treatment and, thus, they should be measured independently.

In our study, we first determined the expression kinetics of TRAIL and its receptors’ isoforms after in vitro stimulation with IFNβ. In agreement with the existing literature, TRAIL isoforms reached a peak 4 h after IFNβ treatment.12 Conversely, TRAIL receptor isoforms begin to upregulate at 8 h and reach a maximum at 24 h. TRAILR-2 isoforms begin to be induced at the 12 h time point and beyond. Expression levels are represented as relative expression compared with the reference gene GAPDH, using the ΔΔCt method. Mean and SD of each time point for each gene isoform is represented.
assess this issue. It remains unknown whether in vivo gene expression of TRAIL receptors after induction with IFN-β shares the same pattern as in vitro, or whether they go through a more complex regulation. We are currently performing a longitudinal study, using blood samples drawn at different time points after injection with IFN-β, to address this question.

Contrary to previous studies, we found that the expression of TRAIL-α was significantly lower in monocytes and

Figure 3 Relative expression of Tumour necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) α, TRAIL-β and TRAILR-1, on in vitro stimulation with interferon β (IFN-β), before treatment onset. Those patients who will become non-responders (NR) to IFN-β therapy showed higher expression of TRAIL-α mRNA in the three cellular subsets, while those who will become responders (R) showed higher baseline expression of TRAIL-β and TRAILR-1. Expression level is represented as relative expression compared with the reference gene GAPDH, using the ΔΔCt method. Figures show box plots: the horizontal bars are the median and lower and upper edges of the boxes represent the 25–75th centiles. Lines extending from the box are 10th and 90th centiles.

Table 3 Sensitivity and specificity of biomarkers to predict the therapeutic response to IFN-β

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cell type</th>
<th>AUC</th>
<th>Cut-off</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL-α</td>
<td>Monocytes</td>
<td>0.736</td>
<td>0.81</td>
<td>88.2</td>
<td>70</td>
<td>62.5</td>
<td>91.3</td>
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<tr>
<td></td>
<td>CD4+ T cells</td>
<td>0.794</td>
<td>0.12</td>
<td>83.3</td>
<td>65.2</td>
<td>55.5</td>
<td>88.2</td>
</tr>
<tr>
<td></td>
<td>CD8+ T cells</td>
<td>0.733</td>
<td>0.055</td>
<td>83.3</td>
<td>61.8</td>
<td>60.6</td>
<td>87.5</td>
</tr>
<tr>
<td>TRAIL-β</td>
<td>CD4+ T cells</td>
<td>0.662</td>
<td>0.095</td>
<td>77.3</td>
<td>47.8</td>
<td>73.9</td>
<td>52.4</td>
</tr>
<tr>
<td></td>
<td>CD8+ T cell</td>
<td>0.683</td>
<td>0.115</td>
<td>71.1</td>
<td>52.6</td>
<td>75</td>
<td>47.6</td>
</tr>
<tr>
<td>TRAILR-1</td>
<td>Monocytes</td>
<td>0.803</td>
<td>0.67</td>
<td>90.5</td>
<td>78.6</td>
<td>79.2</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>CD4+ T cells</td>
<td>0.833</td>
<td>0.29</td>
<td>90</td>
<td>75</td>
<td>85.7</td>
<td>81.8</td>
</tr>
</tbody>
</table>

AUC, area under the curve; IFN, interferon; NPV, negative predictive value; PPV, positive predictive value; TRAIL, Tumour necrosis factor-Related Apoptosis Inducing Ligand.
T cells from untreated patients with MS than from controls. In agreement, Kurne et al. found lower soluble TRAIL concentrations in patients with MS than in HC.23 One possible explanation for this finding may be the lower levels of endogenous IFNβ reported in patients with MS, 24 which are unable to induce TRAIL synthesis to the same extent as they can in healthy subjects.

Cell type-specific induction of TRAIL by IFNβ in patients with MS has been reported. We have shown upregulation of TRAIL-α expression after IFNβ therapy in monocytes, and in CD4+ and CD8+ T lymphocytes. Earlier works found increased levels of TRAIL after IFNβ-1a injection only on the surfaces of monocytes and/or granulocytes.6825 However, upregulation of TRAIL expression in lymphocytes7267 and T cell lines28 from patients with MS and HC has also been reported. These differences could be attributed to the different culture conditions, kinetics and activation stimulus of the cells.

Within the patient group, those patients with MS who will be subsequently considered as responders to IFNβ therapy could be clearly distinguished from non-responders by baseline expression of TRAIL-α and TRAILR-1, indicating underlying differences in transcriptional regulation of these genes. The in vitro addition of IFNβ to previously IFNβ naïve cell subsets triggered a higher expression of TRAIL-α and a lower expression of TRAIL-β in monocytes and T cells from patients who will continue to experience relapses or disease progression while treated with IFNβ, and a higher expression of TRAILR-1 in monocytes and CD4+ T cells from those who will have neither relapses nor progression of the disease. Wândinger et al.25 reported no differences in baseline expression of TRAIL regarding IFNβ responsiveness, but found higher baseline concentrations of soluble TRAIL in responders, findings that could not be replicated by others.29 Conversely, another study found higher levels of TRAIL in monocytes from relapsing–remitting quiescent disease patients (responders).8 Comparison among studies regarding biomarkers for therapeutic response to IFNβ in MS is difficult, due to the unique design and experimental approach of each single study.

It is known that TRAIL is upregulated on activation of monocytes2430 and T cells,2829 while TRAILR-1 and TRAILR-2, in turn, are downregulated on the RNA level when T cells are activated.28 In our ROC analysis, baseline TRAIL-α expression in the three cellular subsets at the cut-off levels described in table 3 predicts suboptimal response with a sensitivity greater than 80% and a specificity around 65%, while baseline TRAILR-1 expression over 0.803 in monocytes and over 0.883 in CD4+ T cells predicts a good response to IFNβ therapy with a sensitivity higher than 90% and a specificity over 75%, promoting the idea of using the expression of TRAIL-α and TRAILR-1 as biomarkers to predict IFNβ treatment response. Probably, the changes in baseline expression of TRAIL-α of such a low magnitude in patients that will become non-responders may not be able to induce the expected changes after a significant increase in TRAIL, such as a strong inhibition of activation of autoreactive T cells, promotion of Tregs, or induction of neurons and oligodendrocytes apoptosis after lymphocyte infiltration.31 They may be more probably reflecting a compensatory mechanism to prevent, in part, the activation of autoreactive T cells in those untreated

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**Figure 4** Effects of long-term treatment with interferon β (IFNβ) on Tumour necrosis factor-Related Apoptosis Inducing Ligand TRAILR-2 isoform 2 and TRAIL-α expression. Expression levels are represented as relative expression compared with the reference gene GAPDH, using the ΔΔCt method. Expression before and after 1 year of IFNβ therapy in the same individual patients is plotted. (A) Long-term therapy with IFNβ significantly decreased mRNA expression of TRAILR-2 isoform 2 in monocytes from patients who will become responders (R) as well as those who will become non-responders (NR) to treatment. (B) TRAIL-α mRNA expression in CD4+ and CD8+ T cells significantly increased along with IFNβ therapy, but only in responders.
patients with an increased state of activation who will continue to experience relapses or progression. Another explanation for this increase in TRAIL-α expression at baseline in non-responders may be attributed to a comparatively higher pretreatment serum concentration of endogenous IFNβ in non-responders than responders, as shown by Axtell et al.

Long-term treatment with IFNβ has been reported to increase TRAIL mRNA expression. In our study, IFNβ therapy significantly increases TRAIL-α expression in both T cell subsets, but exclusively in responder patients, similar to results reported by others, reaching levels of expression that overcome those found in HC. In this sense, upregulation of TRAIL in responders contributes to the deletion of autoreactive lymphocytes, the inhibition of activation of autoreactive lymphocytes and limitation of the production of proinflammatory cytokines, mechanisms that may contribute to the clinical benefit of IFNβ therapy. Moreover, TRAIL-α promotes the induction of endogenous IFNβ, amplifying its effects. Although TRAIL-α is also known to induce apoptosis of oligodendrocytes and neurons, as IFNβ enhances the integrity of the blood–brain barrier (BBB) in responders, the upregulation of TRAIL induced by IFNβ therapy probably comprises immunoregulatory mechanisms outside the central nervous system (CNS) more than apoptosis of brain cells.

However, although non-responders had higher basal TRAIL-α levels than responders, they showed an inability to increase TRAIL-α expression in T cells in response to IFNβ therapy, that is, these patients are already responding to endogenous IFNβ, probably at maximum level, but still show a disease activity that obtains no benefit from further administration of IFNβ. This inability to increase TRAIL-α expression could, on one hand, lead to a failure in the apoptosis of auto-aggressive T cells invading the CNS and, on the other hand, prevent inhibition of the activation of autoreactive T cells contributing to the perpetuation of the inflammatory response and the appearance of relapses and CNS damage.

Moreover, we found that long-term IFNβ therapy decreased the expression of TRAILR-2 isoform 2 in monocytes from patients with MS, regardless of the therapeutic responsiveness to this drug. Expression of TRAILR-2 is constitutive in peripheral blood leucocytes and may be involved in the apoptosis of activated lymphocytes and monocytes. In later stages of tissue pathology, macrophages outnumber T cells in CNS infiltrates and are needed to execute myelin phagocytosis in the demyelinating process. In this sense, downregulation of TRAILR-2 isoform 2 in monocytes could be one of the mechanisms responsible for the clinical benefits of IFNβ, as apoptosis of peripheral monocytes will be prevented, allowing these cells to differentiate into macrophages, cross the BBB and exert their functions.

**CONCLUSIONS**

The present study shows that long-term therapy with IFNβ increased the expression of TRAIL-α in both T cell subsets exclusively from responder patients and decreased the expression of the isoform 2 of the death receptor TRAILR-2 in monocytes from responders as well as non-responders.

ROC analysis revealed that expression of TRAIL-α in the three cellular subsets, before the onset of IFNβ therapy, better predicted those patients who will subsequently become non-responders to treatment while expression of TRAILR-1 on monocytes and CD4+ T cells better predicted those patients who will become responders.

Further studies are necessary in order to elucidate the pathways implicated in this process, which could reveal the precise mechanism by which IFNβ is exerting its beneficial effects on patients with MS, but TRAIL-α and TRAILR-1 should be assessed as predictors in future IFNβ responsiveness studies. However, inconsistent results among different studies regarding therapeutic biomarkers to IFNβ in MS make us evaluate our results with caution. To prove the effectiveness of these predictive markers, studies with larger sample sizes are warranted.

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**Contributors** CL-G, BO-M, M-JP-M and MS performed the cellular subsets isolation, the gene expression analysis and the Nabs test. VR-G, PU and OF recruited the patients, evaluated the response to therapy and participated in the analyses. CL-G and LL designed the study, evaluated the results and wrote the manuscript. OF revised it critically for important intellectual content.

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**Competing interests** OF has received honoraria as consultant in advisory boards and as chairman or lecturer in meetings, and has also participated in clinical trials and other research projects promoted by Biogen-Idec, Bayer-Schering, Merck-Serono, Teva, Novartis, Almirall and Allergan.

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Multiple sclerosis


TRAIL and TRAIL receptors splice variants during long-term interferon β treatment of patients with multiple sclerosis: evaluation as biomarkers for therapeutic response

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