SAGE-Hindawi Access to Research
Autoimmune Diseases
Volume 2011, Article ID 485752, 8 pages
doi:10.4061/2011/485752

Research Article

Effects of IFN-B on TRAIL and Decoy Receptor Expression in Different Immune Cell Populations from MS Patients with Distinct Disease Subtypes

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Received 23 August 2010; Accepted 16 November 2010

Academic Editor: David Dyment

Using quantitative RT-PCR, we compared mRNA levels for TRAIL [tumor necrosis factor (TNF)–related apoptosis-inducing ligand] and its receptors in various immune cell subsets derived from the peripheral blood of untreated normal subjects (NS) and patients with distinct subtypes of multiple sclerosis (MS): active relapsing-remitting MS (RRA), quiescent relapsing-remitting MS (RRQ), secondary-progressive MS (SPMS) or primary-progressive MS (PPMS). Consistent with a role for TRAIL in the mechanism of action of interferon-β (IFN-β), TRAIL mRNA levels were increased in monocytes from patients clinically responsive to IFN-β (RRQ) but not those unresponsive to this therapeutic (RRA). TRAIL-R3 (decoy receptor) expression was elevated in T cells from untreated RRMS patients while IFN-β therapy reversed this increase suggesting that IFN-β may promote the apoptotic elimination of autoreactive T cells by increasing the amount of TRAIL available to activate TRAIL death receptors. Serum concentrations of soluble TRAIL were increased to a similar extent by IFN-β therapy in RRQ, RRA and SPMS patients that had not generated neutralizing antibodies against this cytokine. Although our findings suggest altered TRAIL signaling may play a role in MS pathogenesis and IFN-β therapy, they do not support use of TRAIL as a surrogate marker for clinical responsiveness to this therapeutic.

1. Introduction

Multiple sclerosis (MS) is a chronic neurodegenerative autoimmune disorder characterized by CNS inflammation, demyelination, and axonal injury resulting in clinical relapses and disability [1–3]. MS is considered to be a T cell-mediated disease [4, 5] in which failed apoptotic deletion of autoreactive T cells has been implicated as a pathogenic mechanism [6, 7]. Apoptosis plays an important role in immune system homeostasis by eliminating autoreactive immune cells that might otherwise promote autoimmunity [8]. Tumor necrosis factor- (TNF-)related apoptosis-inducing ligand (TRAIL) plays a key regulatory function in this regard by activating death receptors present on various cellular components of the immune system such as T cells, B cells, and monocytes [9]. As a result, a number of immune cell subtypes have been implicated in autoimmunity subsequent to the loss of TRAIL function [9]. Although $CD4^+$ T cells specific for myelin antigens are thought to initiate and exacerbate MS through secretion of proinflammatory cytokines, peripheral blood monocytes may also contribute to this disease by migrating to the CNS and releasing inflammatory mediators that trigger nerve and tissue damage [1, 2, 10–12]. In the case of B lymphocytes, three lines of evidence suggest these immune cells are involved in MS pathogenesis: increased myelin-specific antibodies, presence of B cells reactive against myelin, and the ability of the anti-CD20 antibody Rituximab to deplete B cells and reduce relapses and disease burden as assessed by MRI [11–14].

TRAIL, also known as Apo2 ligand (Apo2L), is a member of the TNF superfamily that shares 24% amino acid homology with the death receptor CD95 (Fas/ApoL) ligand [15]. TRAIL and CD95L can promote the apoptotic death of a number of cancer cells [15]. Despite TRAIL mRNA being

present in a wide variety of tissue types, most normal cells are resistant to TRAIL cytotoxicity [15]. CNS inflammation in MS is associated with elevated expression of TRAIL, both within the CNS and autoreactive immune cells [16–18]. TRAIL inhibits activated T cell proliferation through intricate interactions with various receptors for this cytokine [19]. The initial TRAIL receptor identified, death receptor 4 (DR4 or TRAIL-R1), transmits proapoptotic signals via a cytoplasmic death domain. DR5 or TRAIL-R2 also contains a DR4-like death domain that conveys apoptotic signaling [15]. TRAIL-R3 and TRAIL-R4 lack the cytoplasmic tails found in TRAIL-R1 and TRAIL-R2 necessary to trigger apoptosis and therefore act as decoy receptors [15]. These decoy receptors prevent TRAIL-induced apoptosis and represent an important mechanism for regulating the apoptotic sensitivity of immune cells. The selective expression of decoy receptors in normal tissues has led to the proposal that TRAIL may be useful for preferentially inducing the apoptosis of cancer cells [15].

TRAIL has been implicated in both MS pathogenesis and the mechanism of action of interferon-beta (IFN-β), a disease modifying therapy that has been used to treat MS for over twenty years [19–21]. Recombinant IFN-β therapy is typically employed for the treatment of relapsing-remitting MS (RRMS). Although the precise mechanism(s) responsible for the beneficial effects of IFN-β in the treatment of MS remain unclear, the abilities of this cytokine to inhibit T-cell activation and proliferation as well as facilitate the apoptotic elimination of autoreactive T cells are thought to be therapeutically relevant [22]. TRAIL/Apo2L-deficient mice subjected to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE) display increased T-cell proliferative responses, more inflammatory lesions in the spinal cord and brain, and elevated clinical scores relative to wild-type littermates, while peripheral administration of recombinant TRAIL reduces EAE severity [23]. Moreover, IFN-β increases circulating levels of soluble TRAIL (sTRAIL) and the expression of membrane-bound TRAIL (mbTRAIL) in immune cells derived from the peripheral blood of MS patients [21, 24]. TRAIL may therefore contribute to the mechanism of action of IFN-β by promoting the apoptosis of autoreactive immune cells in MS patients. IFN-β is not curative but reduces disease progression as evidenced by decreased frequency and severity of relapses. However, some patients are unresponsive to IFN-β therapy and continue to experience relapses and disease progression while treated with this therapeutic [25]. The reasons why some patients respond to IFN-β therapy while others do not benefit remain unclear. The present study therefore sought to determine whether the expression of TRAIL and/or its receptors in peripheral blood immune cells from treated and nontreated MS patients discriminated IFN-β responders from IFN-β nonresponders.

2. Materials and Methods

2.1. Patient Selection and Blood Sampling. One hundred ninety-nine participants were recruited for this study including participants with RRMS ($n = 100$), SPMS ($n = 38$), and PPMS ($n = 30$) as well as thirty-one healthy control subjects from the community, matched for age and gender. Study inclusion criteria included definite MS (RRMS, SPMS, PPMS) according to consensus definitions, 18 years of age or older, and if being treated with IFN-β, patients were on a stable dose for 6 months having had a dose within 8–16 hours prior to blood draw. Several disease modifying therapies (DMTs) have been approved for relapse-onset MS (RRMS and SPMS with superimposed relapses). First line agents, include three different preparations of interferon-β (IFN-β) (IFN-β1a or Avonex, IFN-β1b or Betaseron, and IFN-β1 or Rebif) and glatiramer-acetate (Copaxone). Natilizumab (Tysabri) and mitoxantrone (Novantrone) are considered second line therapies and used for treatment of more aggressive forms of relapse-onset MS [26–28]. For the purpose of this study, RRMS patients were assigned into "active" and "quiescent" groups based on their clinical condition at the time of blood collection. Relapsing-remitting active (RRA) disease was defined in patients that had experienced one or more clinical relapse(s) or had Gd+ enhancing lesion(s) on MRI in the previous year ($n = 41$). Relapsing-remitting quiescent (RRQ) disease was defined in patients that had neither relapses within the previous year nor EDSS progression over the same time period ($n = 59$). These clinical definitions of active and quiescent MS are supported by differences in the expression of members of the Inhibitor of Apoptosis family in peripheral blood mononuclear cells and T cells derived from such patients [29].

Both MS patients and control subjects had an absence of other major medical illness including cancer and autoimmune disease. Study exclusion criteria for all subjects included treatment with immunosuppressive therapy or treatment with intravenous methylprednisolone within 3 months of study participation. All participants provided written informed consent prior to study participation according to the Declaration of Helsinki. All participants meeting inclusion/exclusion criteria provided a blood sample totaling 38 mL into four 8 mL BD Vacutainer sodium citrate Ficoll gradient tubes, one serum tube, and/or one Paxgene (Qiagen) whole blood RNA tube.

2.2. Cell Purification, RNA Extraction, and TRAIL Quantification. Whole blood RNA was isolated using the PAXgene whole blood RNA kit as per manufacturer's instructions and as previously described [29]. For the isolation of highly purified T cells, B cells or monocytes, 400 μL of RosetteSepT, RosetteSepB, or RosetteSep monocytes (Stem Cell Technologies, B.C.), respectively, was added to three separate Ficoll-gradient tubes (Becton, Dickinson and Company; BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate). All tubes (T cells, B cells, monocytes) were centrifuged at 1650–1800 $\times$ g for 25 minutes in a swinging bucket centrifuge. The T cells, B cells, or monocytes were transferred to separate 15 ml conical centrifugation tubes, treated with ammonium chloride (StemCell Technologies, Vancouver, B.C.) to remove any remaining red blood cells, and centrifuged to pellet the cells. RNA was extracted from cell pellets using the RNeasy kit (Qiagen). FACS analyses were performed to confirm the purity (95–98%) of immune cell populations. Total RNA

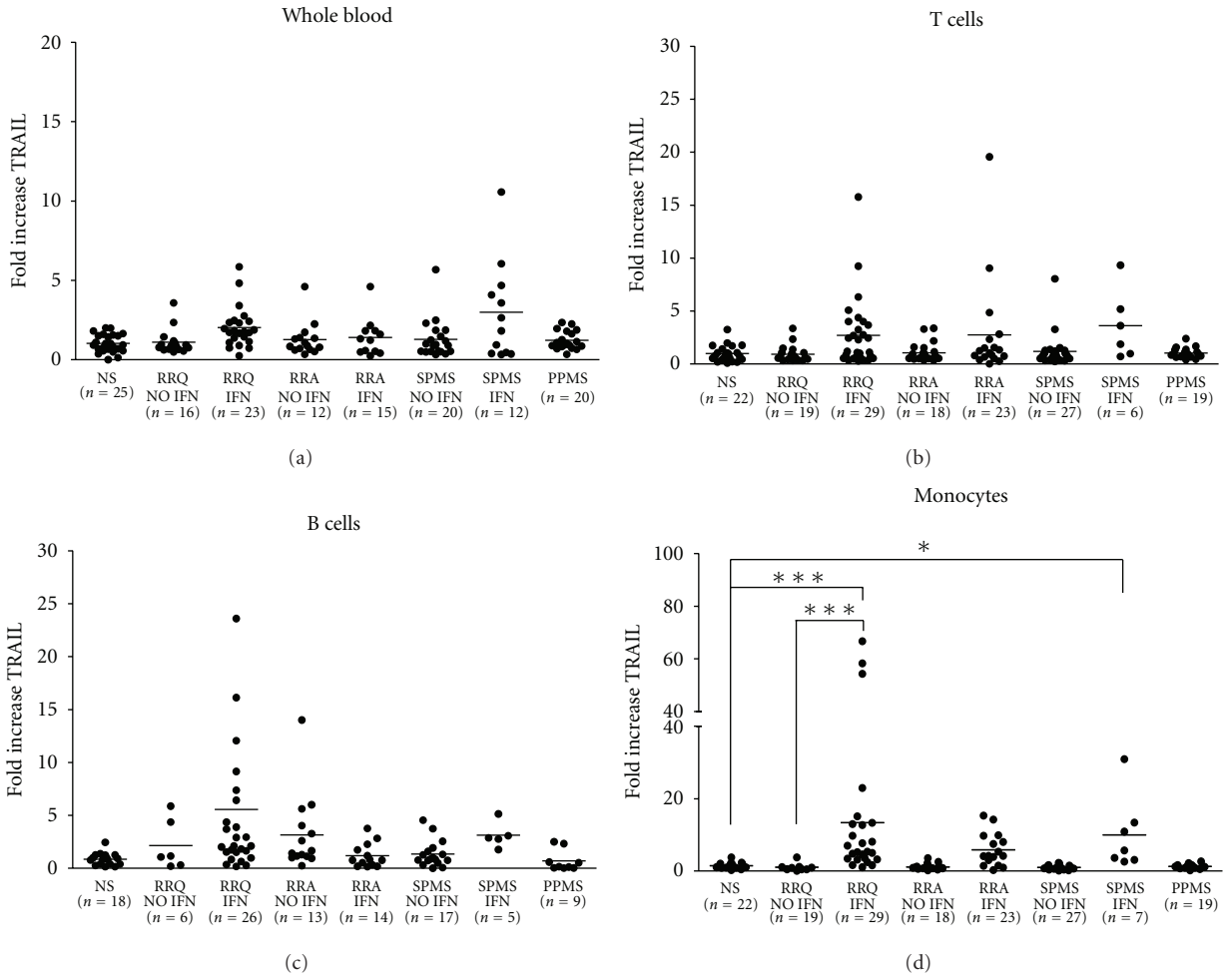

Figure 1: Relative quantification of TRAIL mRNA expression in RNA extracted from whole blood, T cells, B cells, and monocytes employing qRT-PCR. (a) In whole blood, TRAIL gene expression was no different between the various groups**.** (b) In T cells, TRAIL gene expression was also no different among NS and the various patient groups. (c) In B cells, TRAIL gene expression was the same in the various groups examined. (d) In monocytes, TRAIL gene expression was increased in RRQ IFN and SPMS IFN patients relative to NS. TRAIL mRNA was also elevated in RRQ IFN relative to RRQ NO IFN. $^{*}P < .05$,$^{**}P < .01$ and $^{***}P < .001$, Dunn's post hoc test.

yields were measured by ultraviolet (UV) absorbance, and RNA samples were diluted in RNAse free water to give a final concentration of 10 ng/μL.

2.3. Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed to confirm changes in TRAIL, TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (TRAIL decoy receptor), and TRAIL-R4 (TRAIL decoy receptor) mRNA levels in different subtypes of untreated and treated MS patients relative to normal control subjects. Data were first stratified according to MS disease subtype activity and IFN-β treatment. If statistical comparisons between these groups were not significant, data were pooled and analyzed according to overall disease classification (RRMS, SPMS, PPMS). Total RNA (50 ng) was reverse transcribed to yield first-strand cDNA and amplified using the Taqman one-step EZ RT-PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA). The expression of beta (β) actin was used as an endogenous control reference (Applied Biosystems, Foster City, CA, USA). Primers for TRAIL and its receptors were made according to previously published sequences [30–32] (see Table 1). Although the intent was to compare TRAIL profiles amongst all samples collected, in some cases this was precluded due to decreased RNA yields. Results were expressed in Fold change relative to a "calibrator." The calibrator sample was the RNA sample from a normal age-matched female subject [33].

2.4. ELISA. Blood was collected in a 3 ml serum separator tube, allowed to clot at RT for 1 hour and centrifuged at 1000 × g for 20 minutes. Serum was transferred to two 1.5 ml eppendorf tubes and stored at −80°C until analyses. Enzyme linked-immunosorbent assay (ELISA) measured soluble TRAIL as per manufacturer's instructions (R&D systems, Minneapolis, MN USA). Serum (50 μl in duplicate) from normal healthy control subjects (NS, $n = 34$), RRMS and SPMS patients treated with IFN-β (RRMS IFN, $n = 41$; SPMS IFN, $n = 16$), RRMS and SPMS patients not

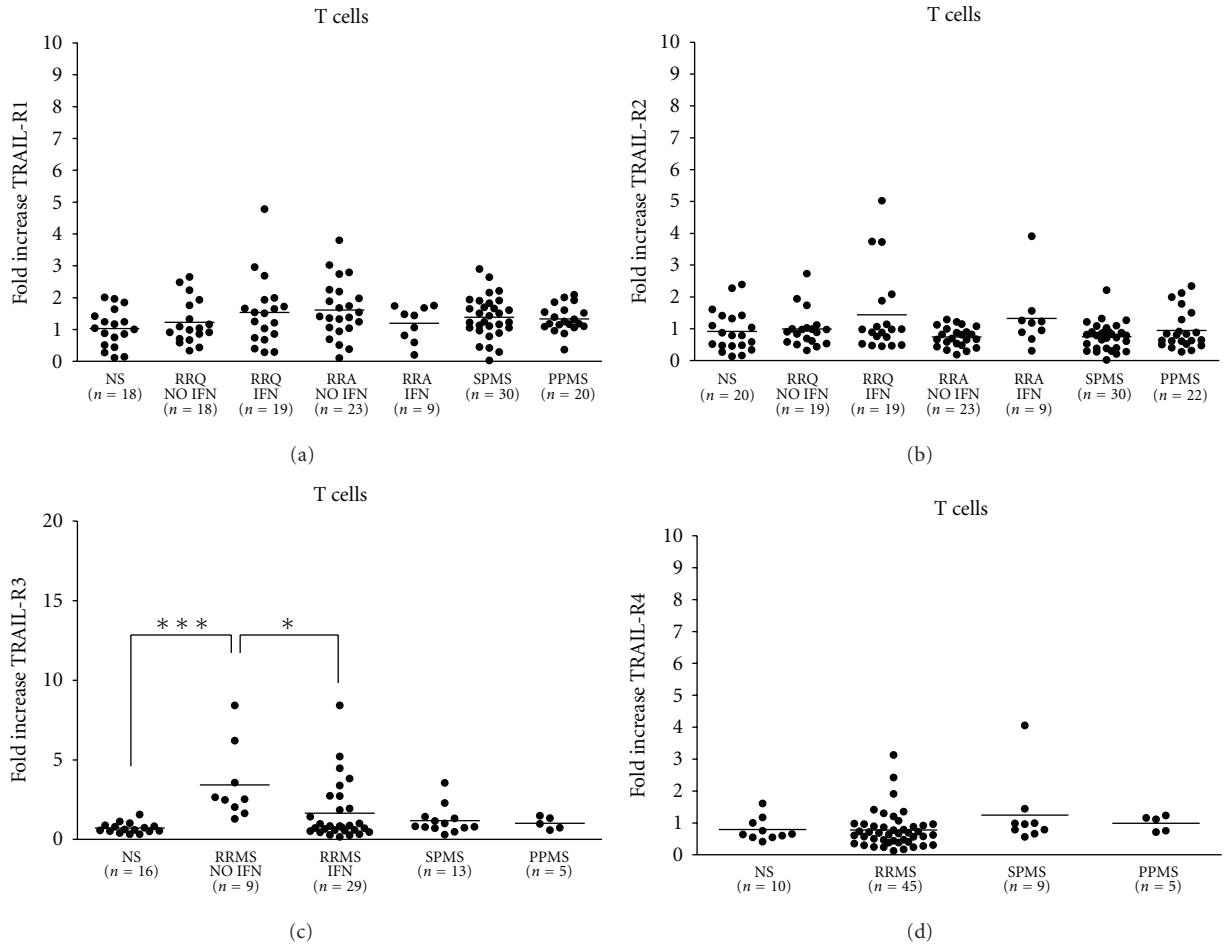

Figure 2: Relative quantification of TRAIL death (TRAIL-R1, TRAIL-R2) and decoy receptor (TRAIL-R3, TRAIL-R4) mRNAs in T cells. (a) In T cells, there were no differences in gene expression of TRAIL-R1 and (b) TRAIL-R2. (c) TRAIL-R3 gene expression was increased in RRMS NO IFN patients relative to the NS group while administration of IFN-β reversed this increase in RRMS patients (RRMS IFN). $^{*}P < .05$ and $^{**}P < .01$, Dunn's posthoc test.

Table 1: Human Taqman Probe and Primer Sequences. Taqman probe and primer sequences for TRAIL and its receptors (R) have been previously published.

Human gene	Human primer and probe sequences
TRAIL	Forward 5′-GCTCTGGGCCGCAAAAT-3′
	Reverse 5′-TGCAAGTTGCTCAGGAATGAA-3′
	Probe 5′-(FAM)ACTCCTGGGAATCATCAAGGAGTGGGC(TAMRA)-3′
TRAIL-R1	Forward 5′-TGTACGCCCTGGAGTGACAT-3′
	Reverse 5′-CACCAACAGCAACGGAACAA-3′
	Probe 5′-(FAM)TGTCCACAAAGAATCAGGCAATGGACATAAT(TAMRA)-3′
TRAIL-R2	Forward 5′-CACTCACTGGAATGACCTCCTTT-3′
	Reverse 5′-GTGCAGGGACTTAGCTCCACTT-3′
	Probe 5′-(FAM)TCACACCTGGTGCAGCGCAAGCAG(TAMRA)-3′
TRAIL-R3	Forward 5′-TCTCCACGCGCACGAAC-3′
	Reverse 5′-CCCCTTGCATCTCTGGTCAA-3′
	Probe 5′-(FAM)CAGCCAACGATTTCTGATAGATTTTTGGGAG(TAMRA)-3′
TRAIL-R4	Forward 5′-TTGGCGCTTTCGATCCAC-3′
	Reverse 5′-CGGTCGGGACGCTTTGT-3′
	Probe 5′-(FAM)CTCCTCCCTTCTCATGGGACTTTGGG(TAMRA)-3′

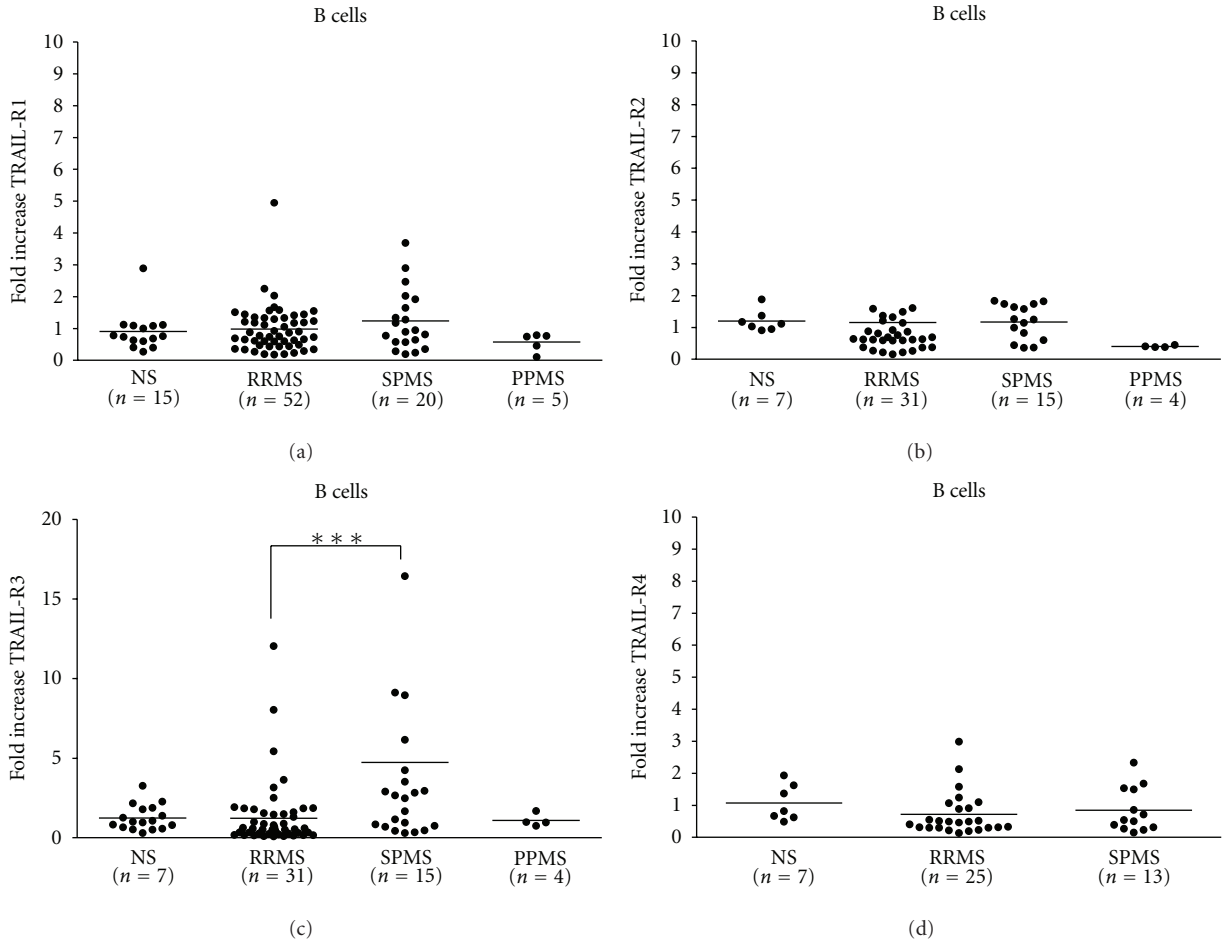

Figure 3: Relative quantification of TRAIL death (TRAIL-R1, TRAIL-R2) and decoy receptor (TRAIL-R3, TRAIL-R4) mRNAs in B cells. (a) TRAIL-R1, (b) TRAIL-R3 and (d) TRAIL-R4 mRNA levels were not altered by IFN-β treatment or different between the various groups (NS group and MS subtypes). Insufficient amounts of B cell RNA were isolated to permit analysis of TRAIL-R4 levels for PPMS patients. (c) TRAIL-R3 gene expression was increased in SPMS patients irrespective of IFN-β treatment condition (SPMS NO IFN and SPMS IFN groups not different and therefore pooled) relative to the RRMS group. $^*P < .05$, Dunn's post-hoc test.

treated with IFN-β (RRMS NO IFN, $n = 32$; SPMS NO IFN, $n = 12$), and PPMS ($n = 23$) were analyzed for soluble TRAIL (sTRAIL) using an ELISA with a monoclonal antibody specific for human TRAIL according to manufacturer's instructions (R&D Systems, Minneapolis, MN). All measurements were performed in duplicate.

2.5. Statistical Analyses. A nonparametric test (Kruskal-Wallis test) was used to compare potential differences in expression of mRNAs encoding TRAIL, TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 in whole blood, T cells, B cells and monocytes derived from the peripheral whole blood of the various groups (NS and patients with subtypes of MS that were either treated or not treated with IFN-β). If significant at an alpha of $P \leq .01$, Dunn's posttest was used to determine whether differences existed between individual groups. The Mann Whitney U test was used to compare soluble TRAIL protein levels in blood serum from NAB(−) and NAB(+) MS patients. Statistical tests were performed using GraphPad Prism version 4 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com/.

3. Results

3.1. IFN-β Increases TRAIL MRNA in Monocytes from RRQ and SPMS Patients. Using RNA derived from whole blood, T cells or B cells, no differences in TRAIL gene expression were observed between the different subtypes of IFN-β-treated and untreated MS patient populations (Figures 1(a)–1(c)). In peripheral monocytes, however, a significant increase in TRAIL gene expression was observed in both RRQ and SPMS patients treated with IFN-β relative to normal subjects (NS) (Figure 1(d)). Relative to untreated RRQ patients (RRQ NO IFN), a significant increase in TRAIL gene expression was also observed in IFN-β-treated RRQ patients (RRQ IFN).

3.2. TRAIL-R3 mRNA Is Elevated in T Cells from RRMS and This Increase Is Reversed by IFN-β Treatment. In T

cells, no differences in either TRAIL-R1 or TRAIL-R2 were observed between the various groups (Figures 2(a) and 2(b)). There was, however, a significant increase in TRAIL-R3 mRNA in T cells from untreated RRMS patients relative to NS (Figure 2(c)). Interestingly, IFN-β-treatment in RRMS patients reversed this increase in TRAIL-R3 mRNA such that expression levels of this gene were significantly decreased in T cells from IFN-β-treated RRMS patients relative to untreated RRMS patients. No differences in TRAIL-R3 mRNA were observed in whole blood or monocytes (data not shown). Quantitative RT-PCR analysis for TRAIL-R4 revealed no differences across disease subtypes in either T cells (Figure 2(d)), monocytes, or whole blood (data not shown).

3.3. TRAIL-R3 mRNA Is Increased in B Cells from SPMS Relative to RRMS Patients. In B cells, no differences in TRAIL-R1, TRAIL-R2, or TRAIL-R4 were observed (Figures 3(a), 3(b), and 3(d)). A significant increase in TRAIL-R3 mRNA expression was observed in SPMS patients relative to RRMS patients (Figure 3(c)). No effects of IFN-β treatment were noted in this analysis (data not shown).

3.4. IFN-β Increases Soluble TRAIL in Serum Regardless of MS Subtype or Disease Activity. The range of soluble TRAIL (sTRAIL) in serum was between 21.6 and 157.2 pg/mL (Figure 4(a)) for the NS group. In the three patient populations treated with IFN-β, (RRQ IFN, RRA IFN, and SPMS IFN), serum concentrations of sTRAIL were significantly increased relative to NS (Figure 4(a)). While not indicated on Figure 4(a), statistical significance ($P < .001$) was also observed between the RRQ NO IFN and RRQ IFN groups as well as the RRA NO IFN and RRA IFN groups. These findings confirm our earlier qRT-PCR results demonstrating that IFN-β-treated patients had elevated TRAIL mRNA in peripheral monocytes suggesting that a major source of sTRAIL is from this particular cell type. When patients were grouped according to the presence of neutralizing antibodies against IFN-β, a significant decrease in sTRAIL was observed in the NAB(+) relative to NAB(−) patients (Figure 4(b)).

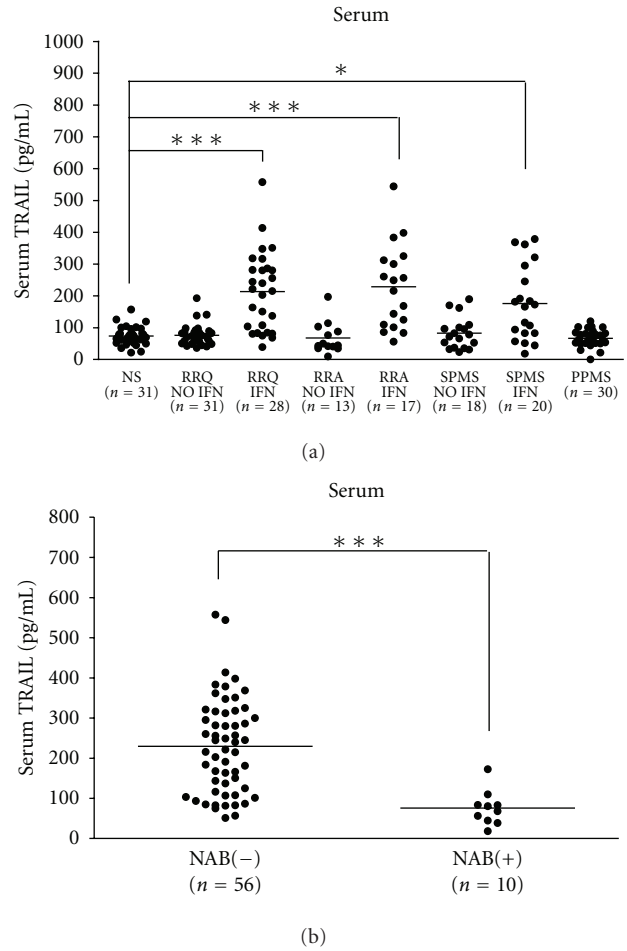

Figure 4: Absolute quantification of soluble TRAIL protein in peripheral blood serum. (a) Levels of soluble TRAIL protein were increased in serum from IFN-β treated-patients irrespective of subgroup (RRMS, SPMS) or disease activity (RRQ, RRA) relative to NS and untreated MS patients. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$, Dunn's post-hoc test. (b) Ten patients (RRQ IFN $n = 4$; RRA IFN $n = 3$; SPMS $n = 3$) that were NAB(+) showed attenuated sTRAIL levels relative to IFN-β NAB(−) MS patients. $^{***}P < .001$, Mann Whitney U test.

4. Discussion

In this retrospective study, we first compared levels of TRAIL mRNA in whole blood, T cells, B cells, and monocytes isolated from the peripheral blood of healthy normal subjects (NS) and MS patients treated with various preparations of IFN-β. Only in monocytes were significant differences in TRAIL gene expression detected between NS and MS patients that received IFN-β. Elevated TRAIL gene expression associated with IFN-β therapy was correlated with clinical responsiveness to this therapeutic in RRMS patients as suggested by higher levels of monocytic TRAIL mRNA in RRQ, but not RRA, patients relative to the NS group. However, TRAIL mRNA levels were not different between RRQ and RRA patients that received IFN-β, indicating that clinical responsiveness to this therapeutic cannot be distinguished purely on this basis. This finding was both supported and extended by our measurements of soluble TRAIL levels in peripheral blood serum. In this case, IFN-β treatment produced a comparable elevation of soluble TRAIL levels in both RRQ and RRA patients relative to the NS group. Furthermore, in patients that were NAB+, the ability of IFN-β to elevate soluble TRAIL levels was lost. Thus, while TRAIL induction appears to depend upon the availability of biologically active IFN-β, blood levels of this death receptor ligand did not distinguish RRA from RRA patients. These results are in agreement with findings from a recent prospective study that reported levels of soluble TRAIL in blood plasma failed to predict clinical responsiveness of RRMS patients to IFN-β [34].

TRAIL-R3 encodes a decoy receptor that suppresses the apoptotic activity of TRAIL [35]. In the present study, we found that expression of TRAIL-R3, but not that for

the death-inducing receptors TRAIL-1 and TRAIL-2 or the second TRAIL decoy receptor known as TRAIL-R4, is to be elevated in T cells from RRMS patients. TRAIL-R3 expression has also been found to be selectively elevated in T cell lines derived from MS patients compared to those from healthy donors [36]. Moreover, we observed in RRMS patients treated with IFN-β that expression of TRAIL-R3 was reduced to levels similar to that detected in healthy controls. We speculate that by reducing TRAIL-3 levels on T cells, IFN-β may enhance the proapoptotic and antiproliferative effects of TRAIL on these immune cells. In keeping with this line of reasoning, we also observed TRAIL-R3 expression in B cells from SPMS patients to be enhanced relative to RRMS patients that may reflect immune mechanisms which could conceivably contribute to disease progression. To the best of our knowledge, this is the first demonstration that TRAIL-R3 may be involved in both the mechanism of action of IFN-β as well as MS disease progression.

Acknowledgments

This work was supported by grants from the Capital Health Research Authority (Capital Health Research Fund), MS Society of Canada, and Dalhousie Medical Research Foundation (Margot Spafford). Postdoctoral fellowship and studentship awards from the MS Society of Canada supported ALOH and CSM, respectively. The authors thank Loretta Stefura, R.N., Trudy Campbell, R.N., Michael Hebb, M.D., and Susan Greek, R.N. for coordination of clinical studies and expert blood collection. The authors also thank Michelle Warhuus, Martine St. Jean, Kay Murphy, and Elizabeth Belland for excellent technical assistance.

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