Published in final edited form as:

Clin Immunol. 2009 October ; 133(1): 27–44. doi:10.1016/j.clim.2009.05.019.

Interferon- β treatment in multiple sclerosis attenuates inflammatory gene expression through inducible activity of the phosphatase SHP-1

George P. Christophi^{1,2}, Michael Panos¹, Chad A. Hudson^{1,2}, Chriso Tsikkou¹, Cornelia Mihai¹, Luis J. Mejico¹, Burk Jubelt^{1,2}, and Paul T. Massa^{1,2,*}

¹ Department of Neurology, SUNY Upstate Medical University, Syracuse NY, USA

² Department of Microbiology & Immunology, SUNY Upstate Medical University, Syracuse NY, USA

Abstract

Interferon- β is a current treatment for multiple sclerosis (MS). Interferon- β is thought to exert its therapeutic effects on MS by down-modulating the immune response by multiple potential pathways. Here, we document that treatment of MS patients with interferon β -1a (Rebif) results in a significant increase in the levels and function of the protein tyrosine phosphatase SHP-1 in PBMCs. SHP-1 is a crucial negative regulator of cytokine signaling, inflammatory gene expression, and CNS demyelination as evidenced in mice deficient in SHP-1. In order to examine the functional significance of SHP-1 induction in MS PBMCs, we analyzed the activity of proinflammatory signaling molecules STAT1, STAT6, and NF- κ B, which are known SHP-1 targets. Interferon- β treatment *in vivo* resulted in decreased NF- κ B and STAT6 activation and increased STAT1 activation. Further analysis *in vitro* showed that cultured PBMCs of MS patients and normal subjects had a significant SHP-1 induction following interferon- β treatment that correlated with decreased NF- κ B and STAT6 activation. Most importantly, experimental depletion of SHP-1 in cultured PBMCs abolished the anti-inflammatory effects of interferon- β treatment, indicating that SHP-1 is a predominant mediator of interferon- β activity. In conclusion, interferon- β treatment upregulates SHP-1 expression resulting in decreased transcription factor activation and inflammatory gene expression important in MS pathogenesis.

Keywords

CNS Demyelination; Autoimmunity; Inflammation; Cytokine Signaling; Signal Transducers and Activators of Transcription STAT; nuclear factor kappa-B (NF- κ B); peripheral immune cells; Interferon-beta therapy; Protein Tyrosine Phosphatase; EAE/TMEV

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that remains a major cause of disability [1;2]. Interferon- β is a current effective treatment

*Corresponding Author: Mailing Address: Department of Neurology, Upstate Medical University, State University of New York, 750 East Adams Street, Syracuse, NY 13210, Phone Number: (315) 464-7606, Fax: (315) 464-6402, massap@upstate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

for relapsing remitting (RR) MS that reduces the frequency of clinical exacerbations and delays the progression of disability [3;4;5]. IFN- β is a pleiotropic cytokine with diverse and cell-specific mechanisms of action including potent anti-viral, immune-stimulating, and immunosuppressive activities [6;7].

Regarding the beneficial role of IFN- β treatment in MS, numerous studies described the role of IFN- β in modulating the immune response [3;8], including downregulation of class II major histocompatibility complex (MHC) molecules [9] and limiting migration of immune cells across the blood brain barrier possibly through the downregulation of inflammatory genes like endothelial adhesion molecules, chemokines, and proteases. In addition, several studies have demonstrated the effect of IFN- β to mitigate the increased blood levels of T cell-derived cytokines IFN- γ , IL-4/IL13, and TNF- α in MS compared to normal subjects [10;11;12;13;14;15;16;17], which otherwise act to increase proinflammatory transcription factors STAT1, STAT6, and NF- κ B observed in MS patients [9;18;19;20;21;22;23]. Inasmuch as IFN- β treatment of MS patients was shown to partially modulate these inflammatory cytokine levels [24;25], the ability of IFN- β to additionally moderate downstream cytokine signaling in target cells is also apparent yet mechanistically unclear [26;27;28;29;30].

Apart from cytokine levels, inflammatory cytokine signaling is modulated by several intracellular negative regulatory proteins, including phosphatases that dampen inflammatory signaling by removing phosphate groups from activated cytokine receptors and transcription factors [31]. Importantly, we have shown that in mice IFN- β treatment induces the expression of the phosphatase SHP-1 both in CNS and immune cells by transcriptional pathways [32]. SHP-1 is a protein tyrosine phosphatase with two SH2 domains which acts as a negative regulator of both innate and acquired immune cytokine signaling via STAT1 [33;34], STAT6 [35;36;37;38] and NF- κ B [39;40;41].

Mice genetically lacking SHP-1 (motheaten mice, *me/me*) are highly susceptible to experimentally-induced demyelinating disease, which may be mediated by increased innate inflammatory mediators in the CNS [42;43;44]. In agreement with these studies, lower levels of SHP-1 have also been implicated in autoimmune-mediated demyelination in rodents [45;46] and in lymphoproliferative diseases in humans [47;48;49;50]. Taken together, these studies indicate that SHP-1 is a key regulator of inflammation that may be relevant to the pathogenesis of MS. Indeed, we have recently reported that SHP-1 expression and function is deficient in leukocytes of MS patients compared to normal human subjects [22;51].

Two distinct promoters are responsible for expression of each of the two known SHP-1 transcripts produced from the SHP-1 gene [52]. These distinct transcripts in turn encode two slightly different SHP-1 isoforms, which have the same catalytic activity [53]. It was previously shown that the two SHP-1 transcripts are differentially expressed in human tissues and cell lines [54;55]. Promoter I transcripts are highly expressed in epithelial cells, while promoter II transcripts are more abundant in hematopoietic cells [55;56]. Several reports show differential regulation of the SHP-1 gene promoters by distinct transcription factors [52;57;58] and we have recently shown that in mice induction of promoter I and promoter II SHP-1 transcripts by IFN- β occurs via IRF-1 and STAT3 transcriptional activity, respectively [59]. These findings are in agreement with studies showing that both IRF-1 and STAT3 are critical for mediating important biological functions of interferons via interferon-inducible genes. Interestingly, promoter-specific regulation of SHP-1 expression has been associated with human disease [47;56;60] and we have shown that SHP-1 promoter II mRNA is specifically deficient in PBMCs of MS patients compared to normal subjects [22].

In this study, we characterize the expression and functions of the phosphatase SHP-1 in PBMCs from multiple sclerosis patients following IFN- β treatment. We demonstrate that a three-month

in vivo treatment of RR MS patients with interferon β -1a (Rebif) results in a significant increase in the levels of SHP-1 protein and mRNA, which coincides with decreased activation of STAT6 and NF- κ B and responsive inflammatory genes. Similarly, *in vitro* treatment of cultured PBMCs of MS patients with IFN- β resulted in increased SHP-1 levels and attenuated signaling via IL-4 and TNF- α pathways. Finally, the ability of IFN- β treatment to down-regulate cytokine signaling and inflammatory gene expression was abolished following experimental depletion of SHP-1 in PBMCs of MS patients. Taken together, these results illustrate that the induction of the phosphatase SHP-1 following IFN- β treatment plays an important role in attenuating the inflammatory immune response in multiple sclerosis by a novel and previously uncharacterized pathway.

MATERIALS AND METHODS

Patient selection

Patients were clinically diagnosed as having definite MS [61]. Patients were clinically diagnosed with either relapsing-remitting (RR) or secondary progressive (SP) MS [62]. All patients selected had not received any disease modifying treatment like IFN- β , glatiramer acetate, steroids, or other immunosuppressive agents at least two months prior to donating blood. For the *in vivo* study, the RR MS gave blood before and after a three-month treatment with recombinant interferon β -1a (Rebif) [63]. For the *in vitro* study, cultured PBMCs of untreated MS patients and normal subjects were treated with recombinant interferon β -1a for 24 hours. Table I provides additional information of the patients and normal subjects used in this study. The Institutional Review Board of SUNY Upstate University approved all studies and both patients and normal controls granted informed consent before providing blood.

PBMC isolation and expansion

Patients and normal subjects donated 60 ml of blood collected in heparinized tubes. Blood was diluted 1:1 with HBSS and overlaid onto lymphocyte separation medium (Cellgro, Herndon, VA). After centrifugation, the plasma was collected and used to quantify cytokine levels, while the 10 ml of the interface containing the PBMCs were collected and washed twice with HBSS. For the *in vivo* studies several samples of the freshly isolated cells were either resuspended in STAT- 60 (Tel-Test, Friendswood, TX) for RNA analysis, RIPA buffer [64] for protein analysis, or fixed for intracellular flow analysis. For the *in vitro* studies, the rest of the PBMCs were cultured for a week in RPMI media with 20 units/ml IL-2 (R & D Systems), and 10% fetal bovine serum. After one week, cells were treated with cytokines and analyzed as outlined in the text.

Cytokine and siRNA Treatment

For the *in vitro* studies, PBMCs of MS patients and normal subjects were cultured for 1 week and pretreated with either 100 U/mL of recombinant human interferon β -1a (PBL, Piscataway, NJ) or control medium for 24 hours and then treated with either 10 ng/mL TNF- α , 10 ng/mL of IL-4, 100 U/mL IFN- γ , or received medium alone (R&D Systems, Minneapolis, MN). A portion of PBMCs were transfected with siRNA against human SHP-1 or scramble siRNA (Dharmacon, Chicago, IL) at a concentration of $1\mu\text{g}/10^6$ cells. The transfection reagent (Dharmafect 4, Dharmacon, Chicago, IL) was used as specified by the manufacturer. Cells were incubated in the transfection media for 24 hours, after which the medium was replaced with complete growth medium for another 48 hr before cytokine treatment. The effectiveness of the SHP-1 siRNA to lower SHP-1 expression was evaluated by real-time RT-PCR, western immunoblot, and flow cytometry.

Real-Time RT-PCR

Total RNA was isolated using RNA STAT-60. RNA was quantified spectrophotometrically and 0.5 µg of total RNA was converted into cDNA. Briefly, total RNA and random primers (Invitrogen, Carlsbad, CA) were incubated at 72 degrees for 10 minutes. Reverse transcription was performed using the Superscript II RT enzyme (Invitrogen, Carlsbad, CA) and followed the specification of the manufacturer. cDNA was diluted to 200 µL with water and 4 µL was used for quantitative real time PCR using SYBR Green kit (Abgene, Epsom, UK). The PCR parameters were 15 minutes for 95 degrees and 35 cycles of 95 degrees for 15 seconds and 60 degrees for 1 minute in ABI Prism 700 (Applied Biosystems, Foster City, CA). The primers were used at 10 nM. Serial dilutions of cDNA containing a known copy number of each gene were used in each quantitative PCR run to generate a standard curve relating copy number with threshold amplification cycle [59]. Gene expression levels were calculated during the logarithmic amplification phase by determining the initial mRNA copy number using the standard curve. Amplification of each gene specific fragment was confirmed both by examination of melting peaks and by agarose gel electrophoresis. The following primer (Forward and Reverse) pairs were used in this study: SHP-1 TGGCGTGGCAGGAGAACAG and GCAGTTGGTCACAGAGTAGGGC, SHP-1 (I) TGGCTTCCCCCTCCCTACAG and CCCTGGTTCCTTGCGACTGG, SHP-1 (II) ATCTGAGGCTTAGTCCCTGAGC and CTGAGGTCTCGGTGAAACCAC, IL-4 α CGTGTATCCCTGAGAACAACG and CGTGTATCCCTGAGAACAACG, TARC CGAGGGACCAATGTGGGC and GGGTGAGGAGGCTTCAAGACC, Arginase I GACCTGCCCTTTGCTGACATC and TTGACTTCTGCCACCTTGCC, ADAM8 ATCCCGAGAGACCCGCTAC and TGATTCACCACCTCCAGCAC, IP-10 TTCAAGGAGTACCTCTCTCTAG and TGTGATTACAGACATCTCTTCTC, Caspase I CATCCTCAGGCTCAGAAGG and TGTGCGGCTTGACTTGTC, COX-2 GCATCTACGGTTTGCTGTG and ACTGCTCATCACCCATTC, CCR4 GGTCTGTTCAAATACAAGCG and AAACCCACTGGTCTGCTGC, CXCR3 TCTGCCTTTTGGGTCTTGTAATA and AGGAAGATGAAGTCTGGGAG, GAPDH ACCACCATGGAGAAGGC and GGCATGGACTGTGGTCATGA.

Cytokine ELISA

The levels of the cytokines IFN- γ , IL-4, IL-13, TNF- α and IL-6 were measured using R&D Systems DuoSet ELISA kits (R&D Systems) following the manufacturer's protocol.

Western Blotting

Whole cell extracts were prepared as previously described [34;39]. Briefly, PBMCs were lysed with RIPA buffer. 20 µg of protein per lane was electrophoresed through a 7.5% polyacrylamide resolving gel and electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Burlington, MA). Membranes were blocked with 5% nonfat dry milk for 1 hour, and then incubated with anti-SHP-1 (Upstate, Lake Placid, NY) antibodies followed by horseradish peroxidase conjugated rabbit IgG antibody (DAKO Corporation, Carpinteria, CA). Blots were subsequently stripped and reprobed with anti-arginase I (BD Biosciences, San Diego, CA) followed by horseradish peroxidase conjugated mouse IgG antibody. Enhanced chemiluminescence (Amersham Life Sciences, Inc., Cleveland, OH) was used to visualize reactive protein bands on X-ray film.

Flow cytometry

The intracellular pSTAT1, pSTAT6 levels were quantified by flow cytometric analysis. PBMCs were treated for 1 hour with 10 ng/mL IL-4 or 100 U/mL IFN- γ . 10^6 cells/mL received 100 µL of 16% stock formaldehyde for fixation and then incubated in 90% methanol at 4°C for half an hour to permeabilize cells for intracellular staining. Cells were washed twice with the

staining media containing 0.5% BSA and 0.02% sodium azide in PBS. Cells were resuspended in 100 μ L of staining media and incubated with 20 μ L of either phosphoSTAT6 (PY-641)-Alexa-488 (612600), pSTAT1 (pY701)-Alexa-647 (612597), or normal mouse IgG-Alexa-488 isotype control (557721) (Becton Dickinson, Mountain View, CA). The intracellular SHP-1 levels were also quantified. Fixed and permeabilized cells were incubated overnight at 4°C with either 1 μ g of rabbit anti-SHP-1 (Upstate, Lake Placid, NY), or normal rabbit polyclonal IgGs (Dako) for the antibody control. After multiple rinses, the cells were incubated for 3 hours in 1 μ g of goat anti-rabbit secondary antibody conjugated to PE (Invitrogen, Carlsbad, CA). Cells were analyzed on an LSRII analyzer (Becton Dickinson, Mountain View, CA) and the mean fluorescence intensity (MFI) was recorded.

PBMC sorting analysis

Cultured PBMCs were sorted into the different cell populations (CD4⁺ and CD8⁺ T cells, B-cells, and NK cells) in order to examine the expression of SHP-1 in individual lymphocyte populations. Cells (2×10^6 /100 μ L) were incubated on ice for 30 minutes with 10 μ L of CD3-FITC (349201), CD4-PE (555347), CD8-APC (555369), CD19-Alexa 488 (340720), or a combination of CD3⁻CD16⁺/CD56⁺ (340042) to stain Natural Killer (NK) cells (Becton Dickinson, Mountain View, CA). Cells were washed and resuspended in 1 mL of Hank's solution and were analyzed by fluorescence-activated cell sorter (FACS Vantage S/E; Becton Dickinson Immunocytometry Systems, Mountain View, CA). The percentage of each cell population was determined and different aliquots labeled CD3⁺CD4⁺ (T-helper cells), CD3⁺CD8⁺ (cytotoxic T-cells), CD19⁺ (B cells), and CD3⁻CD16⁺/CD56⁺ (NK cells) were collected. Total RNA was isolated and quantified from the cells of each aliquot and the SHP-1 mRNA expression was determined.

NF- κ B DNA-binding activity assay

NF- κ B DNA binding activity was analyzed using the TransAMNF-B p65 transcription factor assay kit (Active Motif) following the manufacturer's instructions and as previously described [65;66;67]. Briefly, nuclear extracts were prepared [39] from PBMCs of normal subjects and MS patients that were pretreated with media alone or 10 ng of TNF- α for 1 hour. 2 μ g of nuclear protein was incubated in a 96-well plate coated with oligonucleotide containing the NF- κ B consensus-binding sequence 5'-GGGACTTTCC-3'. Bound NF- κ B was then detected by a p65-specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification at 450 nm. To quantify the amount of NF- κ B, serial dilutions of purified p65 recombinant protein (20 ng - 0.16 ng) were measured to provide a calibration curve between p65 binding and absorbance. The specificity of the assay was further tested by the addition of wild type or mutated NF- κ B consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of nuclear extracts. The addition of the wild-type NF- κ B consensus oligonucleotide completely abolished NF- κ B binding.

Arginase Activity Assay

Arginase enzymatic activity was measured as previously described [68]. Briefly, PBMCs were lysed in RIPA buffer and incubated in 10 mM MnCl₂ and 0.5 M L-arginine at 37°C for 75 min. After the assay was stopped by addition of H₃PO₄, 1-phenyl-1,2-propanedione-2-oxime (Sigma, St Louis, MO) was added and the samples were incubated at 100°C for 60 min. Urea production by arginase was measured by optical density at 540 nm.

Statistical Analysis

Histograms contain statistical means with the standard error values. The number of MS patients and normal subjects used in each experiment are specified in the figure legends. The p-values

were generated using the unpaired Student's t-test and a p-value of less than 0.05 was chosen to indicate statistical significance between two sample means.

RESULTS

***In vivo* IFN- β treatment induces SHP-1 in PBMCs of MS patients**

We have previously shown that IFN- β induces the expression of the phosphatase SHP-1 both in mouse CNS and immune cells by transcriptional pathways [32]. Therefore it became important to examine whether interferon- β , a current effective treatment for MS patients, upregulates SHP-1. RR MS patients who were treated with IFN- β -1a (Rebif) for three months donated blood and the protein and mRNA levels of SHP-1 were quantified in freshly isolated PBMCs before and after treatment. SHP-1 protein expression in freshly isolated PBMCs from normal subjects, untreated RR MS patients and IFN- β -treated RR MS patients was quantified by intracellular flow cytometry and western immunoblot analysis (Figure 1A–C). As previously described, PBMCs of MS patients contained significantly lower levels of SHP-1 protein compared to normal subjects [22]. Importantly, three-month treatment with IFN- β -1a resulted in a substantial and significant increase in SHP-1 protein expression in PBMCs of MS patients compared to PBMC of the same patients before treatment.

Furthermore, SHP-1 mRNA in freshly isolated PBMCs from normal subjects, RR MS patients and IFN- β -treated RR MS patients was quantified by real-time RT-PCR. First, SHP-1 common mRNA transcripts (total SHP-1 mRNA) were quantified using primers flanking the coding region of the gene common to both promoter I and promoter II transcripts (Figure 1D). SHP-1 mRNA levels in freshly isolated PBMCs were significantly lower in MS patients compared to normal subjects and *in vivo* IFN- β treatment resulted in a significant increase in SHP-1 mRNA expression. In order to determine the individual contribution of each of the two transcripts on the expression levels of SHP-1 in PBMCs, promoter I and II transcript copy numbers were measured using promoter-specific RT-PCR primers (Figure 1F & G). *In vivo* IFN- β treatment resulted a significant 4-fold increase in SHP-1 promoter II mRNA levels in the PBMCs of MS patients, but only a modest but insignificant increase in promoter I mRNA levels. Analysis of protein and mRNA of a representative housekeeping gene showed no differences either between MS and normal subject PBMCs or in response to IFN- β . In summary, these data demonstrate that three-month treatment with IFN- β results in a significant induction of SHP-1 levels predominantly through transcriptional pathways.

***In vivo* IFN- β treatment modulates STAT1, STAT6, and NF- κ B activation in PBMCs of MS patients**

SHP-1 regulates phosphorylation of several STATs by enzymatic activity on phosphotyrosine of activated cytokine receptors and STATs [69]. Thus, SHP-1 modulates STAT activation, translocation into the nucleus, and transcriptional activity on responsive genes. In particular, tyrosine phosphorylated STAT1 (pY-STAT1) that mediates interferon signaling was found to be elevated in MS patients [9;18] and is controlled by SHP-1 [33;34;70;71]. Therefore, we characterized STAT1 activation by measuring the level of tyrosine phosphorylated STAT1 (pSTAT1) in freshly isolated PBMCs using intracellular flow cytometry (Figure 2A). In accordance with previous reports, PBMCs of MS patients displayed significantly increased STAT1 activation compared to normal subjects. IFN- β treatment significantly increased STAT1 activation in freshly isolated PBMCs of treated MS patients compared to untreated MS patients.

Because several studies showed that SHP-1 profoundly modulates IL-4R α /STAT6 signaling molecules [35;36;38;72], and STAT6 was shown to be elevated in MS [20;21;22], we examined STAT6 activation in freshly isolated PBMCs from MS patients before and after *in vivo* IFN-

β treatment (Figure 2B). For analysis, we measured the level tyrosine phosphorylated STAT6 (pSTAT6) using intracellular flow cytometry. PBMCs of MS patients demonstrated significantly elevated STAT6 activation compared to normal subjects. Importantly, IFN- β treatment of MS patients resulted in a decrease in the STAT6 activation. These results were in accordance with SHP-1 levels observed in the same patients such that the SHP-1 levels were inversely correlated with STAT6 activation.

Another important transcription factor involved in the pathogenesis of MS is NF- κ B [23;73]. Several reports have documented the negative regulation of NF- κ B by SHP-1 [39;40;71;74;75]. Therefore, it was important to examine NF- κ B activation before and after *in vivo* IFN- β treatment of MS patients (Figure 2C). Nuclear extracts from freshly isolated PBMCs were prepared and allowed to bind an NF- κ B consensus oligonucleotide sequence. Bound NF- κ B was then detected by a p65 (RelA)-specific antibody and quantified based on a calibration using purified p65 recombinant protein. NF- κ B DNA binding activity was significantly increased in PBMCs of MS patients compared to normal subjects and IFN- β treatment resulted in a significant decrease in NF- κ B activation (Figure 2C). Taken together these data indicated that PBMCs of MS patients compared to normal subjects display constitutively increased STAT1, STAT6 and NF- κ B activation, which correlate to the constitutive SHP-1 deficiency seen in MS patients. Furthermore, the SHP-1 induction by IFN- β decreases activation of STAT6 and NF- κ B, yet maintains STAT1 activation via IFN- β signaling pathways.

***In vivo* IFN- β treatment attenuates the inflammatory profile in blood of MS patients**

To further investigate the effects of IFN- β in the inflammatory profile of MS patients, several parameters of the immune response including plasma cytokine levels and PBMC inflammatory gene expression were quantified in normal subjects, untreated RR MS patients, and three-month IFN- β treated RR MS patients (Table II). The level of IFN- γ , a cytokine that signals through STAT1, was significantly higher in the plasma of MS patients compared to normal subjects, yet IFN- β treatment did not significantly alter the concentration of IFN- γ (Table II). Similarly, the levels of IL-4 and IL-13, cytokines that signal through STAT6, were significantly higher in the plasma of MS patients compared to normal subjects. IFN- β treatment of MS patients did not change the levels of IL-4, but significantly decreased plasma levels of IL-13. In addition, we quantified the secretion of the NF- κ B-responsive pro-inflammatory cytokines TNF- α and IL-6, since it was previously shown that a deficiency in SHP-1 leads to higher expression of these cytokines [22;40;68;74;76;77]. The plasma levels of TNF- α and IL-6 were significantly higher in MS patients compared to normal subjects. IFN- β treatment significantly decreased IL-6 levels but not TNF- α levels.

Besides plasma levels of cytokines, we examined the expression of inflammatory genes that are induced via STAT1, STAT6, and NF- κ B activation to corroborate our findings regarding transcription factor activation in freshly isolated PBMCs of MS patients before and after IFN- β treatment (Table II). First, we quantified the mRNA expression of the STAT1-responsive genes IP-10 and caspase-1 by real-time RT-PCR. In accordance with previous reports [27;78] and the present observation of increased STAT1 activation (Figure 2A), the mRNA levels of IP-10 and caspase 1 were higher in PBMCs of MS patients compared to normal subjects and IFN- β treatment significantly increased the expression of these genes.

In addition, we quantified the expression of several STAT6-responsive genes that have been associated with inflammatory demyelination such as the chemokine CCL17/TARC [79;80], arginase I [35;81], and the disintegrin matrix metalloproteinase ADAM8 [82;83]. CCL17, arginase I, and ADAM8 expression were significantly higher in MS patients compared to normal subjects and *in vivo* IFN- β treatment reduced the expression of these genes (Table 2). The functional significance of these changes in mRNA was supported by the finding that

arginase enzymatic activity in these freshly isolated PBMCs corresponded to the expression of arginase I mRNA expression (Table II).

Next, because NF- κ B binding was elevated in PBMCs of MS patients and IFN- β reduced its activation, we examined the expression of NF- κ B-responsive genes in the same patients including NF- κ B inducible cyclooxygenase-2 (COX-2) and the metalloprotease MMP9 (Table II). In accord with previous reports [84;85], COX-2 and MMP9 were significantly higher in PBMCs of MS patients compared to normal subjects and IFN- β treatment decreased the expression of these genes. In contrast, we did not observe any differences in the expression of the chemokine receptors CXCR3 or CCR4 [86], which are not directly regulated by STAT1, STAT6, or NF κ B, between PBMCs of normal subjects, untreated MS patients, and IFN- β -treated MS patients.

***In vitro* IFN- β treatment induces SHP-1 expression in cultured PBMCs**

We have shown so far that interferon β -1a (Rebif) treatment of MS patients increases the expression of SHP-1 in freshly isolated PBMCs, which correlates with decreased activation of transcription factors controlled by SHP-1 and attenuation of the inflammatory profile in blood. In order to examine whether IFN- β directly induces SHP-1 expression in PBMCs and whether this SHP-1 induction mediates the immunomodulatory effects of IFN- β , PBMCs from MS patients and normal subjects were expanded in the presence of IL-2 and were treated *in vitro* with IFN- β for 24 hours. First, SHP-1 protein and mRNA expression were quantified in cultured PBMCs before and after a 24-hour treatment with IFN- β (Figure 3). By intracellular flow cytometry, IFN- β significantly raised SHP-1 protein levels in cultured PBMCs both from normal subjects and MS patients (Figure 3A & B).

In addition, the levels of SHP-1 mRNA in cultured PBMCs of normal subjects, untreated MS patients, SP MS patients, RR MS patients, and *in vivo* IFN- β -1a treated RR MS patients were quantified following a 24-hour *in vitro* treatment with IFN- β (Figure 3C). *In vitro* IFN- β treatment induced the expression of SHP-1 mRNA both in normal subjects and MS patients. Interestingly, PBMCs cultured for 1 week from *in vivo* IFN- β -treated patients displayed decreased SHP-1 relative to levels seen cultured normal control PBMCs suggesting that the induction of SHP-1 following *in vivo* IFN- β treatment was transient returning to abnormally low levels following *in vitro* cultivation. Furthermore, when cultured PBMCs of *in vivo* IFN- β -treated MS patients were treated *in vitro* with IFN- β for 24 hours the levels of SHP-1 were significantly induced to levels seen in normal control PBMCs indicating that the SHP-1 deficiency in MS PBMC can be repeatedly corrected by IFN- β . In addition, the levels of SHP-1 promoter I and promoter II mRNA were significantly induced in cultured PBMCs of normal subjects and untreated MS patients following 24-hour IFN- β treatment (Figure 3D–F).

***In vitro* IFN- β treatment induces the expression of SHP-1 in sorted T-cells, B-cells, and NK cells**

In order to examine whether IFN- β induced the expression of SHP-1 in specific PBMC types, CD4 T-cells, CD8 T-cells, B-cells, and NK cells were sorted by FACS from cultured PBMCs of normal subjects and MS patients before and after 24-hour treatment with IFN- β (Figure 4). The mRNA was isolated from sorted cells and the expression of SHP-1 transcripts was quantified with real-time RT-PCR. First, SHP-1 expression was more abundant in T-cells compared to B-cells or NK cells. Second, as in unsorted PBMCs, SHP-1 transcripts were less abundant in sorted MS cells than in normal control cells. Importantly, IFN- β resulted in a significant increase of SHP-1 transcripts in all cell types such that sorted MS cells no longer displayed SHP-1 deficiency (Figure 4A). Furthermore, both SHP-1 promoter I and promoter II transcripts were increased in CD4 and CD8 T-cells, B-cells, and NK cells following IFN- β treatment (Figure 4B & C).

***In vitro* IFN- β treatment modulates cytokine activation of STAT1, STAT6, and NF- κ B in cultured PBMCs**

Since *in vitro* IFN- β treatment substantially induced SHP-1 expression, we next investigated whether IFN- β increased SHP-1 function. Therefore, activation of the transcription factors STAT1, STAT6, and NF- κ B, that were shown to be tightly controlled by SHP-1, was quantified in cultured PBMCs of normal subjects and MS patients before and after pretreatment with IFN- β (Figure 5). Following 24-hour pretreatment with IFN- β cultured PBMCs were stimulated for 1 hour with IFN- γ , IL-4, or TNF- α to activate STAT1, STAT6, and NF- κ B, respectively. First, STAT1 activation was quantified by measuring pY-STAT1 by intracellular flow cytometry (Figure 5A). IFN- β pretreatment significantly increased STAT1 activation, but was not different in cultured PBMCs of normal subjects and MS patients. IFN- γ treatment resulted in significantly higher STAT1 activation in MS patients compared to normal subjects. When cultured PBMCs were pretreated with IFN- β and then stimulated with IFN- γ the level of STAT1 activation was lower, but was not significant, compared to IFN- γ stimulation alone.

Furthermore, constitutive STAT6 activation was significantly higher in cultured PBMCs of MS patients and pretreatment with IFN- β significantly lowered STAT6 activation (Figure 5B). Additionally, IL-4 stimulation raised pY-STAT6 levels significantly higher in cultured PBMCs of MS patients compared to normal subjects and IFN- β pretreatment before IL-4 stimulation resulted in significantly lower pSTAT6 levels. Next, we examined NF- κ B activation by measuring its DNA binding activity from nuclear extracts of cultured PBMCs (Figure 5C). NF- κ B activation was slightly but significantly higher in PBMCs of MS patients compared to normal subjects and 24-hour IFN- β treatment did not significantly change NF- κ B activation. TNF- α treatment dramatically increased NF- κ B activation, which was significantly higher in MS patients compared to normal subjects. Importantly, pretreatment with IFN- β followed by TNF- α stimulation resulted in significantly lower induction of nuclear NF- κ B binding activity compared to TNF- α stimulation alone. In all, *in vitro* IFN- β pretreatment down-modulates transcription factor activation, which is in agreement with the induction of SHP-1 expression and function.

***In vitro* IFN- β treatment modulates cytokine-induced inflammatory gene expression**

Next, we examined the expression of inflammatory genes that are increased by STAT1, STAT6, and NF- κ B activity to corroborate our findings regarding cytokine-induced transcription factor activation in cultured PBMCs of MS patients following an IFN- β pretreatment (Figure 6). First, we quantified the mRNA expression of the STAT1-responsive genes IP-10 and caspase-1 measured by real-time RT-PCR (Figure 6A & B). IFN- β treatment significantly induced IP-10 and caspase 1 expression to similar levels between normal subjects and MS patients. IFN- γ treatment also induced IP-10 and caspase 1 levels. IP-10 was significantly induced to a higher level in PBMCs of MS patients compared to normal subjects. Importantly, pretreatment with IFN- β followed by IFN- γ stimulation resulted in significantly lower induction of IP-10 compared to IFN- γ stimulation alone. Next, we quantified the expression of the STAT6-responsive genes TARC and ADAM8, which were constitutively higher in cultured PBMCs of MS patients compared to normal subjects (Figure 6C & D). Treatment with IL-4 significantly induced higher levels of TARC and ADAM8 in PBMCs of MS patients compared to normal subjects. IFN- β treatment before IL-4 stimulation resulted in significantly lower levels of TARC and ADAM8 compared to IL-4 stimulation alone. Lastly, the levels of the NF- κ B-responsive genes IL-6 and COX-2 were quantified with ELISA and real-time RT-PCR respectively (Figure 6E & F). *In vitro* IFN- β treatment resulted in a modest but significant increase in IL-6 secretion but did not affect COX-2 levels. TNF- α stimulation substantially increased IL-6 and COX-2 levels which were significantly higher levels in MS patients compared to normal subjects. Importantly, IFN- β pretreatment before TNF- α

stimulation resulted in significantly lower levels of IL-6 and COX-2 compared to TNF- α stimulation alone.

Pharmacologic depletion of SHP-1 diminishes the ability of IFN- β to attenuate cytokine signaling

To examine whether the IFN- β -mediated induction of SHP-1 is directly responsible for the immunomodulatory effects of IFN- β , cultured PBMCs of normal subjects and MS patients were treated with siRNA to deplete SHP-1. Thus, SHP-1 protein was substantially depleted in the PBMCs treated with the SHP-1 siRNA compared to the PBMCs treated with control siRNA of scrambled sequence (Figure 7A & B). As expected, this reduction of SHP-1 inversely correlated with the expression of arginase I, a gene that is tightly regulated by SHP-1 activity [35] (Figure 7A). In the scramble siRNA-treated PBMCs, IFN- β treatment decreased arginase I expression but IFN- β had little effect on arginase I expression in PBMCs that had been transfected with SHP-1 siRNA. Next, activation of the transcription factors STAT1, STAT6, and NF- κ B that were shown to be tightly controlled by SHP-1 was quantified in cultured PBMCs treated with SHP-1 siRNA or control siRNA before and after pretreatment with IFN- β (Figure 7). Following 24-hour pretreatment with IFN- β cultured PBMCs were stimulated for 1 hour with IFN- γ , IL-4, and TNF- α to activate STAT1, STAT6, and NF- κ B respectively. First, in cultured PBMCs treated with scrambled siRNA, STAT1 activation was significantly higher in MS patients compared to normal subjects following IFN- γ treatment (Figure 7C). In contrast, when SHP-1 was depleted, no difference was observed in STAT1 activation following IFN- γ stimulation between PBMCs of normal subjects and MS patients. (Figure 7D). Similar results were obtained when examining the expression of the STAT1-responsive gene IP-10 (Figure 8A & B). Furthermore, SHP-1 depletion abolished the significantly higher STAT6 activation observed both constitutively and following IL-4 treatment in cultured PBMCs of MS patients compared to normal subjects (Figure 7E & F). Importantly, in SHP-1-depleted PBMCs, IFN- β pretreatment was ineffective in decreasing STAT6 signaling. Further, similar data were observed when quantifying the STAT6-responsive gene TARC (Figure 8C & D), lending further support that the IFN- β induction of SHP-1 can downmodulate STAT6 activation.

Lastly, NF- κ B binding activity was quantified in cultured PBMCs that were treated with SHP-1 or control siRNA (Figure 7G & H). SHP-1 depletion abolished the difference in NF- κ B activation between PBMCs of normal subjects and MS patients both constitutively and following TNF- α stimulation. Notably, in the SHP-1 siRNA-treated PBMCs, IFN- β pretreatment did not significantly lower NF- κ B activation, suggesting that the induction of SHP-1 by IFN- β is required for downmodulating NF- κ B activity. In accord with this finding, IFN- β treatment did not significantly lower the induction of the NF- κ B-responsive genes IL-6 following TNF- α stimulation in the SHP-1 siRNA treated PBMCs (Figure 8E & F). In conclusion, SHP-1 induction by IFN- β is directly responsible for the ability of IFN- β to downmodulate inflammatory cytokine signaling.

DISCUSSION

The aim of this study was to document the IFN- β inducible activity of the protein tyrosine phosphatase SHP-1 in human PBMCs and characterize the possible functions of increased SHP-1 levels in the context of mechanisms of efficacy of IFN- β in the treatment of multiple sclerosis. We show that both an *in vivo* three-month treatment with IFN- β -1a (Rebif) and *in vitro* IFN- β treatment raise SHP-1 expression levels in MS PBMCs to levels seen in normal control cells. Furthermore, the increased SHP-1 levels correlated with decreased activation of the transcription factors STAT6 and NF- κ B and decreased expression of corresponding inducible inflammatory gene expression. The effect of increased SHP-1 levels on STAT1 activation is modest by comparison most likely because IFN- β directly activates STAT1.

Notably, experimental depletion of SHP-1 with siRNA indicated that the SHP-1 induction by IFN- β is essential in downregulating inflammatory cytokine signaling. Therefore, this study suggests a novel mechanism by which IFN- β exerts its immunomodulatory effects in MS.

Multiple sclerosis is an immune-mediated disease and numerous reports have demonstrated that leukocytes in the blood and in CNS lesions have increased activation of the transcription factors STAT1, STAT6, and NF- κ B that might be the result of increased plasma levels of the corresponding cytokines IFN- γ , IL-4/IL13, and TNF- α [9;10;11;12;13;14;15;16;18;19;20;21;22;23]. In turn, these elevated transcription factors drive the expression of several inflammatory genes like the chemokines IP-10, TARC, IL-6, the proteases ADAM8 and MMP9, the inflammatory enzymes caspase 1, arginase I, and cyclooxygenase 2 that might contribute to the enhanced demyelinating activity of CNS-infiltrating leukocytes. Recently, we have demonstrated that IFN- β transcriptionally increases SHP-1 expression in mouse CNS and immune cells [32]. Therefore, in the present study, it was of particular interest to characterize the expression and functions of SHP-1 in MS, especially considering that SHP-1 is a potent inhibitor of proinflammatory cytokine signaling [33;34;35;36;37;38;39;40;41]. Indeed, our group has previously demonstrated through experimental depletion with siRNA and overexpression with lentiviral vectors of SHP-1 that SHP-1 is important in modulation of transcription factor activation in human PBMCs [22].

IFN- β is a current effective treatment for MS that is thought to exert therapeutic effects by downregulating the immune response. Here we demonstrate that both *in vivo* and *in vitro* treatment with IFN- β modulates the activation of inflammatory transcription factors and inflammatory gene expression. For example, it was previously shown that IFN- β modulates NF- κ B activation [24;84]. In agreement, we demonstrated that IFN- β treatment significantly decreased the elevated NF- κ B activation seen in PBMCs of MS patients. Furthermore, in accord with studies demonstrating that SHP-1 controls NF- κ B activity [39;40;44], siRNA depletion of SHP-1 in PBMCs of MS patients abolished the ability of IFN- β to decrease NF- κ B activation (Figure 7), suggesting that the IFN- β induction of SHP-1 is essential in downmodulating NF- κ B activity.

Furthermore, in accord with previous studies showing that IFN- β modulates STAT6 activation [87], we demonstrated that IFN- β treatment results in a significant downregulation of STAT6 activation and decrease of STAT6-responsive genes. In contrast, IFN- β treatment alone further elevated STAT1 activation and STAT1-responsive genes. These data are in agreement with several studies documenting that *in vivo* interferon β treatment results in increased expression of STAT-1 responsive genes [26;27;88;89]. Thus, although IFN- β upregulates SHP-1 expression and SHP-1 down regulates STAT1 signaling, simultaneous activation STAT1 by IFN- β ultimately increases STAT1 and STAT-1-responsive genes. Nonetheless, the unique ability of IFN- β to efficiently increase SHP-1 and modulate STAT1 signals was revealed in samples treated additionally with IFN- γ in which IFN- β modulated IFN- γ -inducible STAT1-responsive genes. This modulation of IFN- γ -inducible STAT1 responsive genes by IFN- β was in general agreement with STAT1 activity although the degree of modulation pY-STAT1 was not significant. Interestingly, the increase levels of STAT-1 responsive genes like the chemokine IP-10 following IFN- β treatment closely correlate with the flu-like symptoms in treated MS patients [27]. Therefore, one possible drawback of interferon- β treatment in MS is that it directly activates STAT1 and STAT-1 responsive genes, which could be partly responsible for the side effects and/or incomplete response of interferon- β therapy in MS.

The cytokine signaling of PBMCs of MS patients both *in vivo* and *in vitro* appeared very similar. MS patients had high levels of plasma cytokines that were modulated by the *in vivo* IFN- β treatment and certainly contributed to the enhanced transcription factor activation in the freshly isolated PBMCs of MS patients. Based on our studies, it is likely that SHP-1 controls both the

production of and responsiveness to proinflammatory cytokines in leukocytes. With respect to responsiveness, culturing PBMCs of MS patients and normal subjects offered the advantage of stimulating the cells with equal amounts of the cytokines IFN- γ , IL-4, and TNF- α and examining intrinsic differences in cytokine signaling. Our results show that PBMCs of MS patients have a stable deficiency in SHP-1 expression, which is responsible for enhanced cytokine signaling in these cells. Importantly, IFN- β pretreatment of PBMCs *in vitro* substantially raised SHP-1 levels that accounted for the anti-inflammatory effects of IFN- β treatment. Therefore, we propose that the plasma levels of the IFN- γ , IL-4/IL-13, and TNF- α serve as the pro-inflammatory stimulus in MS patients and these activities are opposed by the IFN- β -inducible SHP-1 in treated MS patients. Ultimately, the combination and intensity of both the proinflammatory and inhibitory signals result in a specific inflammatory homeostasis and therapeutic outcome of IFN- β treatment of an individual MS patient (Figure 9).

Apart from modulating transcription factor activation, the IFN- β -mediated induction of SHP-1 can modulate several disparate arms of the immune response that are dysregulated in MS patients [3;49]. For instance, multiple signaling events of antigen receptors in lymphocytes involve tyrosine kinases and SHP-1 was shown to control the activity of these kinases including Src and Lyn in B-cells and Lck, Fyn, and Zap-70 in T-cells [90]. As a result, decreased SHP-1 levels have been shown to contribute to lower activation threshold in T-cells [46;91] and prevent T-cell anergy [92;93;94]. The present studies suggest that defects in antigen receptor signaling in T and B cells signaling may be reversed in lymphocytes of IFN- β -treated MS patients in an SHP-1-dependent manner. This is in agreement with results demonstrating that *in vivo* IFN- β treatment decreases the IFN- γ - and IL-4-producing T-cells in MS patients [17]. Additionally, IFN- β -mediated induction of SHP-1 in B-cells is expected to result in decreased antigen receptor signaling, polyclonal B-cell activation, and autoimmunity [95;96;97]. Furthermore, deficiency of SHP-1 enhances leukocyte responsiveness to chemokines that could play an important role in leukocyte migration into the CNS [98;99;100]. In the light that much of the CNS damage seen in MS may be mediated through myelin reactive T-cells and increased auto-antibody production by B cells, the SHP-1 induction by IFN- β is likely to be extremely important in attenuating the lymphocyte-mediated immune responses that cause demyelination. In support of this hypothesis, in both viral and the autoimmune mouse models of MS, lack of SHP-1 leads to more severe disease progression and demyelination [44;45; 46].

Two distinct promoters drive the expression of two different SHP-1 transcripts from the SHP-1 gene. Previously we demonstrated that promoter II transcripts, which are more abundantly expressed in hematopoietic cells, are selectively lower in the PBMCs of MS patients compared to normal subjects [22]. This study demonstrates that *in vivo* IFN- β significantly induces SHP-1 promoter II transcripts and *in vitro* IFN- β treatment significantly increases both SHP-1 promoter I and promoter II transcripts. Previously we characterized the promoter activities of the mouse SHP-1 gene, which have considerable homology with the human promoters [32]. Both mouse and human genes have a similar interferon-response factor element (IRF-E) consensus sequence in promoter I and have five STAT3-binding sites in promoter II. Using knockout mice, we demonstrated in CNS and immune cells that IFN- β induced SHP-1 promoter I and promoter II transcripts through IRF-1 and STAT3, respectively [32]. In agreement, it was shown that in human T-cell lines the SHP-1 promoter II binds STAT3 [101] and several studies have shown that IFN- β is potent inducer of STAT3 activation in human leukocytes [102; 103]. Based on our current findings in human PBMC, we propose that IFN- β induction of SHP-1 promoter II might be mediated via STAT3 activation but that the induction of promoter I is mediated by the activation of the transcription factor IRF-1. With respect to our present findings on the transient nature of IFN- β effects on SHP-1 promoter activity in MS PBMCs, we propose that promoter II in MS patients contain stable genetic or epigenetic alterations that lower constitutive transcriptional activity. We further hypothesize that IFN- β treatment of

patients transiently induces promoter activity above constitutive levels possibly via distinct promoter elements consistent with our studies in the mouse. Consequently, instances in which IFN- β may wane over time in patients or disappear for instance between IFN- β administrations or after isolation of PBMC in vitro as in the present study, SHP-1 promoter activities are expected to return to abnormally low constitutive levels. Further examination of the role of IFN- β on SHP-1 expression and function will lead to an increased understanding of essential regulatory circuits in MS patients and the role of IFN- β in correcting the functional deficits of SHP-1.

Acknowledgments

We thank Carol Ozark for collection of normal subject blood samples. We would also like to thank Dr. Nick J. Gonchoroff for his expertise in flow cytometry/cell sorting performed in this study. This work was supported in part by research grants from the National Multiple Sclerosis Society (RG2569C5) to Paul T. Massa, NIH grant (NS041593) to Paul T. Massa, and Serono, Inc. to Burk Jubelt, Cornelia Mihal, and Paul T. Massa.

References

1. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. *N Engl J Med* 2000;343:938–52. [PubMed: 11006371]
2. Kornel B, Lassmann H. Neuropathology of multiple sclerosis-new concepts. *Brain Res Bull* 2003;61:321–6. [PubMed: 12909302]
3. Javed A, Reder AT. Therapeutic role of beta-interferons in multiple sclerosis. *Pharmacol Ther* 2006;110:35–56. [PubMed: 16229894]
4. Ann Marrie R, Rudick RA. Drug Insight: interferon treatment in multiple sclerosis. *Nat Clin Pract Neurol* 2006;2:34–44. [PubMed: 16932519]
5. Kinkel RP, Kollman C, O'Connor P, Murray TJ, Simon J, Arnold D, Bakshi R, Weinstock-Gutman B, Brod S, Cooper J, Duquette P, Eggenberger E, Felton W, Fox R, Freedman M, Galetta S, Goodman A, Guarnaccia J, Hashimoto S, Horowitz S, Javerbaum J, Kasper L, Kaufman M, Kerson L, Mass M, Rammohan K, Reiss M, Rolak L, Rose J, Scott T, Selhorst J, Shin R, Smith C, Stuart W, Thurston S, Wall M. IM interferon beta-1a delays definite multiple sclerosis 5 years after a first demyelinating event. *Neurology* 2006;66:678–84. [PubMed: 16436649]
6. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 2005;5:375–86. [PubMed: 15864272]
7. van Boxel-Dezaire AH, Rani MR, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 2006;25:361–72. [PubMed: 16979568]
8. Axtell RC, Steinman L. Type I interferons cool the inflamed brain. *Immunity* 2008;28:600–2. [PubMed: 18482563]
9. Gobin SJ, Montagne L, Van Zutphen M, Van Der Valk P, Van Den Elsen PJ, De Groot CJ. Upregulation of transcription factors controlling MHC expression in multiple sclerosis lesions. *Glia* 2001;36:68–77. [PubMed: 11571785]
10. Link J, Soderstrom M, Olsson T, Hojeberg B, Ljungdahl A, Link H. Increased transforming growth factor-beta, interleukin-4, and interferon-gamma in multiple sclerosis. *Ann Neurol* 1994;36:379–86. [PubMed: 8080246]
11. Hermans G, Stinissen P, Hauben L, Van den Berg-Loonen E, Raus J, Zhang J. Cytokine profile of myelin basic protein-reactive T cells in multiple sclerosis and healthy individuals. *Ann Neurol* 1997;42:18–27. [PubMed: 9225681]
12. Rohowsky-Kochan C, Molinaro D, Cook SD. Cytokine secretion profile of myelin basic protein-specific T cells in multiple sclerosis. *Mult Scler* 2000;6:69–77. [PubMed: 10773850]
13. Ochi H, Osoegawa M, Wu XM, Minohara M, Horiuchi I, Murai H, Furuya H, Kira J. Increased IL-13 but not IL-5 production by CD4-positive T cells and CD8-positive T cells in multiple sclerosis during relapse phase. *J Neurol Sci* 2002;201:45–51. [PubMed: 12163193]

14. Hohnoki K, Inoue A, Koh CS. Elevated serum levels of IFN-gamma, IL-4 and TNF-alpha/unelevated serum levels of IL-10 in patients with demyelinating diseases during the acute stage. *J Neuroimmunol* 1998;87:27–32. [PubMed: 9670842]
15. Ishizu T, Osoegawa M, Mei FJ, Kikuchi H, Tanaka M, Takakura Y, Minohara M, Murai H, Mihara F, Taniwaki T, Kira J. Intrathecal activation of the IL-17/IL-8 axis in opticospinal multiple sclerosis. *Brain* 2005;128:988–1002. [PubMed: 15743872]
16. Killestein J, Den Drijver BF, Van der Graaff WL, Uitdehaag BM, Polman CH, Van Lier RA. Intracellular cytokine profile in T-cell subsets of multiple sclerosis patients: different features in primary progressive disease. *Mult Scler* 2001;7:145–50. [PubMed: 11475436]
17. Franciotta D, Zardini E, Bergamaschi R, Andreoni L, Cosi V. Interferon gamma and interleukin 4 producing T cells in peripheral blood of multiple sclerosis patients undergoing immunomodulatory treatment. *J Neurol Neurosurg Psychiatry* 2003;74:123–6. [PubMed: 12486283]
18. Frisullo G, Angelucci F, Caggiula M, Nociti V, Iorio R, Patanella AK, Sancricca C, Mirabella M, Tonali PA, Batocchi AP. 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;84:1027–36. [PubMed: 16865709]
19. Feng X, Petraglia AL, Chen M, Byskosh PV, Boos MD, Reder AT. Low expression of interferon-stimulated genes in active multiple sclerosis is linked to subnormal phosphorylation of STAT1. *J Neuroimmunol* 2002;129:205–15. [PubMed: 12161037]
20. Zeis T, Graumann U, Reynolds R, Schaeren-Wiemers N. Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection. *Brain* 2008;131:288–303. [PubMed: 18056737]
21. Cannella B, Raine CS. Multiple sclerosis: cytokine receptors on oligodendrocytes predict innate regulation. *Ann Neurol* 2004;55:46–57. [PubMed: 14705111]
22. Christophi GP, Hudson CA, Gruber RC, Christophi CP, Mihai C, Mejico LJ, Jubelt B, Massa PT. SHP-1 deficiency and increased inflammatory gene expression in PBMCs of multiple sclerosis patients. *Lab Invest* 2008;88:243–55. [PubMed: 18209728]
23. Eggert M, Goertsches R, Seeck U, Dilk S, Neeck G, Zettl UK. Changes in the activation level of NF-kappa B in lymphocytes of MS patients during glucocorticoid pulse therapy. *J Neurol Sci* 2008;264:145–50. [PubMed: 17889033]
24. Ozenci V, Kouwenhoven M, Huang YM, Kivisakk P, Link H. Multiple sclerosis is associated with an imbalance between tumour necrosis factor-alpha (TNF-alpha)- and IL-10-secreting blood cells that is corrected by interferon-beta (IFN-beta) treatment. *Clin Exp Immunol* 2000;120:147–53. [PubMed: 10759776]
25. Wiesemann E, Deb M, Hemmer B, Radeke HH, Windhagen A. Early identification of interferon-beta responders by ex vivo testing in patients with multiple sclerosis. *Clin Immunol* 2008;128:306–13. [PubMed: 18539537]
26. Wandinger KP, Sturzebecher CS, Bielekova B, Detore G, Rosenwald A, Staudt LM, McFarland HF, Martin R. Complex immunomodulatory effects of interferon-beta in multiple sclerosis include the upregulation of T helper 1-associated marker genes. *Ann Neurol* 2001;50:349–57. [PubMed: 11558791]
27. Buttmann M, Merzyn C, Hofstetter HH, Rieckmann P. TRAIL, CXCL10 and CCL2 plasma levels during long-term Interferon-beta treatment of patients with multiple sclerosis correlate with flu-like adverse effects but do not predict therapeutic response. *J Neuroimmunol* 2007;190:170–6. [PubMed: 17884184]
28. Jensen J, Krakauer M, Sellebjerg F. Cytokines and adhesion molecules in multiple sclerosis patients treated with interferon-beta1b. *Cytokine* 2005;29:24–30. [PubMed: 15579375]
29. Hedegaard CJ, Krakauer M, Bendtzen K, Sorensen PS, Sellebjerg F, Nielsen Ct. The effect of beta-interferon therapy on myelin basic protein-elicited CD4+ T cell proliferation and cytokine production in multiple sclerosis. *Clin Immunol* 2008;129:80–9. [PubMed: 18653385]
30. Prinz M, Schmidt H, Mildner A, Knobloch KP, Hanisch UK, Raasch J, Merkler D, Detje C, Gutmacher I, Mages J, Lang R, Martin R, Gold R, Becher B, Bruck W, Kalinke U. Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. *Immunity* 2008;28:675–86. [PubMed: 18424188]

31. Xu D, Qu CK. Protein tyrosine phosphatases in the JAK/STAT pathway. *Front Biosci* 2008;13:4925–32. [PubMed: 18508557]
32. Christophi GP, Hudson CA, Gruber R, Christophi CP, Massa PT. Promoter-specific induction of the phosphatase SHP-1 by viral infection and cytokines in CNS glia. *J Neurochem*. 2008
33. David M, Chen HE, Goelz S, Larner AC, Neel BG. Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. *Mol Cell Biol* 1995;15:7050–8. [PubMed: 8524272]
34. Massa PT, Wu C. The role of protein tyrosine phosphatase SHP-1 in the regulation of IFN-gamma signaling in neural cells. *J Immunol* 1996;157:5139–44. [PubMed: 8943425]
35. Bonaparte KL, Hudson CA, Wu C, Massa PT. Inverse regulation of inducible nitric oxide synthase (iNOS) and arginase I by the protein tyrosine phosphatase SHP-1 in CNS glia. *Glia* 2006;53:827–35. [PubMed: 16565987]
36. Hanson EM, Dickensheets H, Qu CK, Donnelly RP, Keegan AD. Regulation of the dephosphorylation of Stat6. Participation of Tyr-713 in the interleukin-4 receptor alpha, the tyrosine phosphatase SHP-1, and the proteasome. *J Biol Chem* 2003;278:3903–11. [PubMed: 12459556]
37. Huang Z, Coleman JM, Su Y, Mann M, Ryan J, Shultz LD, Huang H. SHP-1 regulates STAT6 phosphorylation and IL-4-mediated function in a cell type-specific manner. *Cytokine* 2005;29:118–24. [PubMed: 15613279]
38. Kamata T, Yamashita M, Kimura M, Murata K, Inami M, Shimizu C, Sugaya K, Wang CR, Taniguchi M, Nakayama T. src homology 2 domain-containing tyrosine phosphatase SHP-1 controls the development of allergic airway inflammation. *J Clin Invest* 2003;111:109–19. [PubMed: 12511594]
39. Massa PT, Wu C. Increased inducible activation of NF-kappaB and responsive genes in astrocytes deficient in the protein tyrosine phosphatase SHP-1. *J Interferon Cytokine Res* 1998;18:499–507. [PubMed: 9712366]
40. Khaled AR, Butfiloski EJ, Sobel ES, Schiftenbauer J. Functional consequences of the SHP-1 defect in motheaten viable mice: role of NF-kappa B. *Cell Immunol* 1998;185:49–58. [PubMed: 9636682]
41. Croker BA, Lawson BR, Berger M, Eidenschenk C, Blasius AL, Moresco EM, Sovath S, Cengia L, Shultz LD, Theofilopoulos AN, Pettersson S, Beutler BA. Inflammation and autoimmunity caused by a SHP1 mutation depend on IL-1, MyD88, and a microbial trigger. *Proc Natl Acad Sci U S A* 2008;105:15028–33. [PubMed: 18806225]
42. Massa PT, Saha S, Wu C, Jarosinski KW. Expression and function of the protein tyrosine phosphatase SHP-1 in oligodendrocytes. *Glia* 2000;29:376–85. [PubMed: 10652447]
43. Massa PT, Wu C, Fecenko-Tacka K. Dysmyelination and reduced myelin basic protein gene expression by oligodendrocytes of SHP-1-deficient mice. *J Neurosci Res* 2004;77:15–25. [PubMed: 15197735]
44. Christophi GP, Hudson CA, Panos M, Gruber RC, Massa PT. Modulation of macrophage infiltration and inflammatory activity by the phosphatase SHP-1 in virus-induced demyelinating disease. *J Virol* 2009;83:522–39. [PubMed: 18987138]
45. Massa PT, Ropka SL, Saha S, Fecenko KL, Beuler KL. Critical role for protein tyrosine phosphatase SHP-1 in controlling infection of central nervous system glia and demyelination by Theiler's murine encephalomyelitis virus. *J Virol* 2002;76:8335–46. [PubMed: 12134038]
46. Deng C, Minguela A, Hussain RZ, Lovett-Racke AE, Radu C, Ward ES, Racke MK. Expression of the tyrosine phosphatase SRC homology 2 domain-containing protein tyrosine phosphatase 1 determines T cell activation threshold and severity of experimental autoimmune encephalomyelitis. *J Immunol* 2002;168:4511–8. [PubMed: 11970996]
47. Zhang Q, Raghunath PN, Vonderheid E, Odum N, Wasik MA. Lack of phosphotyrosine phosphatase SHP-1 expression in malignant T-cell lymphoma cells results from methylation of the SHP-1 promoter. *Am J Pathol* 2000;157:1137–46. [PubMed: 11021818]
48. Oka T, Yoshino T, Hayashi K, Ohara N, Nakanishi T, Yamaai Y, Hiraki A, Sogawa CA, Kondo E, Teramoto N, Takahashi K, Tsuchiyama J, Akagi T. Reduction of hematopoietic cell-specific tyrosine phosphatase SHP-1 gene expression in natural killer cell lymphoma and various types of lymphomas/leukemias: combination analysis with cDNA expression array and tissue microarray. *Am J Pathol* 2001;159:1495–505. [PubMed: 11583976]

49. Shultz LD, Rajan TV, Greiner DL. Severe defects in immunity and hematopoiesis caused by SHP-1 protein-tyrosine-phosphatase deficiency. *Trends Biotechnol* 1997;15:302–7. [PubMed: 9263478]
50. Wu C, Sun M, Liu L, Zhou GW. The function of the protein tyrosine phosphatase SHP-1 in cancer. *Gene* 2003;306:1–12. [PubMed: 12657462]
51. Christophi GP, Panos M, Hudson CA, Christophi RL, Mersich AT, Blystone SD, Jubelt B, Massa PT. Macrophages of multiple sclerosis patients display deficient SHP-1 expression and enhanced inflammatory phenotype. *Lab Invest*. 2009In Press
52. Banville D, Stocco R, Shen SH. Human protein tyrosine phosphatase 1C (PTPN6) gene structure: alternate promoter usage and exon skipping generate multiple transcripts. *Genomics* 1995;27:165–73. [PubMed: 7665165]
53. Martin A, Tsui HW, Shulman MJ, Isenman D, Tsui FW. Murine SHP-1 splice variants with altered Src homology 2 (SH2) domains. Implications for the SH2-mediated intramolecular regulation of SHP-1. *J Biol Chem* 1999;274:21725–34. [PubMed: 10419485]
54. Yi TL, Cleveland JL, Ihle JN. Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. *Mol Cell Biol* 1992;12:836–46. [PubMed: 1732748]
55. Tsui HW, Hasselblatt K, Martin A, Mok SC, Tsui FW. Molecular mechanisms underlying SHP-1 gene expression. *Eur J Biochem* 2002;269:3057–64. [PubMed: 12071971]
56. Ruchusatsawat K, Wongpiyabovorn J, Shuangshoti S, Hirankarn N, Mutirangura A. SHP-1 promoter 2 methylation in normal epithelial tissues and demethylation in psoriasis. *J Mol Med* 2006;84:175–82. [PubMed: 16389548]
57. Xu Y, Banville D, Zhao HF, Zhao X, Shen SH. Transcriptional activity of the SHP-1 gene in MCF7 cells is differentially regulated by binding of NF-Y factor to two distinct CCAAT-elements. *Gene* 2001;269:141–53. [PubMed: 11376946]
58. Wlodarski P, Zhang Q, Liu X, Kasprzycka M, Marzec M, Wasik MA. PU.1 activates transcription of SHP-1 gene in hematopoietic cells. *J Biol Chem*. 2007
59. Christophi GP, Isackson PJ, Blaber S, Blaber M, Rodriguez M, Scarisbrick IA. Distinct promoters regulate tissue-specific and differential expression of kallikrein 6 in CNS demyelinating disease. *J Neurochem* 2004;91:1439–49. [PubMed: 15584920]
60. Leon F, Cespon C, Franco A, Lombardia M, Roldan E, Escribano L, Harto A, Gonzalez-Porque P, Roy G. SHP-1 expression in peripheral T cells from patients with Sezary syndrome and in the T cell line HUT-78: implications in JAK3-mediated signaling. *Leukemia* 2002;16:1470–7. [PubMed: 12145687]
61. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY, Wolinsky JS. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol* 2001;50:121–7. [PubMed: 11456302]
62. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology* 1996;46:907–11. [PubMed: 8780061]
63. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group. *Lancet* 1998;352:1498–504. [PubMed: 9820297]
64. Massa PT, Wu H. Interferon regulatory factor element and interferon regulatory factor 1 in the induction of major histocompatibility complex class I genes in neural cells. *J Interferon Cytokine Res* 1995;15:799–810. [PubMed: 8536108]
65. Renard P, Ernest I, Houbion A, Art M, Le Calvez H, Raes M, Remacle J. Development of a sensitive multi-well colorimetric assay for active NFkappaB. *Nucleic Acids Res* 2001;29:E21. [PubMed: 11160941]
66. Zhu L, Zou W, Yu C, Lin J, He X. Human recombinant PLD2 can repress p65 activity of guinea pigs of chronic asthma in vivo. *Cell Mol Immunol* 2006;3:307–10. [PubMed: 17083197]

67. Sun J, Ramnath RD, Tamizhselvi R, Bhatia M. Neurokinin A engages neurokinin-1 receptor to induce NF-kappaB-dependent gene expression in murine macrophages: implications of ERK1/2 and PI 3-kinase/Akt pathways. *Am J Physiol Cell Physiol* 2008;295:C679–91. [PubMed: 18596216]
68. Hudson CA, Christophi GP, Cao L, Gruber RC, Massa PT. Regulation of avoidant behaviors and pain by the anti-inflammatory tyrosine phosphatase SHP-1. *Neuron Glia Biol* 2006;2:235–246. [PubMed: 18250891]
69. Platanias LC, Fish EN. Signaling pathways activated by interferons. *Exp Hematol* 1999;27:1583–92. [PubMed: 10560905]
70. Yetter A, Uddin S, Krolewski JJ, Jiao H, Yi T, Platanias LC. Association of the interferon-dependent tyrosine kinase Tyk-2 with the hematopoietic cell phosphatase. *J Biol Chem* 1995;270:18179–82. [PubMed: 7629131]
71. Zhang Z, Jimi E, Bothwell AL. Receptor activator of NF-kappa B ligand stimulates recruitment of SHP-1 to the complex containing TNFR-associated factor 6 that regulates osteoclastogenesis. *J Immunol* 2003;171:3620–6. [PubMed: 14500659]
72. Haque SJ, Harbor P, Tabrizi M, Yi T, Williams BR. Protein-tyrosine phosphatase Shp-1 is a negative regulator of IL-4- and IL-13-dependent signal transduction. *J Biol Chem* 1998;273:33893–6. [PubMed: 9852037]
73. Bonetti B, Stegagno C, Cannella B, Rizzuto N, Moretto G, Raine CS. Activation of NF-kappaB and c-jun transcription factors in multiple sclerosis lesions. Implications for oligodendrocyte pathology. *Am J Pathol* 1999;155:1433–8. [PubMed: 10550297]
74. Zhao J, Brooks DM, Lurie DI. Lipopolysaccharide-activated SHP-1-deficient motheaten microglia release increased nitric oxide, TNF-alpha, and IL-1beta. *Glia* 2006;53:304–12. [PubMed: 16265671]
75. Wu L, Iwai M, Li Z, Shiuchi T, Min LJ, Cui TX, Li JM, Okumura M, Nahmias C, Horiuchi M. Regulation of inhibitory protein-kappaB and monocyte chemoattractant protein-1 by angiotensin II type 2 receptor-activated Src homology protein tyrosine phosphatase-1 in fetal vascular smooth muscle cells. *Mol Endocrinol* 2004;18:666–78. [PubMed: 14684844]
76. Thrall RS, Vogel SN, Evans R, Shultz LD. Role of tumor necrosis factor-alpha in the spontaneous development of pulmonary fibrosis in viable motheaten mutant mice. *Am J Pathol* 1997;151:1303–10. [PubMed: 9358756]
77. An H, Hou J, Zhou J, Zhao W, Xu H, Zheng Y, Yu Y, Liu S, Cao X. Phosphatase SHP-1 promotes TLR- and RIG-I-activated production of type I interferon by inhibiting the kinase IRAK1. *Nat Immunol* 2008;9:542–50. [PubMed: 18391954]
78. Huang WX, Huang P, Hillert J. Increased expression of caspase-1 and interleukin-18 in peripheral blood mononuclear cells in patients with multiple sclerosis. *Mult Scler* 2004;10:482–7. [PubMed: 15471361]
79. Wirsberger G, Hebenstreit D, Posselt G, Horejs-Hoeck J, Duschl A. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *Eur J Immunol* 2006;36:1882–91. [PubMed: 16810739]
80. Narikawa K, Misu T, Fujihara K, Nakashima I, Sato S, Itoyama Y. CSF chemokine levels in relapsing neuromyelitis optica and multiple sclerosis. *J Neuroimmunol* 2004;149:182–6. [PubMed: 15020078]
81. Xu L, Hilliard B, Carmody RJ, Tsabary G, Shin H, Christianson DW, Chen YH. Arginase and autoimmune inflammation in the central nervous system. *Immunology* 2003;110:141–8. [PubMed: 12941151]
82. King NE, Zimmermann N, Pope SM, Fulkerson PC, Nikolaidis NM, Mishra A, Witte DP, Rothenberg ME. Expression and regulation of a disintegrin and metalloproteinase (ADAM) 8 in experimental asthma. *Am J Respir Cell Mol Biol* 2004;31:257–65. [PubMed: 15087305]
83. Amour A, Knight CG, English WR, Webster A, Slocombe PM, Knauper V, Docherty AJ, Becherer JD, Blobel CP, Murphy G. The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. *FEBS Lett* 2002;524:154–8. [PubMed: 12135759]
84. Bosca L, Bodelon OG, Hortelano S, Casellas A, Bosch F. Anti-inflammatory action of type I interferons deduced from mice expressing interferon beta. *Gene Ther* 2000;7:817–25. [PubMed: 10845718]
85. Yushchenko M, Mader M, Elitok E, Bitsch A, Dressel A, Tumani H, Bogumil T, Kitzke B, Poser S, Weber F. Interferon-beta-1 b decreased matrix metalloproteinase-9 serum levels in primary progressive multiple sclerosis. *J Neurol* 2003;250:1224–8. [PubMed: 14586607]

86. Fox RJ, Kivisakk P, Lee JC, Tucky B, Lucchinetti C, Rudick RA, Ransohoff RM. Chemokine receptors as biomarkers in multiple sclerosis. *Dis Markers* 2006;22:227–33. [PubMed: 17124344]
87. Dickensheets HL, Donnelly RP. Inhibition of IL-4-inducible gene expression in human monocytes by type I and type II interferons. *J Leukoc Biol* 1999;65:307–12. [PubMed: 10080532]
88. Reder AT, Velichko S, Yamaguchi KD, Hamamcioglu K, Ku K, Beekman J, Wagner TC, Perez HD, Salamon H, Croze E. IFN-beta1b induces transient and variable gene expression in relapsing-remitting multiple sclerosis patients independent of neutralizing antibodies or changes in IFN receptor RNA expression. *J Interferon Cytokine Res* 2008;28:317–31. [PubMed: 18547162]
89. Dayal AS, Jensen MA, Lledo A, Arnason BG. Interferon-gamma-secreting cells in multiple sclerosis patients treated with interferon beta-1b. *Neurology* 1995;45:2173–7. [PubMed: 8848188]
90. Unkeless JC, Jin J. Inhibitory receptors, ITIM sequences and phosphatases. *Curr Opin Immunol* 1997;9:338–43. [PubMed: 9203414]
91. Johnson KG, LeRoy FG, Borysiewicz LK, Matthews RJ. TCR signaling thresholds regulating T cell development and activation are dependent upon SHP-1. *J Immunol* 1999;162:3802–13. [PubMed: 10201897]
92. Kilgore NE, Carter JD, Lorenz U, Evavold BD. Cutting edge: dependence of TCR antagonism on Src homology 2 domain-containing protein tyrosine phosphatase activity. *J Immunol* 2003;170:4891–5. [PubMed: 12734331]
93. Wasserman HA, Beal CD, Zhang Y, Jiang N, Zhu C, Evavold BD. MHC variant peptide-mediated anergy of encephalitogenic T cells requires SHP-1. *J Immunol* 2008;181:6843–9. [PubMed: 18981103]
94. Stefanova I, Hemmer B, Vergelli M, Martin R, Biddison WE, Germain RN. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol* 2003;4:248–54. [PubMed: 12577055]
95. Cyster JG, Goodnow CC. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity* 1995;2:13–24. [PubMed: 7600299]
96. Huang ZY, Hunter S, Kim MK, Indik ZK, Schreiber AD. The effect of phosphatases SHP-1 and SHIP-1 on signaling by the ITIM- and ITAM-containing Fcgamma receptors FcgammaRIIB and FcgammaRIIA. *J Leukoc Biol* 2003;73:823–9. [PubMed: 12773515]
97. Pao LI, Lam KP, Henderson JM, Kutok JL, Alimzhanov M, Nitschke L, Thomas ML, Neel BG, Rajewsky K. B cell-specific deletion of protein-tyrosine phosphatase Shp1 promotes B-1a cell development and causes systemic autoimmunity. *Immunity* 2007;27:35–48. [PubMed: 17600736]
98. Christophi GP, Hudson CA, Panos M, Gruber R, Massa PT. Modulation of macrophage infiltration and inflammatory activity by the phosphatase SHP-1 in virus-induced demyelinating disease. *J Virol* 2008
99. Kim CH, Qu CK, Hangoc G, Cooper S, Anzai N, Feng GS, Broxmeyer HE. Abnormal chemokine-induced responses of immature and mature hematopoietic cells from motheaten mice implicate the protein tyrosine phosphatase SHP-1 in chemokine responses. *J Exp Med* 1999;190:681–90. [PubMed: 10477552]
100. Forget G, Matte C, Siminovitch KA, Rivest S, Pouliot P, Olivier M. Regulation of the Leishmania-induced innate inflammatory response by the protein tyrosine phosphatase SHP-1. *Eur J Immunol* 2005;35:1906–17. [PubMed: 15902687]
101. Zhang Q, Wang HY, Marzec M, Raghunath PN, Nagasawa T, Wasik MA. STAT3- and DNA methyltransferase 1-mediated epigenetic silencing of SHP-1 tyrosine phosphatase tumor suppressor gene in malignant T lymphocytes. *Proc Natl Acad Sci U S A* 2005;102:6948–53. [PubMed: 15870198]
102. Tanabe Y, Nishibori T, Su L, Arduini RM, Baker DP, David M. Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes. *J Immunol* 2005;174:609–13. [PubMed: 15634877]
103. Yang CH, Shi W, Basu L, Murti A, Constantinescu SN, Blatt L, Croze E, Mullersman JE, Pfeffer LM. Direct association of STAT3 with the IFNAR-1 chain of the human type I interferon receptor. *J Biol Chem* 1996;271:8057–61. [PubMed: 8626489]

Figure 1.

SHP-1 protein and mRNA expression in freshly drawn PBMCs. SHP-1 levels were quantified in PBMCs of normal subjects ($n=32$), untreated RR MS patients ($n=30$), and *in vivo* three-month treated RR MS patients with interferon β -1a (Rebif). A. SHP-1 levels were measured with intracellular flow cytometry and the overlay histograms are labeled as follows: filled histogram for isotype control, solid line for normal subject, dashed line for MS patient, and dotted line for IFN- β treated MS patient. B. SHP-1 expression in normal subjects (C), MS patients (MS), and IFN- β treated MS patients (MS-Tx) was measured by flow cytometry and was quantified using the Mean Fluorescence Intensity (MFI). C. Western blot analysis of SHP-1 and actin in freshly isolated PBMCs from normal subjects, MS patients, and IFN- β treated MS

patients. Five MS patients are shown and SHP-1 protein expression was quantified in each patient before and after IFN- β treatment. D–G. Similarly, SHP-1 mRNA levels were quantified in freshly isolated PBMCs of normal subjects, MS patients, and IFN- β treated MS patients. The absolute mRNA transcript copy numbers per 10ng of total RNA were quantified using real time RT-PCR and were normalized to the levels of normal subjects that are shown as 1. D. Using primers in the mid-region of the SHP-1 gene that would amplify both SHP-1 transcripts, the levels of the common SHP-1 transcripts were measured. E. The mRNA levels of the housekeeping gene GAPDH were quantified as a loading control. Sequence-specific primers to the SHP-1 gene were used to quantify the expression of the F. SHP-1 promoter I transcripts and D. the SHP-1 promoter II transcripts.

Figure 2.

The activation of the transcription factors STAT1, STAT6, and NF- κ B was quantified in freshly isolated PBMCs from normal subjects ($n=32$), untreated RR MS patients ($n=30$), and *in vivo* three-month treated RR MS patients with interferon β -1a (Rebif). A & B. Phosphorylated STAT1 (pSTAT1) and phosphorylated STAT6 (pSTAT6) levels were quantified in freshly isolated PBMCs normal subjects (C), MS patients (MS), and IFN- β treated MS patients (MS-Tx) with intracellular flow cytometry. Cells were fixed, permeabilized, and stained with antibodies against either against pSTAT1 or pSTAT6 and Mean Florescence Intensity (MFI) was measured with flow cytometry. The overlay histograms are labeled as follows: filled histogram for isotype control, solid line for normal subject, dashed line for MS patient, and

dotted line for IFN- β treated MS patient. pSTAT1 and pSTAT6 levels in normal subjects, MS patients, and IFN- β treated MS patients were measured by flow cytometry and was quantified using the MFI. C. The NF- κ B activation was also quantified in normal subjects, MS patients, and IFN- β treated MS patients. Nuclear extracts were isolated and allowed to bind an NF- κ B consensus-binding sequence. Bound NF- κ B was then detected by a p65 specific antibody and quantified based on a calibration curve generated by using a purified p65 recombinant protein. The NF- κ B DNA binding activity is reported as ng of bound p65 protein per 10 μ g of nuclear extracts.

Figure 3.

SHP-1 protein and mRNA expression in cultured PBMCs of normal subjects ($n=32$) and MS patients (untreated RR and SP MS patients, $n=40$) before and after 24hr in vitro treatment with IFN- β . A. SHP-1 levels in cultured PBMCs before and after IFN- β treatment were measured with intracellular flow cytometry and the overlay histograms are labeled as follows: filled histogram for isotype control, solid line for normal subject, dashed line for MS patient, and dotted line for IFN- β treated MS patient. B. SHP-1 expression in normal subjects and MS patients was quantified using the Mean Fluorescence Intensity (MFI). C. The common SHP-1 transcripts levels were quantified in cultured PBMCs of normal subjects and MS patients before and after 24hr IFN- β treatment using real time RT-PCR and were normalized to the levels of

normal subjects that are shown as 1. In addition, the common SHP-1 transcript mRNA levels in cultured PBMCs of patients with secondary progressive (SP) MS, relapsing-remitting (RR) MS, and in vivo IFN- β -treated RR MS patients are shown before and after 24 hour *in vitro* treatment with IFN- β . D & E. Sequence-specific primers to the SHP-1 gene were used to quantify the expression of the SHP-1 promoter I and promoter II transcripts. F. RT PCR of control housekeeping gene GAPDH.

Figure 4.

CD4 and CD8 T-cells, B-cells, and NK cells were sorted from cultured PBMCs of normal subjects (n=12) and MS patients (n=18) using a fluorescence-activated cell sorter (FACS). Before sorting, cultured PBMCs were treated with media alone or with IFN-β for 24 hours. Total RNA was extracted from the sorted aliquots the absolute levels of the SHP-1 mRNA were quantified with real-time RT-PCR. A. The levels of the common SHP-1 transcripts and B & C. the SHP-1 promoter I and promoter II transcripts were quantified. (a indicates significance of $P < 0.05$ versus the untreated normal subjects and b indicates significance of $P < 0.05$ versus the untreated MS patients).

Figure 5.

The activation of the transcription factors STAT1, STAT6, and NF-κB was quantified in cultured PBMCs from normal subjects (n=32) and untreated MS patients (n=40). Cultured PBMCs were pre-treated with IFN-β for 24 hours followed by 1-hour cytokine treatment with IFN-γ, IL-4, or TNF-α. A & B. Phosphorylated STAT1 (pSTAT1) and phosphorylated STAT6 (pSTAT6) levels were quantified in cultured PBMCs with intracellular flow cytometry. Cells were fixed, permeabilized, and stained with antibodies against either pSTAT1 or pSTAT6 and Mean Fluorescence Intensity (MFI) was quantified. C. NF-κB activation was also quantified in cultured PBMCs before and after IFN-β pre-treatment and following TNF-α stimulation. Nuclear extracts were isolated and allowed to bind an NF-κB consensus-binding

sequence. Bound NF- κ B was then detected by a p65 specific antibody and quantified based on a calibration curve generated by using a purified p65 recombinant protein. The NF- κ B DNA binding activity is reported as ng of bound p65 protein per 10 μ g of PBMC nuclear extracts.

Figure 6.

The expression of STAT1-, STAT6-, and NF- κ B-responsive genes was quantified in cultured PBMCs from normal subjects (n=32) and untreated MS patients (n=40). Cultured PBMCs were pre-treated with IFN- β or media for 24 hours followed by 18-hour cytokine treatment with IFN- γ , IL-4, or TNF- α . Then total RNA was isolated and the mRNA levels of several inflammatory genes were quantified by real-time RT-PCR. The mRNA levels were normalized to the levels of PBMCs of untreated normal subjects that are shown as 1. A & B. The levels of the STAT1-responsive chemokine IP-10 and caspase 1 are shown following IFN- γ treatment. C & D. The levels of the STAT6-responsive chemokine TARC and the protease ADAM8 are shown following IL-4 treatment. E. The levels of the NF- κ B-responsive cytokine IL-6 were quantified with ELISA and are shown following TNF- α treatment. F. The levels of the NF- κ B responsive cyclooxygenase 2 are shown following TNF- α treatment.

Figure 7.

The activation of the transcription factors STAT1, STAT6, and NF-κB was quantified in SHP-1 siRNA or control scramble sequence siRNA treated cultured PBMCs from normal subjects (n=17) and untreated MS patients (n=19). SHP-1 or control siRNA treated cultured PBMCs were pre-treated with IFN-β for 24 hours followed by 1-hour cytokine treatment with IFN-γ, IL-4, or TNF-α. A. Western blot analysis of SHP-1, arginase-I, and actin in scramble/control or SHP-1 siRNA treated PBMCs that were treated with IFN-β for 24 hours. B. SHP-1 protein expression in scramble/control or SHP-1 siRNA treated PBMCs that were treated with IFN-β for 24 hours was further quantified with intracellular flow and was quantified using the MFI. C – F. Tyrosine phosphorylated STAT1 (pSTAT1) and tyrosine phosphorylated STAT6 (pSTAT6) levels were quantified in cultured PBMCs with intracellular flow cytometry. Cells were fixed, permeabilized, and stained with antibodies against either against pSTAT1 or pSTAT6 and Mean Fluorescence Intensity (MFI) was quantified. G & H. NF-κB activation was also quantified in cultured PBMCs before and after IFN-β pre-treatment and following TNF-α stimulation. Nuclear extracts were isolated and allowed to bind an NF-κB consensus-binding sequence. Bound NF-κB was then detected by a p65 specific antibody and quantified based on a calibration curve generated by using a purified p65 recombinant protein. The NF-κB DNA binding activity is reported as ng of bound p65 protein per 10μg of PBMC nuclear extracts.

Figure 8.

The expression of IP-10, CCL17, and IL-6 was quantified in SHP-1 siRNA or control scramble sequence siRNA treated cultured PBMCs from normal subjects (n=17) and untreated MS patients (n=19). SHP-1 or control siRNA treated cultured PBMCs were pre-treated with IFN- β for 24 hours followed by 18-hour cytokine treatment with IFN- γ , IL-4, or TNF- α . The mRNA levels were normalized to the levels of PBMCs of scramble siRNA treated normal subjects that are shown as 1. A & B. The levels of the STAT1-responsive chemokine IP-10 are shown following IFN- γ treatment. C & D. The levels of the STAT6-responsive chemokine TARC are shown following IL-4 treatment. E & F. The levels of the NF- κ B-responsive cytokine IL-6 were quantified with ELISA and are shown following TNF- α treatment.

Figure 9.

Schematic summarizing the findings of this study and depicting the possible role of SHP-1 in multiple sclerosis following interferon- β treatment. MS patients display increased levels of the pro-inflammatory cytokines TNF- α , IL-4/IL-13, and IFN- γ that in turn can promote activation of the transcription factors NF- κ B, STAT6, and STAT1. This transcription factors can be pathogenic in MS through the upregulation of several inflammatory genes. On the other hand, interferon- β treatment in MS was shown to attenuate this inflammatory response and this study demonstrates that the induction of the phosphatase SHP-1 by interferon- β in leukocytes could mediate a large part of the anti-inflammatory effects of interferon- β . Increased SHP-1 levels can negatively regulate NF- κ B, STAT6, and STAT1 signaling and their corresponding inflammatory gene expression. One possible drawback of interferon- β treatment in MS is that it directly activates STAT1 and STAT-1 responsive genes, which could be partially responsible for the side effects and/or incomplete response of interferon- β therapy in MS.

Biometric data of MS patients and normal subjects used in the study. MS patients were subdivided based on the clinical sub classification, Relapsing Remitting (RR), or Secondary Progressive (SP) multiple sclerosis. The data are shown in mean value $\pm$ SD. For the gender F stands for females and M stands for males. The age at onset of clinically diagnosed MS and disease duration are shown in years. The clinical symptoms were measured on Kurtzke's Expanded Disability Status Scale (EDSS) at the time the blood was drawn. For the *in vivo* study, patients with RR MS gave blood before and after a three-month treatment with recombinant interferon β -1a (Rebif) and EDSS scores shown here were calculated before treatment with interferon β -1a.

Table 1

Patient Category	Number of Subjects	Age (Years)	Gender	Age at Onset	Disease Duration	EDSS Score
Normal Subjects	32	41 $\pm$ 12	22F, 10M	---	---	---
Total MS Patients	40	45 $\pm$ 12	28F, 12M	36 $\pm$ 9	8.8 $\pm$ 5.6	3.6 $\pm$ 2.3
RR MS	30	43 $\pm$ 10	20F, 10M	37 $\pm$ 9	5.1 $\pm$ 3.4	2.5 $\pm$ 1.4
SP MS	10	51 $\pm$ 13	8F, 2M	29 $\pm$ 4	17 $\pm$ 8	6.3 $\pm$ 2.7

Table II

Inflammatory profile of normal subjects, untreated RR MS patients, and *in vivo* three-month treated RR MS patients with IFN- β -1a. Several inflammatory indicators were quantified in the plasma or in freshly isolated PBMCs of normal subjects (n=32), untreated RR MS patients (n=30), and interferon β -treated RR MS patients (Rebif). The levels of the cytokines IFN- γ , TNF- α , IL-6, IL-4, and IL-13 were quantified in the plasma by ELISA and are recorded in pg cytokine per mL plasma. The mRNA levels of chemokine CXCL10/IP-10, Caspase 1, the chemokine CCL17/TARC, the protease ADAM8, cyclooxygenase 2 (COX-2), the protease MMP9, and the chemokine receptors CXCR3 and CCR4 were quantified by real-time RT-PCR in freshly isolated PBMCs of normal subjects, MS patients, and IFN- β treated MS patients. The mRNA levels were normalized to the levels of normal subjects that are shown as 1. The levels of the arginase enzymatic activity was quantified in freshly isolated PBMCs and the levels of nitrate (indicative of nitric oxide) were quantified in the plasma and are shown relative to the levels of normal subjects designated as 1.

	Gene	Normal Subjects	MS Patients	IFN- β Tx MS Patients
Cytokine Levels in Plasma (pg/mL)	IFN- γ	10 $\pm$ 4	569 $\pm$ 207 [†]	467 $\pm$ 262
	IL-4	12 $\pm$ 7	201 $\pm$ 81 [†]	207 $\pm$ 102
	IL-13	67 $\pm$ 26	500 $\pm$ 175 [†]	232 $\pm$ 96 [*]
	TNF- α	33 $\pm$ 8	390 $\pm$ 156 [†]	243 $\pm$ 92
	IL-6	18 $\pm$ 5	152 $\pm$ 28 [†]	97 $\pm$ 25 [*]
mRNA levels (relative to normal subjects)	IP-10	1 $\pm$ 0.2	3.2 $\pm$ 1.3 [†]	12.4 $\pm$ 3.3 [*]
	Caspase 1	1 $\pm$ 0.3	2.5 $\pm$ 0.6	6.3 $\pm$ 2.0 [*]
	CCL17	1 $\pm$ 0.2	3.2 $\pm$ 0.5 [†]	1.7 $\pm$ 0.4 [*]
	ADAM8	1 $\pm$ 0.4	4.9 $\pm$ 1.0 [†]	2.3 $\pm$ 1.2 [*]
	IL-4 α	1 $\pm$ 0.3	3.2 $\pm$ 1.4 [†]	1.9 $\pm$ 0.8
	Arginase I	1 $\pm$ 0.2	3.7 $\pm$ 0.6 [†]	2.1 $\pm$ 0.9 [*]
	COX-2	1 $\pm$ 0.1	6.7 $\pm$ 2.4 [†]	3.8 $\pm$ 1.3
	MMP9	1 $\pm$ 0.2	4.2 $\pm$ 0.9 [†]	2.2 $\pm$ 0.7 [*]
	CXCR3	1 $\pm$ 0.2	1.7 $\pm$ 0.5	1.3 $\pm$ 0.2
	CCR4	1 $\pm$ 0.3	1.5 $\pm$ 0.4	1.2 $\pm$ 0.7
Enzyme Activity	Arginase	1 $\pm$ 0.1	2.1 $\pm$ 0.3 [†]	1.3 $\pm$ 0.2 [*]
Plasma levels	Nitric oxide	1 $\pm$ 0.3	1.4 $\pm$ 0.6	1.9 $\pm$ 0.5

The values indicate statistical means $\pm$ standard error values,

[†] indicates significance of P<0.05 between normal subjects and untreated MS patients and

^{*} indicates significance of P<0.05 between untreated and *in vivo* IFN- β treated MS patients