

## Original Article

# IFN- $\beta$ alters neurotrophic factor expression in T cells isolated from multiple sclerosis patients - implication of novel neurotensin/NTSR1 pathway in neuroprotection

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**Abstract:** Inflammation in relapsing remitting multiple sclerosis (RRMS) is hypothesized to provide neuroprotective effects via altered cytokine/neurotrophin homeostasis. The distinct neurotrophin production from specific cell populations has not been systematically studied and is likely of high yield in understanding the complex regulation of MS pathogenesis. Here, we describe how the mainstream therapy interferon- $\beta$  (IFN- $\beta$ ) modulates neurotrophin expression in T cells isolated from RRMS patients and characterize the neuroprotective capabilities of these factors. We utilize SuperArray gene screen technology to investigate the neurotrophin expression profile of T cells. We demonstrate that IFN- $\beta$  induces an anti-inflammatory cytokine expression pattern in T cells. Additionally, IFN- $\beta$  upregulates the expression of a novel neurotrophin receptor, the neurotensin high affinity receptor 1 (NTSR1). NTSR1 is expressed in active demyelinating lesions. Furthermore, we demonstrate that the receptor agonist neurotensin is a potent inducer of human neural stem/progenitor cell survival. Our findings highlight the importance of neurotrophin receptors in RRMS and offer insight into disease pathogenesis as well as the mechanisms of action of IFN- $\beta$ .

**Keywords:** Multiple sclerosis, interferon-beta, T cells, neurotensin high affinity receptor 1, neural progenitor cells, neuroprotection

## Introduction

Although T cell dense inflammatory attacks are hypothesized to drive neurodegeneration in relapsing remitting multiple sclerosis (RRMS) [1], recent evidence suggests that inflammation may also be beneficial and neuroprotective via altered neurotrophin regulation [2, 3]. Elevated neurotrophin levels have been identified in MS inflammatory processes, but studies are limited to *in situ* lesion slices [4-6], whole peripheral blood monocytes (PBMC) and serum samples [7-12], and cerebrospinal fluid (CSF) [13-16]. The distinct neurotrophin contributions from specific cell populations has not been systematically studied and is likely of high yield in understanding the complex regulation of RRMS pathogenesis. For example, T cells may participate in the destructive RRMS inflammatory response, yet T cell infiltration is also required

for the process of nerve injury and repair [17]. Characterizing the molecular basis of this seemingly paradoxical phenomenon may exploit key regulatory pathways that drive disease progression as well as reveal insight into mechanisms that promote neural protection and repair.

Interferon-beta (IFN- $\beta$ ) is a mainline therapy in RRMS and is hypothesized to function by modulating the immune response [18]. Altered neurotrophin homeostasis is likely one of the predominating mechanisms behind these actions as elevated levels of brain-derived neurotrophic factor (BDNF), glia cell-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) are seen in IFN- $\beta$  responding patients [8, 19-22]. However, the identity of the cells that produce these factors remains elusive. T cells are potential candidates as a main mechanism of action of IFN- $\beta$  is to reduce the level of T cell

activation thereby decreasing inflammation [18]. Investigating how IFN- $\beta$  modulates T cell neurotrophin expression therefore represents a novel approach to exploit key factors that are functioning to reduce inflammation as well as promote neuroprotection during RRMS inflammatory attacks. If the anti-inflammatory factors are coming from T cells, it is likely that IFN- $\beta$  is acting directly on and inhibiting the elusive pathways that initiate inflammation; however if the cytokines and neurotrophins are coming from elsewhere their increased production is likely to be a complex response of other cells to the T cell driven inflammation.

In this study, we isolate a pure T cell population from RRMS patients treated and untreated with IFN- $\beta$  to investigate the direct role of IFN- $\beta$  in altering T cell neurotrophin homeostasis. We use SuperArray screening to characterize neurotrophin production at the mRNA level. Our study goals are two-fold. First, we aim to confirm previous findings that describe how IFN- $\beta$  alters T cell neurotrophin expression through the use of a pure T cell population. Second, we aim to identify novel neurotrophins produced by T cells and test if these factors promote neuroprotection. We confirm the anti-inflammatory neurotrophin profile of T cells induced by IFN- $\beta$  and we identify the neurotensin pathway as a potent inducer of neural progenitor cell survival, cells capable of remyelination. Our findings highlight the interesting roles that neurotrophin receptors may play in RRMS.

### Materials and methods

#### *Patient population*

The University of Vermont IRB approved the study and all subjects gave informed consent. IFN- $\beta$  treated RRMS patients [N=8, 6 females and 2 males, Age: 35  $\pm$  6, EDSS: 2.5  $\pm$  1.1, Disease Duration (years): 5.6  $\pm$  3.1], non-treated RRMS patients [N=6, 5 females and 1 male, Age: 34  $\pm$  5, EDSS: 2.2  $\pm$  0.9, Disease Duration: 5.0  $\pm$  3.2], and 5 healthy controls [N=5, 4 female and 1 male, Age 33  $\pm$  12] were recruited for this study from the Multiple Sclerosis Center at Fletcher Allen Health Care, affiliated with the University of Vermont in Burlington, VT. Diagnosis of MS was established based on clinical and MRI findings.

Heparinized whole blood was obtained from each patient. No patient received corticoste-

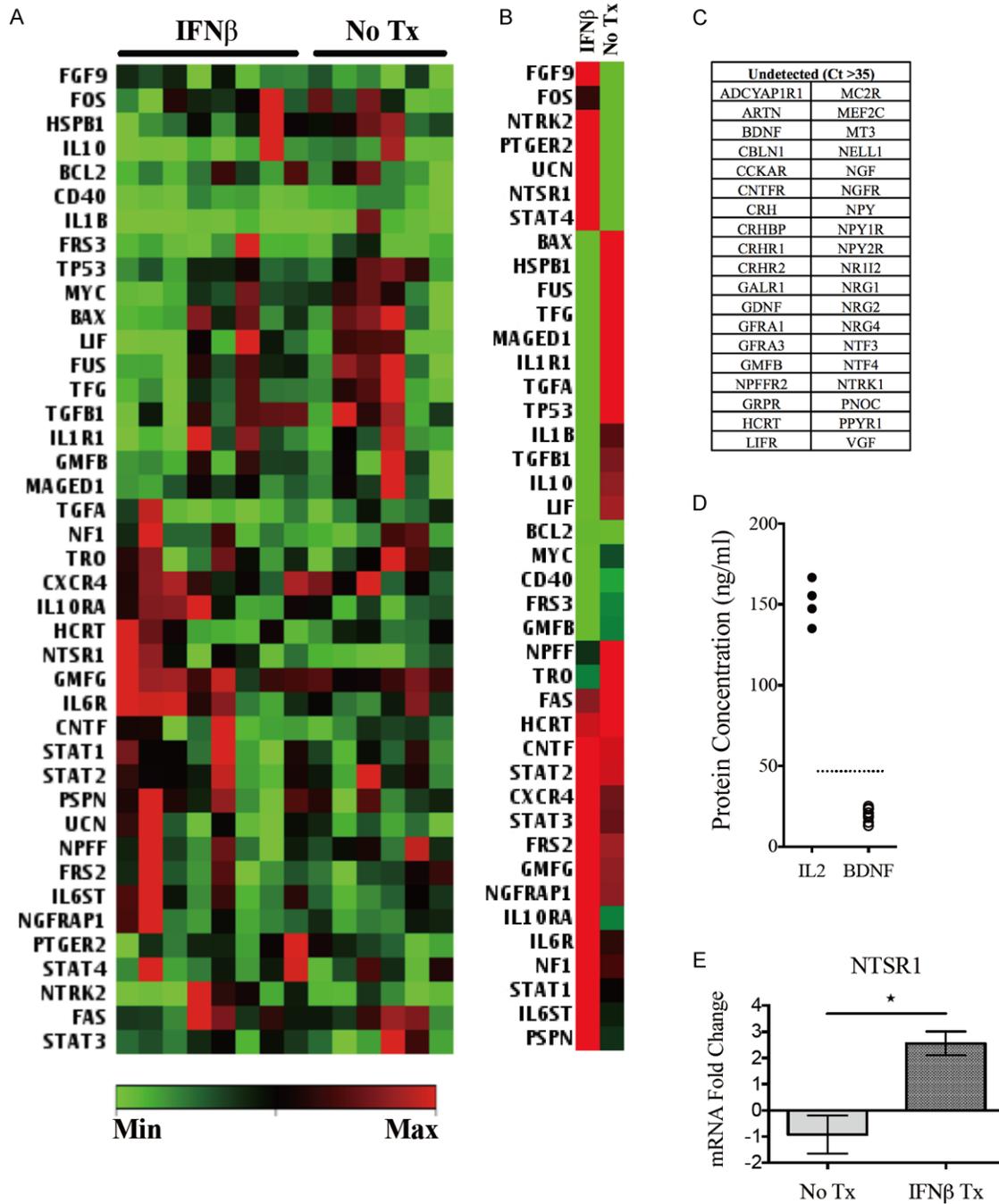
roid treatment prior to the blood draw. IFN- $\beta$  treated patients had received high dose IFN- $\beta$ -1a (Rebif<sup>®</sup>, EMD Serono), 44 mcg three times weekly for at least one year; untreated patients had been without IFN- $\beta$ -1a or other immunotherapy for at least one year. Blood was drawn between 20-24 hours after the patient's last IFN- $\beta$ -1a injection.

#### *Isolation, activation and culture of T cells from PBMC*

PBMCs were obtained by standard Ficoll gradient centrifugation from whole blood. T cells were isolated from this population using a Pan T cell isolation kit (Miltenyi Biotech) according to manufacturer protocol. In brief, non-T cells were bound with biotin conjugated antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and glycophorin A. These cells were captured with anti-biotin microbeads and magnetically depleted via column pass-through (negative selection) to produce a highly pure (90-97%) population of T cells. Population purity was confirmed by flow cytometry using human T cell receptor (TCR) staining (FITC-conjugated mouse anti-human TCR $\alpha\beta$ , 1:5, BD Pharmingen). Isolated T cells were cultured in T cell complete media (HPMI media, 10% FCS, 2.5 g/L glucose, 2 nM glutamine, 10  $\mu$ g/ml folate, 1 mM pyruvate, 50  $\mu$ M 2-mercaptoethanol). Cultures were stimulated with anti-human CD3 and CD28 monoclonal antibodies (5  $\mu$ g/10<sup>6</sup> cells) and cultured for 48 hours, at 37°C and 5% CO<sub>2</sub>. Prior to RNA extraction, all cells were collected via centrifugation and the supernatants immediately frozen at -80°C for protein analysis.

#### *mRNA isolation and profiling*

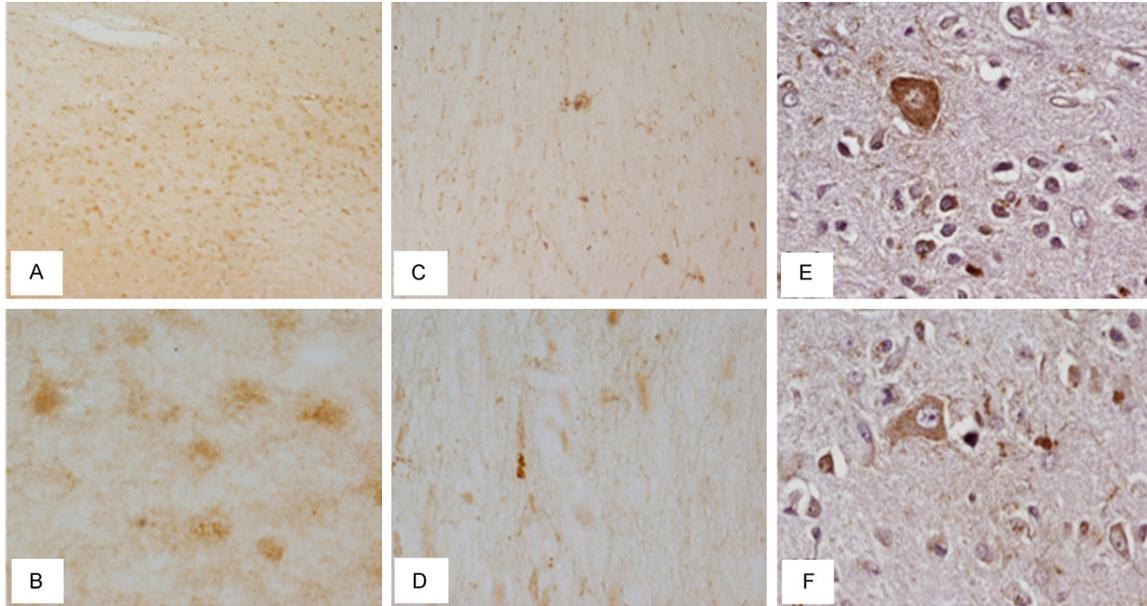
RNA was extracted from T cells using the RNeasy RNA Extraction Kit (Qiagen) via manufacturer protocol. RNA integrity was assessed with using Agilent 2100 Bioanalyzer. RNA samples were reverse-transcribed to cDNA using the SuperArray RT<sup>2</sup> First Strand Kit (Invitrogen), according to manufacturer protocol. Absence of genomic DNA contamination was confirmed by RTq-PCR. cDNA samples were run on the SuperArray plate "Neurotrophins and Receptors" (PAHS-31A, SABiosciences) using Applied Biosystems 7900HT, assaying for 84 neurotrophic factors and receptors, and 5 house-keeping genes. Results were quantified using



**Figure 1.** *NTSR1* is upregulated in IFN β-treated RRMS patients. T cell mRNA “Neurotrophin and Receptor” SuperArray analysis of MS patients treated and untreated with IFN-β. mRNA was isolated from pure T-cells from MS patients treated (N=8) and untreated (N=6) with IFN-β. SuperArray analysis characterized mRNA expression of neurotrophins and their receptors after 48 hours in CD3/CD28 stimulated culture, and results for each sample are expressed as the fold regulation of the specified gene compared to healthy controls (N=5). (A) Individual sample results; (B) Results collapsed by treatment group showing general trend; (C) Undetected genes, defined as Ct>35; Confirmation of culture activation was done via IL2 ELISA (D). (E) RT-qPCR analysis of *NTSR1* expression in T cells from MS patients treated (N=8) and untreated (N=6) with IFN-β. \*denotes significance, p<.05.

the  $2^{-\Delta\Delta Ct}$  method and expressed as a fold difference with respect to the healthy control

group. For all analysis, a Ct cutoff of >35 was used to define a gene as undetectable.



**Figure 2.** *NTSR1* is expressed in actively demyelinating lesions. An active demyelinating lesion was stained to characterize *NTSR1* expression. (A, B) Macrophages expressed high levels of *NTSR1* within the center of an actively demyelinating white matter lesion (A: 100x, B: 200x). Staining on CNS tissue was similar to normal white matter areas (C, D) (C: 100x, D: 200x). (E, F) Within the cortex, *NTSR1* was expressed predominantly by neurons in both cortical lesions (E) and in normal appearing cortex (F).

#### *BDNF and IL2 assay with enzyme-linked immunosorbent binding (ELISA)*

ELISA assays were performed to detect the presence of BDNF and IL2 in our cultures (R&D Systems, DY248 and BD550611), per manufacturer protocol. BDNF was not detectable above background levels. Standard curves were made using recombinant BDNF or IL2.

#### *Human brain tissue immunohistochemistry*

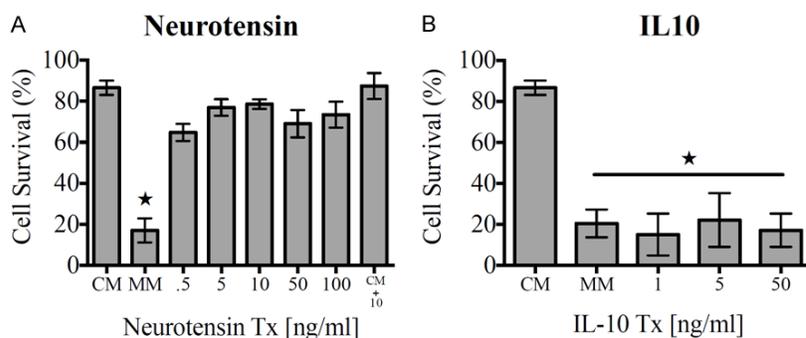
Sections of brain tissue from MS patients were processed as previously described [23]. Briefly, tissue sections were deparaffinized and hydrated with histoclear and a graded alcohol series. Sections were quenched (0.3% H<sub>2</sub>O<sub>2</sub>) and blocked, and then incubated with *NTSR1* primary antibody (Lifespan Biosciences #LS-A938; 1:50 dilution) overnight. A biotinylated secondary antibody solution was applied, and stain intensity was developed using VECTA-STAIN® ABC reagent and 3, 3'-diaminobenzidine (Vector Labs). Demyelinating lesions and normal appearing white matter [24, 25] were identified and representative pictures were obtained with a Nikon Eclipse TE 300 microscope.

#### *Human neural progenitor/stem cell culture*

Human neural progenitor cells (hNPCs) were cultured as previously described [26]. Human NPCs were expanded in “complete media” which is composed of *NeuroCult® NS-A Basal Medium* supplemented with both *NeuroCult® NS-A Proliferation Supplement*, 10 ng/ml bEGF, 10 ng/ml EGF and 0.0002% Heparin (all StemCell Technologies). Cultures were maintained at 37°C and 5% CO<sub>2</sub>. To assess the impact of neurotensin on cell viability, the expanded hNPCs were washed twice in EBSS to remove any remaining growth factors and the cells were treated with either IL10 (Peprotech; 1, 5, 10, and 50 ng/ml) or neurotensin (Sigma; 5, 10, 50 and 100 ng/ml) in minimal media (MM), which is *NS-A Basal media* supplemented only with *NeuroCult® NS-A Proliferation Supplement*, void of EGF and bFGF.

#### *Cell viability assay*

hNPCs were subjected to harsh, MM conditions for 2 days and cell survival and viability was assessed using a UV/Annexin flow cytometry assay [27]. UV live/dead dye (Invitrogen) labels necrotic cells while Annexin-alexa fluor 647 (Invitrogen) labels apoptotic cells. Staining was



**Figure 3.** *Neurotensin is a potent stimulator of hNPC survival.* hNPC cell viability was assessed using a UV/Annexin flow cytometry assay in growth factor deprived media (MM) as well as complete growth medium (CM), with or without treatment. Treatments were performed in MM conditions for 48 hours and began immediately after CM growth factor withdrawal. A: Neurotensin rescues hNPCs from undergoing apoptosis. Neurotensin does not exert any additional protective effect in the presence of EGF/FGF (far right, CM + 10). B: IL10 treatment does not rescue cells from undergoing apoptosis. \* $p < .05$ .

quantified via flow cytometry and cell survival is represented as the percent of UV and Annexin double negative cells over the total number of cells in the population. The assay was repeated for a total of N=4 biologic replicates.

#### Statistical analysis

SuperArray statistical analysis was done with the PCR Data Array Web Portal provided by SABiosciences. Pair-wise comparisons between groups of experimental replicates were performed. A  $p$ -value of  $< .05$  is considered to be potentially significant. Data was exported and significance tested for multiple comparisons with Dunnet's ANOVA (GraphPad Prism 6).

## Results

### *IFN- $\beta$ induces an anti-inflammatory cytokine and neurotrophin expression in T cells*

We used SuperArray gene screen technology to identify the neurotrophin mRNA profile of T cells isolated from RRMS patients treated and untreated with IFN- $\beta$  (**Figure 1A**- individual samples; **1B**- group averages; **1C**- undetectable genes). Our key finding is that IFN- $\beta$  treatment induces gene expression of neurotensin high affinity receptor 1 (NTSR1, **Figure 1E**). The second critical observation is that IFN- $\beta$  induced an anti-inflammatory cytokine profile, particularly on neurotrophin receptors (**Figure 1A, 1B**). Other observations include no T cell gene expression of BDNF, GDNF, and NGF in

any group and increased levels of the GDNF-like molecule persephin in many IFN- $\beta$  treated patients. While most genes formed distinct clusters between treated and untreated groups, we curiously observed similar clustering of a small number of upregulated genes in a subset of both treated and untreated patients. IL2 detection confirmed culture activation (**Figure 1D**).

### *NTSR1 is expressed in inflammatory demyelinating lesions*

We investigated NTSR1 expression in demyelinating lesions to further explore the clinical significance of this receptor. Cortical lesions and normal appearing white matter from actively demyelinating lesions were stained for NTSR1. In actively demyelinating areas, high NTSR1 expression localized to macrophages (**Figure 2A, 2B**) while lower expression was seen on some CNS cells in a pattern similar to normal appearing white matter (**Figure 2C, 2D**). Further investigation revealed these cells to be neurons (**Figure 2E, 2F**).

### *NTSR1 activation promotes neural stem/progenitor cell survival*

Neuroregeneration through neural progenitor/stem cell (NPC) activation has significant therapeutic potential as NPCs are multipotent and can differentiate into all CNS lineages [28]. Since T cell infiltration is necessary for efficient neuronal repair [17], we aimed to further characterize how our upregulated factors influence NPC physiology. Interestingly, NTSR1 expression is also upregulated on human NPCs when treated *in vitro* with IFN- $\beta$  [26]. We hypothesized that the global upregulation of this receptor may be an IFN  $\beta$ -induced protective response. Indeed, we demonstrate that the NTSR1 ligand, neurotensin (NT), potently induces hNPC survival at several doses (**Figure 3A**) in growth-factor deprived media that mimics the harsh MS inflammatory environment, characterized using a UV/annexin assay. NT does not have pro-apoptotic effects on hNPCs as cells

grown under optimal proliferative conditions showed no decrease in survival upon NT treatment (**Figure 3A**, far right CM + 10). In contrast, IL10 treatment was not able to rescue hNPCs from undergoing apoptosis (**Figure 3B**). We selected IL10 as this is an anti-inflammatory cytokine that plays a large role in reducing inflammation. Although IL10 is not consistently upregulated in our gene array, it is nonetheless expressed in our T cell population.

### Discussion

This study investigates the specific contributions of T cells to the production of neurotrophins during IFN- $\beta$  treatment in RRMS patients. Our critical finding highlights the neurotrophic capabilities of the NTSR1/NT pathway.

We have previously reported the neurotrophin expression profile of hNPCs when treated with IFN- $\beta$  in vitro [26]. We were intrigued to note that IFN- $\beta$  induced NTSR1 on hNPCs as well as T cells here. NTSR1 is also expressed in demyelinating lesions suggesting its potential role in the disease process (**Figure 2**). Based on these observations, the NTSR1 system was selected as our candidate gene for further investigation. We hypothesized that NTSR1 may play some neuroprotective role in RRMS pathogenesis during IFN- $\beta$  treatment. To test this hypothesis, we treated hNPCs with the NTSR1 agonist neurotensin (NT) in an assay that otherwise induces cell death. NT is a 13 amino-acid neurohormone/neuromodulator that is expressed mainly in the CNS and intestinal tract of mammalian species. In the assay, NT significantly promoted hNPC survival. Our findings demonstrate neurotensin as a potent inducer of hNPC survival.

This report is to our knowledge the first characterization of the NT/NTSR1 interaction in RRMS, and only a few reports examine NT in other models. NT inhibits the migration of CD4+ circulating T-lymphocytes in Sezary syndrome, and it is also known that NT is capable of inducing a response in T cells and PBMCs [29, 30]. These reports suggest that NT may also play an uncharacterized role in regulating the T cell response in RRMS. Additionally, we have previously demonstrated that NPCs modulate T cell survival and vice versa through neurotrophin and cytokine signaling [31]. The specific neurotrophins mediating this cross-talk may have therapeutic relevance in RRMS. Expanding

these findings here, we demonstrate that NT, but not IL10, promotes hNPC survival. Collectively, our findings support that the NT/NTSR1 may play novel roles in T cell and NPC interactions and understanding how the NT/NTSR1 system is involved in this feedback loop may reveal additional insights into RRMS pathogenesis. In summary, our results are consistent with the few reports in other models, and our identification of NTSR1/NT interactions between immune and CNS cells is deserving of future investigation.

As described above, NTSR1 was selected as our candidate gene for further study. However, we were able to detect additional gene expression changes in our SuperArray. Importantly, we detected statistically significant gene expression changes that parallel observations in PBMC populations [7-12] as well as novel T cell-specific neurotrophin changes in a healthy T cell culture (**Figure 1**). We are therefore confident that our reported data represent true observations. We observed two key findings amongst the other genes, which will be investigated further in additional studies. First, RNA and protein expression levels of BDNF were low (below assay sensitivity levels) in purified T cells (**Figure 1C** and **1D**). This suggests that BDNF is mainly produced from monocytes, B cells, or other cells in PBMC. We examined mRNA and protein after 48 hours in culture, which does not rule out earlier expression but supports that any T cell contribution of BDNF is tightly regulated. Although BDNF expression was low, NTRK2, the cognate receptor for BDNF, was upregulated. Second, we observed induction of a general anti-inflammatory response. For example, IFN- $\beta$  treatment upregulated STAT1, STAT4, IL10RA, IL6R and IL6ST expression. In contrast, the pro-inflammatory cytokine IL1B and its receptor IL1R1 were down-regulated. These observations support that IFN- $\beta$  acts through an immunomodulatory mechanism.

It is interesting that the most prominent neurotrophin changes in our study were generally observed on receptors, rather than soluble ligands. One hypothesis that describes this observation is that the critical neurotrophic receptors that promote immune cell regulation or neural protection in RRMS may always have some lower level of expression/activity. When this activity on CNS and immune cells eventu-

ally exhausts, the delicate balance between inflammation and neuroprotection is lost and a more progressive disease course ensues. Thus, IFN- $\beta$  may upregulate these factors to prolong their time to depletion.

In conclusion, we examined how IFN- $\beta$  regulates neurotrophin expression of a pure T cell population isolated from RRMS patients and identified the NT/NTSR1 system as a potent inducer of hNPC survival. Our findings support that IFN- $\beta$  plays an immunomodulatory role in RRMS, and support future studies that examine the additional roles of NT/NTSR1 signaling as well as other neurotrophin receptors in RRMS.

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### Disclosure of conflict of interest

None.

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