Type I interferon signature is high in lupus and neuromyelitis optica but low in multiple sclerosis

Xuan Feng, Nicholas P. Reder, Mounica Yanamandala, Addie Hill, Beverly S. Franek, Timothy B. Niewold, Anthony T. Reder, Adil Javed

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Objective: Neuromyelitis optica (NMO) is characterized by selective inflammation of the spinal cord and optic nerves but is distinct from multiple sclerosis (MS). Interferon (IFN)-β mitigates disease activity in MS, but is controversial in NMO, with a few reports of disease worsening after IFN-β therapy in this highly active disease. In systemic lupus erythematosus (SLE), IFNs adversely affect disease activity. This study examines for the first time whether serum IFN-α/β activity and IFN-β-induced responses in peripheral blood mononuclear cells (MNC) are abnormally elevated in NMO, as they are in SLE, but contrast to low levels in MS.

Methods: Serum type I IFN-α/β activity was measured by a previously validated bioassay of 3 IFN-stimulated genes (RT-PCR sensitivity, 0.1 U/ml) rather than ELISA, which has lower sensitivity and specificity for measuring serum IFNs. IFN responses in PBMC were assessed by in vitro IFN-β-induced activation of phospho-tyrosine-STAT1 and phospho-serine-STAT1 transcription factors, and MxA proteins using Western blots.

Results: Serum IFN-α/β activity was highest in SLE patients, followed by healthy subjects and NMO, but was surprisingly low in therapy-naïve MS. In functional assays in vitro, IFN-β-induced high levels of P-S-STAT1 in NMO and SLE, but not in MS and controls. IFN-β-induced MxA protein levels were elevated in NMO and SLE compared to MS.

Conclusions: Serum IFN activity and IFN-β-induced responses in PBMC are elevated in SLE and NMO patients versus MS. This argues for similarities in pathophysiology between NMO and SLE and provides an explanation for IFN-induced disease worsening in NMO.

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1. Introduction

Neuromyelitis optica (NMO) is an autoimmune disease characterized by selective inflammation of the spinal cord and optic nerves with sequelae of paralysis and blindness. It has been considered a variant of multiple sclerosis and is often treated with the same agents, but is presumed to be from enhancing autoantibody responses, particularly as it has been noted with drug-induced SLE-like syndromes [16,17]. In addition, growing evidence linking IFN pathway activation to aberrant gene activation, murine models, and results from therapeutic interventions underscore the pathological role for IFNs in connective tissue diseases [23].
There are no controlled studies showing whether IFN-β-induced responses in NMO are abnormally elevated. The studies herein systematically examine type I serum IFN activity and IFN-β-induced responses in NMO, SLE, MS, and healthy controls, demonstrating for the first time that IFN responses in NMO are more akin to those of SLE than to MS and healthy controls.

2. Methods

2.1. Patients

Serum type I IFN activity was examined in healthy controls (HC), partially treated NMO (NMO-P), fully treated NMO (NMO-Full), SLE, and RRMS patients (Fig. 1). Demographic information for these subjects is shown in Table 1. All NMO patients were positive for NMO-IgG. Patients in Fig. 1 had never been treated with interferons. Partially-treated NMO patients were those who had recently started (<1 month) immunosuppressive agents such as methotrexate or mycophenolate mofetil plus low-dose prednisone (20–40 mg) or those who had evidence of substantial lymphocyte recovery (>80%) from immunosuppressive agents such as rituximab. Fully-treated NMO patients were those who had been on several days of high-dose steroids (1 g methylprednisolone IV) or on rituximab therapy with absolute CD19 count of zero for the prior 6 months. All SLE patients were grouped together regardless of the intensity of immunosuppression, including therapeutic doses of mycophenolate mofetil, azathioprine, methotrexate, IV steroids or plasmapheresis that could affect IFN signaling. Stable RRMS (RRMS-s) patients were free from exacerbation or clinical disease progression for >6 months plus had no new activity on MRI. The demographic information for patient groups in Figs. 2–4 is shown in Table 1.

In all analyses subsequent to those shown in Fig. 1, NMO patients were classified as stable versus active disease rather than as partially and fully treated. Since high-dose IV steroids can inhibit IFN-α production in MS [24], the intent of this latter grouping was to exclude active patients that had just received acute treatments such as steroids or plasmapheresis that could affect IFN signaling. Stable NMO (NMO-s) patients were defined as those who had been free

![Image of Fig. 1](image1.png)

![Image of Table 1](table1.png)

![Image of Fig. 2](image2.png)
from any relapse and had been on a consistent therapeutic regimen, excluding steroids, for at least 3 months prior to blood draw. Active NMO (NMO-a) patients were undergoing a relapse at the time of analysis. Note all NMO-active patients were treated with high-dose steroids at the time of analysis, which likely decreasing IFN induced responses.

(B) SLE patients had higher P-Y-STAT1 than RRMS-stable (p=0.008) and NMO-stable (p=0.03). p-Values are comparisons to RRMS-stable. Significant lower P-S-STAT1 than RRMS-stable.

2.2. Serum IFN-α/β activity assay

Serum IFN-α/β activity was measured using a highly sensitive assay with a limit of detection of 0.1 U/ml, that allows detection of levels well below the typical 10–20 U/ml threshold used in ELISA. In addition, ELISA specificity for detecting serum IFN can be lower than the bioassay system used herein because ELISA detects cross-reacting serum proteins that have no IFN function [25]. Sera from 8 healthy controls, 14 therapy-naïve RRMS-stable patients, 9 clinically stable NMO patients, 7 clinically active NMO patients, and 8 SLE patients. For all in vitro stimulation experiments, IFN-β was used as the type I IFN because of its therapeutic relevance in treating MS, and sometimes the NMO spectrum of disorders. Stable NMO patients were analyzed as a group, despite differences in prophylactic treatments. This is because the intensity of IFN-β-induced protein expression did not depend on the type of immunosuppression.

MNCs were isolated with Ficoll density gradients. Transcription factors and IFN-regulated proteins were quantitated immediately ex vivo. In vitro, 5 × 10⁵ MNC/ml were in media alone or induced with IFN-β-1b at 160 U/ml for 0–2 h (STAT1) or 0–24 h (MxA) [26]. 0.33 million cells for each condition were lysed in Laemmlı buffer plus protease inhibitors (Sigma), and 1 mM Na orthovanadate, and then analyzed by Western blot as described previously [26]. P-Y-STAT1, P-S-STAT1, and MxA levels were normalized to actin expression and quantified with Genetools software (Philomath, OR).

IFN-β-induced MxA mRNA [29] and MxA protein on Western blots [26] are well-correlated. However, MxA protein rather than mRNA was used to examine IFN-β-induced responses because mRNA has a relatively short half-life and fluctuations are more likely to be missed. Antibodies: Actin (sc-1615, Santa Cruz Biotechnology), P-Y701-STAT1 (sc-7988, Santa Cruz Biotechnology), P-Ser-STAT1 (sc-16570-R), and MxA (Biogen).

Fig. 4. In vitro IFN-β-induced MxA protein at 0 h and 24 h in SLE (n=8), NMO-s (n=9), NMO-a (n=7), RRMS (n=14), and HC patients (n=8). All groups showed significant induction of MxA from 0 h to 24 h, with SLE and NMO-s showing the most robust differences from 0 h.

2.3. Dose-response and kinetics of IFN response

IFN-β-induced responses were examined in cultures of MNCs from 8 healthy controls, 14 therapy-naïve RRMS-stable patients, 9 clinically stable NMO patients, 7 clinically active NMO patients, and 8 SLE patients. For all in vitro stimulation experiments, IFN-β was used as the type I IFN because of its therapeutic relevance in treating MS, and sometimes the NMO spectrum of disorders. Stable NMO patients were analyzed as a group, despite differences in prophylactic treatments. This is because the intensity of IFN-β-induced protein expression did not depend on the type of immunosuppression.

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Fig. 3. Kinetics of IFN-β-induced P-S-STAT1 and P-Y-STAT1 in SLE (n=8), NMO-stable (n=9), NMO-active (n=7), and RRMS-stable (n=14). (A) Both SLE (p=0.0004) and NMO-stable (p<0.02) patients showed significantly higher P-S-STAT1 than RRMS-stable. NMO-active patients had significantly lower P-S-STAT1 than RRMS-stable (p<0.04). Note all NMO-active patients were treated with high-dose steroids at the time of analysis, likely decreasing IFN induced responses. (B) SLE patients had higher P-Y-STAT1 than RRMS-stable (p=0.0085) and NMO-stable (p<0.03). P-Values are comparisons to RRMS-stable using SAS PROC MIXED.
2.4. Statistics

SAS version 9.2 (SAS Institute, Inc., Cary, NC) was used for all statistical analyses. P-S- and P-Y-STAT1 measurements were square root-transformed and MxA measurements were log-transformed to satisfy normality requirements. Proc MIXED was used to compare repeated measure data between groups, as with IFN-α-induced P-S-STAT1 and P-Y-STAT1 kinetic responses. This approach is more appropriate and efficient than repeated measures ANOVA because it first models the within-subject variance and correlation structure of each repeated measure over time [43]. The estimated covariance structure is then used to correct for within-subject correlation when obtaining least squares estimates of treatment and time differences. Within-subject Pearson correlation coefficients between time points for P-S-STAT1 and P-Y-STAT1 were all above 0.60 and MxA was above 0.30, indicating that a regression approach that corrected for within-subject correlation was required. Multiple covariance structures (constant, exponential decay, etc.) were tested to determine best model fit. The compound symmetry covariance structure was chosen for modeling with Proc MIXED, as it yielded the best model fit, measured by AIC and BIC statistics. Each subject group was modeled separately to account for the possibility of different covariance structures among different disease states. Also, Pearson correlation coefficients were calculated using the maximum value for P-S-STAT1, P-Y-STAT1, or MxA for each subject. Correlations were also performed using P-S- and P-Y-STAT1 measurements at each time point, and similar correlation coefficients and p-values were produced regardless of the time point used.

3. Results

Serum type I IFN activity was much higher in SLE than in HC, untreated MS, and partially- and fully-treated NMO patients (Fig. 1). Partially-treated NMO patients had higher serum IFN activity than untreated MS patients (p = 0.015) and maximally immunosuppressed NMO patients (p = 0.021). Untreated MS patients had the lowest mean IFN activity compared to all other groups, and demonstrated significantly lower serum IFN activity than healthy donors (p < 0.0001). Overall, the results show markedly enhanced serum IFN activity in SLE, but a striking deficiency of serum IFN activity in MS. Serum IFN activity was abnormally elevated in NMO, despite partial immunosuppression.

In the next set of experiments, responses to IFN-β were examined by measuring intracellular P-S-STAT1, P-Y-STAT1, and MxA protein levels. P-S-STAT1 enhances IFN signaling. Levels of P-S-STAT1 and MxA protein are subnormal in immune cells from clinically-active, therapy-naïve MS patients [26]. Even though significant differences between the SLE and NMO groups versus MS were seen using the serum IFN activity assay, a more dramatic effect among these groups was noted in these intracellular assays.

First, IFN-β-induced P-S-STAT1 and P-Y-STAT1 responses in SLE and NMO were compared to HC. IFN-β-stimulated expression of P-S-STAT1 was significantly elevated in SLE (p = 0.0001) and clinically stable NMO patients (p = 0.0022) compared to HC (Fig. 2A). IFN-β-induced expression of P-Y-STAT1, however, was significantly elevated only in patients with SLE (p = 0.01), and not in NMO patients, versus HC (Fig. 2B).

Next, IFN-β-induced P-S-STAT1 and P-Y-STAT1 responses in SLE and NMO were compared to RRMS. IFN-β-induced P-S-STAT1 levels were higher in both SLE (p = 0.0004) and NMO-stable (p = 0.02) than in RRMS-stable (Fig. 3A). IFN-β-induced P-Y-STAT1 levels were even higher in SLE than in NMO-stable patients (p = 0.03). In contrast, patients in the NMO-active group had the lowest levels of IFN-β-induced P-S-STAT1 (Fig. 3A). A separate cohort of 14 therapy-naïve SPMS patients also had low P-S-STAT1 levels that were comparable to the NMO-active group and less than healthy controls (data not shown), confirming our previous observations of low intrinsic IFN activity in SPMS patients [26]. Finally, IFN-β-stimulated P-Y-STAT1 levels were significantly elevated in SLE (p = 0.008), but not in NMO-stable (p = 0.85) compared to RRMS (Fig. 3B).

MxA protein is downstream from P-S-STAT1 and P-Y-STAT1 and is a specific marker for responses to IFN-β therapy. IFN-β-induced robust expression of MxA protein in SLE, NMO, MS, and HC (Fig. 4). However, the magnitude of IFN-β-induced MxA responses was significantly higher in SLE (p = 0.02) and NMO-stable (p = 0.03) patients versus HC. IFN-β-induced MxA responses in the RRMS-stable group were not statistically different from HC (p = 0.08). In terms of absolute difference from baseline levels, both SLE (p = 0.01) and NMO-stable (p < 0.05) had a larger induction of IFN-β-induced MxA protein than RRMS-stable, but no difference was seen between the SLE and NMO groups (Fig. 5).

Three different outcome variables, P-S-STAT1, P-Y-STAT1, and MxA, were used to assess IFN-β-induced responses. These outcome variables are interdependent because they all take part in the signaling cascade for IFN-β-induced responses. Pearson correlations demonstrated a highly significant, though moderate correlation (r values from 0.48 to 0.57) among all three outcome variables.

4. Discussion

IFNs can alleviate certain diseases such as MS but worsen connective tissue diseases such as SLE. There is much interest in identifying biomarkers for diagnosis or predicting response to treatment early in the disease course. For example, there is preliminary evidence that increased serum IL-17F levels in MS before therapy are biomarkers for non-responsiveness to IFN-β treatment and disease worsening despite treatment with IFN-β [28]. Herein, type I IFN responses in NMO and MS were explored using both serum IFN-α/β levels and functional assays of IFN-α/β-induced responses in MNC. We identify a new approach for detecting the interferon signature in NMO and MS that can be further explored as a sensitive biomarker in these disease states.

Serum type I IFN activity was examined using a highly sensitive bioassay, an improvement over ELISA-based IFN detection assays, which have lower sensitivity and lower specificity because of serum contaminants [25]. SLE patients had the highest serum type I IFN activity (Fig. 1). This activity reflects high endogenous IFN-α levels because it can be blocked by antibodies to IFN-α [27]. In contrast, the untreated RRMS population had the lowest levels of endogenous IFN-α/β activity. For analysis of serum IFN activity in NMO, patients were divided into two groups, based on the intensity of immunosuppression they had received (as described in the Methods section). Serum IFN activity was higher in partially-treated NMO patients...
who had limited immune suppression compared to fully-treated NMO patients who were more completely immunosuppressed and to treatment-naïve MS patients. This suggests that in NMO patients, more complete immunosuppression can further reduce serum IFN activity.

In all subsequent analyses, NMO patients were grouped into stable versus active disease, rather than partial and fully treated groups (Figs. 2 and 3 vs. Fig. 1). High-dose steroids can inhibit IFN responses [24]. The intent of this grouping was to exclude NMO active patients who had just received acute treatments with steroids that could affect IFN signaling and depress cellular responses to IFN-β induction. IFN-β-induced cellular responses in NMO patients not exposed to recent steroids may more reflect intrinsic disease characteristics. All NMO-stable patients had been on a continuous therapeutic regimen (mycophenolate mofetil or rituximab) and none had received recent steroids for at least 3 months prior to blood draw. IFN-β-induced P-S-STAT1 and MxA were the lowest in NMO-active group compared to NMO-stable patients, supporting the hypothesis that steroids reduce IFN responses in NMO.

IFN-β-induced P-S-STAT1 was significantly higher in both SLE and NMO-stable patients compared to normal controls and MS patients. When MxA was used as a downstream marker of IFN activity, enhanced IFN-β-induced activity in SLE and NMO-stable patients was again seen in comparison to healthy controls. Elevated IFN-β-induced P-Y-STAT1 activity, however, was restricted only to SLE patients. The lack of statistically increased IFN-β-induced P-Y-STAT1 in the NMO group versus MS and controls could be due to a high degree of variability in IFN-β-induced responses in P-Y-STAT1 expression versus P-S-STAT1. A more likely possibility is that alteration in P-S-STAT1 expression is specific for MS due to low endogenous IFN α/β levels, with occasional exceptions [26,28]. Elevated P-Y-STAT1 expression may be more reflective of SLE disease state due to chronically high endogenous IFN α/β activity [9]. It is unlikely that the differences in the IFN-β-induced responses seen among SLE and NMO versus MS are due to changes in the IFN receptor expression on cell surface (Reder and Croze, 2005, unpublished). In NMO and MS, differential responses to IFN-β are observed in P-S-STAT1 and MxA levels, but receptor-induced P-Y-STAT1 is not altered, arguing against functional changes in receptor expression among SLE, NMO, and MS disease states. IFN receptor expression on the cell surface is decreased by chronic receptor stimulation, as may be the case with chronic IFN-β therapy [40–42]. The patients in this study were never treated with IFNs. Chronically elevated endogenous type I interferon levels as seen in SLE and NMO patients would be expected to down-regulate type IFN α/β receptors (IFNARs) and thereby reduce IFN-β-induced responses rather than enhance them [42].

In the MS patients, the basal IFN levels and IFN-β-induced MxA responses were very low (Figs. 1 and 4). We have previously shown that during disease activity and progression, MS patients have a further decline in IFN-β-induced P-S-STAT1 and MxA levels [26] and these observations were reconfirmed in the current study (data not shown). IFN therapy is likely to correct subnormal IFN responses in MS in many patients. This concept parallels studies where lower baseline IFN levels and lower MxA levels predict better responses to IFN therapy [28,29]. In this study, a dichotomy in the serum IFN α/β activity or the IFN-β-induced signature within the RRMS group was not seen as would be expected from a recent study showing worsening of MS disease despite IFN-β therapy in a small number of patients with endogenously elevated serum IFN-β and IL-17F levels measured by ELISA [28].

IFNs play an important role in the pathogenesis of connective tissue diseases such as SLE and Sjögren’s syndrome. Increased endogenous IFN activity is associated with more severe disease in SLE [11]. IFN-β treatment induces elevated levels of serum B-cell activating factor (BAFF) in NMO and MS [39]. Overexpression of BAFF in autoimmune diseases and in mice is linked to SLE and Sjögren-like disease [37,38]. IFN-induced BAFF could in turn elevate serum aquaporin-4 IgG (NMO-IgG) levels. IFNs, when used as treatment for NMO, have been reported to result in a severe relapse [18–22], although small series in this highly active disease may be misleading. The present findings of elevated type I IFN activity in NMO provide an explanation for IFN-induced disease worsening in NMO and caution against the use of IFNs as a treatment for confirmed NMO.

The studies herein describe type I serum IFN activity and IFN-β–induced cellular responses in a mixed cohort of treated and partially treated NMO patients. Given the urgency of treating NMO relapses and rarity of this illness, it is very challenging to find a large number of NMO patients naïve to any treatment. Despite immunosuppression used in these patients, type I serum IFN activity and IFN-β–induced cellular responses were consistently elevated in SLE, stable NMO, and partially-treated NMO patients. Without any immunosuppression, it is likely that these responses in NMO patients would be further augmented. In SLE, stable patients on the low-dose immunosuppressive therapy described in the Methods section did not influence the elevated serum type I IFN activity. SLE patients with active disease and treated with high-dose IV steroids and/or plasma exchange were not examined in this study, and would be analogous to the active NMO cohort. It remains speculative whether type I IFN responses are altered in SLE patients during intense acute versus low-dose chronic therapy. Nonetheless, a lowering of type I IFN responses during active NMO relapses cannot be entirely excluded.

In clinical practice, occasional patients are classified as NMO based on the initial presentation of longitudinally extensive myelitis (LEM) or severe optic neuritis, but have neither NMO-IgG antibodies nor additional brain lesions that resemble those seen in MS. There are also patients who have relapsing LEM and some features of Sjögren’s disease such as sicca symptoms, elevated SSA/B levels, or positive minor salivary gland biopsy, but negative NMO-IgG titers [7]. Patients who are difficult to classify as having NMO disease may benefit from their IFN activity evaluated prior to therapy, especially if IFN therapy is considered. A small subset of relapsing-remitting MS patients also has an excessive IFN-β signature and does not respond well to IFN-β therapy [28,30]. In addition to the cellular IFN responses described herein, IFN-induced gene responses determined by MxA responses [26,29,31] or Affymetrix or multiplex RNA/protein analysis [32,33] could predict response to IFN therapy in NMO and MS patients.

In summary, SLE and NMO patients have abnormally elevated serum IFN activity and in vitro cellular IFN-β–induced responses compared to MS patients. This further distinguishes NMO from MS, could also explain some pathological and clinical differences in the two types of diseases, and could aid in therapeutic or diagnostic decisions.

Author contribution

AJ had full access to the data and takes full responsibility for the integrity of the data and accuracy or data analysis. Study concept and design: AJ, ATR, TBN, and XF. Experimental procedures and analysis: XF, MY, AH, and BSF. Statistical analysis: NPR. Manuscript preparation: AJ, ATR, XF, and TBN. Study supervision: AJ, ATR, and XF.

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