Enhanced in Vivo Efficacy of a Type I Interferon Superagonist with Extended Plasma Half-life in a Mouse Model of Multiple Sclerosis

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Background: IFNβ constitutes an approved drug to treat multiple sclerosis (MS), but it has limited efficacy. Results: A modified human IFN variant, which exhibits both superagonist properties and 10-fold increased lifespan, outperforms IFNβ in an animal MS model. Conclusion: This drug candidate has potential to supersede IFNβ for the treatment of MS. Significance: Protein engineering allows development of more effective drugs to treat autoimmune diseases.

IFNβ is a common therapeutic option to treat multiple sclerosis. It is unique among the family of type I IFNs in that it binds to the interferon receptors with high affinity, conferring exceptional biological properties. We have previously reported the generation of an interferon superagonist (dubbed YNSo8) that is built on the backbone of a low affinity IFNα receptor (dubbed YNS1) but modified to exhibit higher receptor affinity than even for IFNβ. Here, YNSo8 was fused with a 600-residue hydrophilic, unstructured N-terminal polypeptide chain comprising proline, alanine, and serine (PAS) to prolong its plasma half-life via “PASylation.” PAS-YNSo8 exhibited a 10-fold increased half-life in both pharmacodynamic and pharmacokinetic assays in a transgenic mouse model harboring the human receptors, notably without any detectable loss in biological potency or bioavailability. This long-lived superagonist conferred significantly improved protection from MOG35–55-induced experimental autoimmune encephalomyelitis compared with IFNβ, despite being injected with a 4-fold less frequency and at an overall 16-fold lower dosage. These data were corroborated by FACS measurements showing a decrease of CD11b+/CD45hi myeloid lineage cells detectable in the CNS, as well as a decrease in IBA+ cells in spinal cord sections determined by immunohistochemistry for PAS-YNSo8-treated animals. Importantly, PAS-YNSo8 did not induce antibodies upon repeated administration, and its biological efficacy remained unchanged after 21 days of treatment. A striking correlation between increased levels of CD274 (PD-L1) transcripts from spleen-derived CD4+ cells and improved clinical response to autoimmune encephalomyelitis was observed, indicating that, at least in this mouse model of multiple sclerosis, CD274 may serve as a biomarker to predict the effectiveness of IFN therapy to treat this complex disease.

Multiple Sclerosis (MS) is a chronic immune disease of the CNS characterized by inflammation and demyelination within the brain and the spinal cord. This can lead to pathologies including visual disturbance, cognitive impairment, and paralysis (1). Epidemiological evidence supports that environmental factors play a significant role in triggering or facilitating the development of MS, although a genetic component is also evident for a subset of individuals (2). Genome-wide association studies have identified more than 100 potential MS risk alleles with a strong predisposition to genes involved in immune function, revealing a bias of genes relating to human leukocyte antigen and T-helper cell development or signaling (3, 4). These genetic findings provide support to a large body of immunological data that define MS as largely a T-cell-driven immune disease of the CNS (5).

IFNβ was the first drug to be approved for the treatment of relapsing-remitting MS and, to this day, remains a major therapeutic option for MS patients. Meta-analyses of clinical trials indicate that IFNβ therapy results in a significant reduction of MS relapses, and although its efficacy is less optimal than some

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The abbreviations used are: MS, multiple sclerosis; PAS, proline, alanine, and serine; EAE, autoimmune encephalomyelitis; qPCR, quantitative PCR; PK, pharmacokinetic; MOG, myelin oligodendrocyte glycoprotein; DPI, days post-induction; PD, pharmacodynamic; SPR, surface plasmon resonance; IFNAR, type I interferon receptor; HyBNAR, HyBrid IFNAR.
of the newer drugs that have recently entered the market, its safety profile is good. Although efficacy correlates with higher administered dose and increased injection frequency (6, 7), there is clear evidence that the response to IFNβ therapy differs greatly between individual patients. Therefore, much effort has been invested in determining predictive factors to separate good from bad responders but with limited success (8). IFNβ is a member of the type I interferon (IFN-I) family, which includes also 13 IFNα subtypes as well as IFNλα, IFNλε, and IFNε in humans. All IFN-I subtypes activate signaling through binding to a heterodimeric receptor complex comprising the cell surface subunits of IFNAR1 and IFNAR2 (9–12). IFNβ is unique in that it binds to the IFNARs with much higher affinity than the other IFN-I counterparts, conferring superior biological properties to this cytokine. For example, in certain cancer cell lines, IFNβ can induce potent antiproliferative and proapoptotic responses at concentrations 2 or 3 orders of magnitude lower than required for IFNαs (13). It seems that this high affinity interaction of IFNβ with its receptor is critical for its anti-inflammatory function in MS; in fact, clinical trials using a low affinity IFNα failed to suppress clinical disease progression (14).

It has long been recognized that the short half-life of interferons in the blood may hamper their efficacy as injected drugs. IFN PEGylation has been developed to counter this problem and has been widely used in several clinical applications. In particular, two long-lived IFNα2 variants conjugated with 12 and 40 kDa PEGs (PegIntron™ and Pegasys™) have been approved to treat hepatitis C infections. Both are prescribed as once weekly injections in patients, demonstrating improved efficacy and patient compliance compared with their non-PEGylated counterparts (the latter are typically injected three times per week) (15, 16). More recently, a 20-kDa N-terminal PEG conjugate of human IFNβ (PLEGGRIDY™) with prolonged pharmacodynamics has been developed for MS therapy (17–19). This new PEGylated IFNβ variant injected either once every 2 weeks or once every 4 weeks was shown to significantly reduce disease relapse rates over a 48-week trial period in MS patients compared with placebo controls (20). However, this trial was not designed to test for relative drug efficacy in comparison with conventional IFNβ therapies for MS treatment, and accordingly, relative effectiveness of PEG-IFNβ in providing improved clinical response still remains to be established.

In our laboratory, we have used structural insight to engineer a tight receptor-binding variant named YNSα8 from the backbone of low affinity IFNα2 (21–23). This variant is an extremely potent IFNβ-like molecule according to its very similar gene activation profile, supporting the notion that the tightness of binding to the IFNAR receptor complex is the overwhelming biophysical factor that distinguishes different interferons with regard to their biological activities. However, in studying the effect of these superagonists in a mouse model, we are faced with a dilemma, because the human and mouse IFNAR sequences have diverged considerably, and cross-species activation of human IFN-I subtypes in mouse cells is weak and nonphysiological. To allow the study of human IFN-I in mice, we have recently generated transgenic mice harboring humanized IFNAR receptors (HyBNAR) that sensitively transduce human IFN-I signaling (24). Here, we examine a human IFN-I superagonist with a greatly improved pharmacological lifespan through PASylation (25), a biological alternative to PEGylation, using the HyBNAR transgenic mouse model. We compared the pharmacodynamic properties of PAS-YNSα8 with the non-PASylated counterpart and with that of human IFNβ, demonstrating superior efficacy in a mouse EAE model that mimics MS in humans.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids for the Production of PASylated YNSα8—The previously described plasmid pASK-IBA4-IFN (25), which encodes human IFNα2b as fusion protein with a N-terminal Strep-tag II, was subjected to QuikChange site-directed mutagenesis (Agilent Technologies, Waldbronn, Germany) using appropriate oligodeoxynucleotide primers to introduce the following six amino acid substitutions: H57Y, E58N, Q61S, E159K, S160R, and R162K. After that, the N-terminal Strep-tag II was replaced by a His6 tag, and one PAS#1(200) cassette was inserted in a consecutive manner downstream of the His6 tag using the single SapI restriction site directly in front of the IFNα2b coding region as described (25), thus yielding the expression plasmid pASK75-His6-PAS#1(1600)-YNSα8. An equivalent vector was prepared for the human wild type IFNα2b (pASK75-His6-PAS#1(600)-IFN).

Recombinant Protein Production and Purification—Recombinant protein production was performed according to a published procedure (25). The IFN variants were produced by periplasmic secretion in Escherichia coli KS272 (26) in the presence of the helper plasmid pTUM4 (27) as needed. Bacteria were cultivated either in shake flasks containing 2 liters of LB medium supplemented with 100 mg/liter ampicillin, 30 mg/liter chloramphenicol (for pTUM4), 1 g/liter proline, and 5 g/liter glucose or, alternatively, in a 4- or 8-liter bench top fermenter with a synthetic glucose mineral salt medium supplemented with the same antibiotics, as well as proline, following a published procedure (28). In the shake flask, recombinant gene expression was induced with 200 μg/liter anhydrotetracycline at A550 = 0.5 for up to 3 h at 22 °C. In case of fermenter production, induction was achieved by addition of 500 μg/liter anhydrotetracycline as soon as the culture reached A550 = 20 for a period of up to 2.5 h. Immediately thereafter, the cells were harvested by centrifugation, and a periplasmic extract was prepared in a buffer containing 500 mM sucrose, 200 mM sodium borate, pH 8.0, 1 mM (15 mM for 8 liters fermentation) EDTA and 1 mM 2,2'-dithiodipyridine (Sigma-Aldrich). PASylated YNSα8 and IFN were initially purified from the periplasmic E. coli extract via the His6 tag using a Ni2+-charged HisTrap HP column (GE Healthcare). Then cation exchange chromatography was performed on a Resource S column (GE Healthcare) using 20 mM Tris-HCl, pH 7.0, as running buffer and a NaCl concentration gradient for elution. All proteins were finally polished by size exclusion chromatography on a Superdex 200 pg HiLoad 16/60 column (GE Healthcare) in PBS (4 mM KH2PO4, 16 mM Na2HPO4, 115 mM NaCl). Protein purity was checked by SDS-PAGE, and protein concentrations were determined via UV absorption at 280 nm using calculated extinction coefficients of 19,180 μM−1 cm−1 for PAS-YNSα8 and
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17,900 m⁻¹ cm⁻¹ for PAS-IFN. Note that the PAS sequence shows no absorption at this wavelength (25). Final endotoxin content was typically below 20 endotoxin units/mg as measured with an Endosafe-PTS system using cartridges with 0.1–10 units/ml sensitivity (Charles River Laboratories, Wilmington, MA). Protein identity was confirmed by ESI/qTOF-MS on an mAXis mass spectrometer (Bruker Daltonics, Bremen, Germany) in positive ion mode after dialysis against 10 mM ammonium acetate, pH 6.6. Directly prior to measurement, the solution was supplemented with 20% (v/v) acetonitrile and 0.5% (v/v) formic acid.

Surface Plasmon Resonance (SPR) Measurements—SPR real time affinity measurements were performed on a BIAcore 2000 system (BIAcore, Uppsala, Sweden) as described (25) with a human IFNAR2-Fc chimera (R&D Systems, Minneapolis, MN) immobilized via an amine-coupled anti-Fc antibody (Jackson ImmunoResearch, West Grove, UK) on a CMDP sensor chip (Xantec, Düsseldorf, Germany). The purified PAS-YNSo8 was injected in an appropriate concentration series using PBS containing 0.05% (v/v) Tween 20 as running buffer at a flow rate of 25 µl/min. The kinetic parameters were determined by fitting the raw data to a Langmuir binding model for bimolecular complex formation using BIAevaluation software version 4.1 (BIAcore).

Cell Culture—Measurements of antiproliferation and anti-viral activity of IFNs on human WISH cells were described previously (22). In both assays, the EC₅₀ values were calculated using KaleidaGraph version 4.1, according to the formula

\[ y = A_0 + A \left(\frac{1}{1 + s/EC_{50}}\right) \]

where \( y \) represents the absorbance corresponding to the relative number of cells, \( A_0 \) is the offset, \( A \) is the amplitude, \( c \) is the IFN concentration, and \( s \) is the slope (23).

Quantitative PCR (qPCR)—Gene induction levels using qPCR were performed according to the protocol detailed in Ref. 24. Measurements were made using either an Agilent 7300 real time PCR system (96-well setup) or for some of the studies (see Fig. 7) and 10 µM ammonium acetate, pH 6.6. Directly prior to measurement, the solution was supplemented with 20% (v/v) acetonitrile and 0.5% (v/v) formic acid.

For time qPCR system (96-well setup) or for some of the studies (see Fig. 7), and the EC₅₀ values of antiviral activity relative to an IFNα2 standard were determined in WISH cells. Pharmacokinetics (PKs) were calculated by fitting the EC₅₀ values to a single exponential decay.

Human MX1 Transcript Quantification—The data were extracted from a microarray study performed by Fernald et al. (29). The individual microarray signals were first normalized by subtraction of the average signal for each spot measurement against the average value of signals given by five reference genes (HMBS, HPRT1, POLR2A, TAF1, and TAF2), thus yielding the “\( \Delta \) signal.” To determine relative change in expression of MX1, the signal for the IFNβ-treated patients was further subtracted against the average MX1 signal determined from four untreated control patients, yielding the “\( \Delta \Delta \) signal.” Fold change in gene expression was hence determined by the equation \( 2^{-\Delta \Delta CT} \).

Experimental Autoimmune Encephalomyelitis Studies—EAE was induced in 9–11-week-old wild type and homozygous HyBNAR (C57BL/6) female mice (Harlan Laboratories Israel/Weizmann Institute animal facilities) by injecting a peptide comprising residues 35–55 of mouse myelin oligodendrocyte glycoprotein (MOG35–55; Polypeptide Laboratories, Strasbourg, France). Mice were injected subcutaneously above the lumbar spinal cord with 100 µl of emulsion containing 200 µg/mouse of the encephalitogenic peptide in complete Freund’s adjuvant (BD-Difco) enriched with 250 µg/mouse of heat-inactivated Mycobacterium tuberculosis (BD-Difco) at 0 days post-induction (DPI). Pertussis toxin (Enzo Life Sciences) at a dose of 300 ng per mouse was injected intraperitoneally immediately after the encephalitogenic injection, as well as at 2 DPI. IFN treatments commenced by intraperitoneal injection 3–4 h before EAE induction. EAE disease was scored using a five-point grading with 0 for no clinical disease; 1, tail weakness; 2, paraparesis (incomplete paralysis of one or two hindlimbs); 3, paraplegia (complete paralysis of one or two hindlimbs); 4, paraplegia with forelimb weakness or paraparesis; 5, moribund or dead animals. The mice were examined daily. Graphics and statistical analyses were performed using KaleidaGraph version 4.1 or Microsoft Excel (data analysis tool pack add-in).

Western Blot Analysis—0.5 µg (excluding PAS component) of each indicated protein was run by SDS-PAGE and transferred to PVDF membranes (Millipore). Subsequently, the membranes were incubated with sera collected from mice (1:1000 diluted in TBST) or with a positive control anti-His₆ antibody (1:8000; Qiagen). After washing, each membrane was incubated with the appropriate HRP-conjugated secondary antibody conjugate and stained by ECL.

FACS Analysis—Brain-infiltrating leukocytes: Minced brain and spinal cord tissue were processed in C-tubes (Miltenyi Biotec). Brain lymphocytes were isolated from the interface of a Percoll density gradient. The isolated lymphocytes were washed with cold PBS and resuspended in PBS containing 1% BSA and 0.1% NaN₃ for direct cell surface staining. Single-cell suspensions were stained with antibodies for 30 min on ice.
mAbs against CD8, CD4, CD11b, CD45, CD11c, CD205, and GR1 were from BD Biosciences or Biolegend. Nonspecific binding to cell surface Fc receptors was blocked with unlabeled FcγRII/FcγRIII-specific antibody (clone 2.4G2). Stained cells were acquired on a ten-LSRII cytometer and were analyzed with Kaluza software. Spleens were minced and cells were dissociated (BD-Falcon 40 μm cell strainer) before staining with indicated antibodies and live sorted using a SORP FACSARiaII device. Sorted cells were processed for qPCR as described above.

**Immunohistochemistry**—Animals were anesthetized and perfused transcardially with Dulbecco’s PBS. Spines were dissected, fixed with paraformaldehyde (4% w/v) for 3 days, and decalcified (6% w/v) trichloroacetic acid; 5 days) as described before. The lumbar and thorax SC segments were paraffin-embedded and sectioned coronally (4 μm) with a microtome (Leica, Nussloch, Germany). Sections from the fourth lumbar vertebra (L4) region were chosen for staining. Paraffin sections were deparaffinized; antigen—retrieved in 10 mM sodium citrate, pH 6, or with 10 mM Tris-HCl, 1 mM EDTA, pH 9, in the case of CD3 staining; next preincubated in PBS containing 20% (w/v) normal horse serum (Vector Laboratories, Burlingame CA) and 0.2% (w/v) Triton X-100 for 1 h; and then incubated overnight with rabbit anti-IBA1 (Wako Chemicals) or rat anti-myelin basic protein (Abcam, Cambridge, UK) primary antibodies. Then the sections were incubated with species—specific highly cross-absorbed Cy2- or Cy3-conjugated antibodies for 50 min (Jackson ImmunoResearch). Sections were counterstained with Hoechst 33258 (Molecular Probes, Eugene, OR) for nuclear labeling. Stained sections were examined in a fluorescence microscope (E600; Nikon, Tokyo, Japan) equipped with Plan Fluar objective connected to a CCD camera (DMX1200F; Nikon). Digital images were analyzed by Image-Pro Plus 4.5 software (Media Cybernetics, Bethesda, MD).

**RESULTS**

**Molecular Design of PASylated YNS80a: A Long-lived Human Type I IFN Superagonist**—Introduction of three side chain substitutions into IFNα2b at the IFNAR1 binding interface via protein engineering, i.e. H57Y, E58N, and Q61S (YNS), results in a 60-fold higher affinity to the receptor. Exchange of the five C-terminal amino acids to those from human IFNα8 increases binding to IFNAR2 by 15-fold (23). Combination of both sets of mutations led to the variant YNS80α, which exhibits significantly higher potency than IFN-β in cell culture systems (22–24). To enhance the plasma half-life of this molecule, we appended to the N terminus a PAS polypeptide comprising 600 residues of Pro, Ala, and Ser, which forms a natively disordered, uncharged biopolymer with large hydrodynamic volume, i.e. with biophysical properties very similar to the chemical polymer PEG (25). The resulting long-lived superagonist has been named PAS-YNS80α (Fig. 1a).

PAS-YNS80α was produced in *E. coli* in a soluble state via periplasmic secretion and purified to homogeneity by means of immobilized metal ion affinity chromatography, cation exchange chromatography, and size exclusion chromatography. The correct formation of the two disulfide bridges was verified by SDS-PAGE under nonreducing conditions. In this assay, PAS-YNS80α showed a generally slower electrophoretic mobility caused by low binding of SDS to the hydrophilic PAS polymer, giving rise to an apparent size of ~140 kDa in comparison with a calculated mass of 70.3 kDa (Fig. 1b). The correct mass and monodisperse composition of the recombinant protein preparation was confirmed by ESI-MS both for PAS-YNS80α (Fig. 1c) and for the PASylated original IFNα2b.

**PASylated YNS80α Retains High Biological Activity**—Binding of PAS-YNS80α to the extracellular domain of IFNAR2 was measured by SPR real time (BIAcore) analysis, and the results are shown in Fig. 1d and Table 1. The observed affinity of 0.43 nM to the receptor was similar to that previously measured for YNS80α (0.4 nM) (23), indicating no loss of binding activity to IFNAR2. PAS-YNS80α was then subjected to two *in vitro* biological assays using human WISH cells (22). In an antiproliferative dose—response assay (Fig. 1f) PAS-YNS80α induced a 50% growth inhibition at 7.7 pM, essentially the same value as for YNS80α (6.6 pM). Notably, this corresponds to approximate 4- and 360-fold higher potency than that for human IFNβ and IFNα2, respectively (Fig. 1f and Table 1). Unlike antiproliferative activity, anti-viral potency is generally similar, whether using low or high affinity IFN-Is (31). This is shown in Fig. 1e, where IFN—induced protection from challenge with vesicular stomatitis virus took place for all IFNs tested with EC₅₀ between 0.4 and 0.7 pM. These data support that the PAS tag has no effect on the biological activity of IFNs *in vitro* (Table 1).

We next tested the ability of different IFN-Is, including PAS-YNS80α, to induce the expression of IFN response genes. WISH cells were stimulated with low dose IFN-Is (1 pM) for either 24 h without interruption or alternatively, for 6 h followed by washing and further incubation without IFN for the remaining 18 h. As expected, after 24 h of uninterrupted cytokine exposure, the amplitude of activation was found to be dependent on the type of IFN, with higher levels of gene induction measured for YNS80α and IFNβ in comparison with the lower affinity IFNα2. The gene activation profile of PAS-YNS80α was very similar to the ones of IFNβ and YNS80α, again indicating no loss of potency (Fig. 1g). For samples subjected to a 6-h pulse of IFN-Is, gene expression levels returned to nearly baseline at t = 24 h, demonstrating that the PASylated version of YNS80α does not inherently induce prolonged IFN-I signaling *in vitro* (Fig. 1g). Thus, at least at the cellular signaling level, PAS-YNS80α behaves in a manner very similar to that of its non—PASylated high affinity IFN counterpart.

**PASylated YNS80α Activates a Long Lasting IFN-I Response in Mice—HyBNAR transgenic mice that harbor humanized IFNAR1 and IFNAR2 receptors and that respond sensitively to human IFN-I injection (24) were used to evaluate the *in vivo* activity of PAS-YNS80α. These mice were further crossed with a transgenic mouse strain expressing the luciferase reporter under the control of the IFN-I-responsive MX2 promoter (24, 32) to follow the course of human IFN-I activation in real time. These double transgenic mice were injected intraperitoneally with 1 μg of YNS80α or either 0.25 or 1.0 μg of PAS-YNS80α (the described weight of PAS-YNS80α relates only to the active IFN component of the chimeric protein). The luciferase signal was followed in live animals in the course of 1 week (three mice per group). Representative images for two mice injected with either
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FIGURE 1. Development and evaluation of PAS-YNSα8. a, PAS-YNSα8 was depicted using PyMOL, also showing IFNAR1, IFNAR2, and the PAS tag (here with 200 residues in one exemplary random coil conformation) as cartoons in green, magenta, and gray, respectively, using two orientations rotated by 180°. Mutations H57Y, E58N, and Q61S (YNS) and E159K, S160R, and R162K (α8-tail) are depicted on the IFN surface facing IFNAR1 and IFNAR2, respectively. b, analysis of PAS-YNSα8, purified from the periplasmic cell fraction of E. coli, by Coomassie-stained 12% SDS-PAGE under reducing (lane 1) and nonreducing (lane 2) conditions. Lane M, protein size marker. c, ESI-MS analysis of PAS-YNSα8, confirming monodisperse composition and the calculated average molecular mass of 70,271.8 Da (ExPASy ProtParam tool). d, 200 residues in one exemplary random coil conformation) as cartoons in green, magenta, and gray, respectively, using two orientations rotated by 180°. Mutations H57Y, E58N, and Q61S (YNS) and E159K, S160R, and R162K (α8-tail) are depicted on the IFN surface facing IFNAR1 and IFNAR2, respectively. c, ESI-MS analysis of PAS-YNSα8, confirming monodisperse composition and the calculated average molecular mass of 70,271.8 Da (ExPASy ProtParam tool). d, representative real time SPR analysis of PAS-YNSα8 binding to the soluble human IFNAR2-F, chimera immobilized at RU = 200–250 on a Xantec CMDP sensor chip measured on a BIAcore 2000 instrument and fitted to a 1:1 Langmuir model. The resulting kinetic and affinity parameters are listed in Table 1. e, anti-viral (e) and antiproliferative (f) dose-response curves in human WISH cells for different IFN subtypes. g, transcript expression levels of representative IFN-I response genes after stimulation with 1 pM of different human IFN-Is. The cells were exposed to the different IFNs for 24 or 6 h (followed by 18 h in IFN-free medium) before harvest and gene transcript analysis.

TABLE 1

| IFN | Anti-proliferationa | Antiviral activitya | BIACore (SPR)b |
|-----|-------------------|-------------------|---------------|
|     | EC50 Ratio | EC50 Ratio | Kd Ratio |
| IFNα2 | 36004 | 1× | 0.6 | 1× | 2.2 | 1× |
| IFNβ | 30.6 | 120× | 0.42 | 1.4× | 0.4 | 5.5× |
| YNS | 235 | 160× | 0.42 | 3× | 0.4 | 1.1× |
| YNSα8 | 6.6 | 540× | 0.46 | 1.3× | 0.4 | 5.5× |
| PAS-YNSα8 | 7.7 | 470× | 0.50 | 1.2× | 0.435 | 5.0× |

a Antiviral and antiproliferative potencies were determined in WISH cells as described. The experimental error (σ) for these biological assays was 35%. Therefore, a confidence level of 2× S.E. would suggest that differences smaller than 2-fold between interferons are within the experimental error.

b Kd values against IFNAR2 were determined by SPR using either the ratio of kΓ/k0 or mass action over five different concentrations of the analyte.

c Ratios are relative to wild type IFNα2.

d An error of 35% was calculated for the Kd measurements. The indicated values were determined as described elsewhere (21, 22).

1 μg of PASylated or non-PASylated YNSα8 are shown in Fig. 2a.

A similar rapid rise in signal for both IFN-I variants was noted at t = 6 h, the first time point tested, indicating similar early stage bio-distribution. Thereafter, the pharmacodynamic response of PASylated and non-PASylated YNSα8 was dramatically different: mice injected with the non-PASylated IFN already exhibited a loss in activation signal at 12 h post-injection, further decreasing to nearly baseline at t = 24 h (II, right panels in Fig. 2a). In contrast, mice injected with PAS-YNSα8 exhibited prolonged activation of the luciferase signal, with a sustained biological response over >120 h (I, left panels in Fig. 2a). The live mouse luciferase images were quantified and fitted to a double exponential according to the Bateman function, i.e. one exponential representing increase of the signal during the distribution phase and the second exponential representing decay during elimination (Fig. 2b). The fitted pharmacodynamic half-life of the luciferase signal for non-PASylated YNSα8 was 3 h versus 30 h for PAS-YNSα8. Furthermore, injecting a 4-fold higher initial dose (1.0 μg instead of 0.25 μg of active component) of PASylated YNSα8 translated into a corresponding elevation of MX2-Luc levels during the entire period of the experiment, suggesting both that PAS-YNSα8 is stable in circulation and that its pharmacological activity is linearly dose-dependent.

To test whether our pharmacodynamic (PD) readouts correlate directly with PK properties of the PASylated IFN superagonist, we injected mice intraperitoneally with either 0.25 or 1.0 μg of PAS-YNSα8, and serum was collected 6, 24, and 48 h thereafter. The concentration of active IFN was then quantitatively determined by measuring its antiviral potency in the serum, showing a decay half-life of ~24 h (Fig. 2c). In sharp
contrast, when we injected 1 μg of human IFNβ intraperitoneally into mice, although high levels of IFN activity were detected in the serum at the 6-h time point, no detectable signal remained at 24 h (Fig. 2c). Thus, both our PK and PD studies indicate a similarly extended biological lifespan upon PASylation of our IFN superagonist without observable loss of bioactivity in relation to its non-PASylated counterpart.

To examine the effects of PASylation on tissue distribution of IFN, PAS-YNS8 and a panel of other IFNs were injected intraperitoneally into HyBNAR/MX2-Luc double transgenic mice, and after 6 h a panel of organs was extracted for analysis. MX2-driven luciferase signals were shown to be most highly up-regulated in the liver but gave strong activation in all tissues tested with exception of the brain (Fig. 3a). Importantly, the activation profile did not differ between Mu-IFNβ and both the PASylated and non-PASylated forms of YNS8, demonstrating that neither the tight binding of the superagonist to IFNARs nor the addition of the PAS tag results in an altered biodistribution or bioactivity at this early time interval postinjection.

Next, we tested the effects of unmodified IFN-I and PAS-YNS8 on induction of IFN-I response genes. To this end, HyBNAR mice were injected intraperitoneally with 1 μg of Mu-IFNβ or either 0.25 or 1 μg of PAS-YNS8 (active component). Then tissues were dissected, and activation of six IFN-I-controlled genes is analyzed by qPCR. The IFN-I response was found to be both tissue- and gene-specific (Fig. 3b). Whereas gene induction by mouse IFNβ lasted less than 24 h in all tissues and for all genes tested, a strong and prolonged induction was observed upon PAS-YNS8 treatment even at the low dose of 0.25 μg. Interestingly, gene induction was remarkably tissue-specific, with the liver displaying the highest level of IFN-I induced gene activation as already seen in the in vivo luciferase study. Although all six genes were robustly activated in the liver (both by IFNβ and PAS-YNS8), CMPK2 and RSDA2 were poorly induced in the kidney and spleen, indicating that the IFN-I activation profile is subject to tissue-specific epigenetic differences. Follow-up measurements of gene expression 24 and 48 h after PAS-YNS8 injection demonstrated durable activation of these genes (Fig. 3b). Overall, this analysis of six IFN-I response genes in three tissues reveals a complex pharmacodynamic profile of gene activation, which is influenced by both gene and tissue specific parameters.

Single-dose intramuscular injection of IFNβ into humans also results in the activation of IFN response genes, as well as of response markers such as the metabolite neopterin. These responses can be measured by monitoring either their transcriptional or translational induction (18, 29, 33). In one of these studies, transcription microarray data were made available from two volunteers injected intramuscularly with single dose IFNβ, as well as from noninjected control patients (29).
From these data, we determined the expression levels of the IFN-I response gene MX1 (Fig. 3c), revealing elevated activation over 3 days, in line with claims of others (18). Thus, IFNβ remains in circulation in humans ~10 times longer than in mice, which is in accordance with the rules of allometric PK scaling (34). Consequently, IFNβ in humans has a half-life similar to that measured by us for PAS-YNSa8 in mice. This finding is of importance when planning the IFN treatment regime for a mouse disease model, which will be discussed next.

**Therapeutic IFN Response in a Mouse Model of MS: EAE Induction**—Induction of EAE with a MOG35–55 peptide is an extensively used mouse model to emulate chronic relapsing MS in humans (35, 36). Because IFNβ treatment is a major therapeutic option offered to MS patients, we were particularly interested in learning how well HyBNAR mice respond to human IFN therapy and how this response compares with that with PAS-YNSa8. We adjusted the system in a manner to induce a relatively severe form of EAE and then tested different forms of IFN showed therapeutic efficacy to different degrees. Both Mu-IFNβ and Hu-IFNβ led to a less severe clinical score (Fig. 5a) and protection from inflammation-induced cachexia (Fig. 5b). In contrast, PAS-YNSa8 exerted a much stronger suppression of clinical symptoms and weight loss relative to both human and mouse IFNβ therapies during the course of the experiment, despite being administered at one-sixteenth of the cumulated protein dose, with merely one-quarter the frequency of injection (Fig. 5, a and b). A statistical comparison of cumulative EAE scores revealed that the clinical efficacy of PAS-YNSa8 in the treatment of the EAE mice was clearly significant (Fig. 5c). Similar to the previous experiment, after cessation of therapy, a worsening of disease soon followed. Our findings are consistent with previous reports on exacerbated clinical symptoms in both EAE and in MS patients after discontinuation of IFNβ therapy (37, 38). In summary, we demonstrate superior clinical response using our engineered long-lived human IFN variant compared with that of IFNβ in an EAE model using the transgenic HyBNAR mouse strain.

Next, we sought to test whether raising the dose of PAS-YNSa8 could further protect mice from EAE clinical symptoms. Indeed, clinical data using IFNβ to treat human MS indicate that increased drug dosage improves disease protection (7). Despite these findings, we found that maximum therapeutic efficacy was already observed with the lower dose of 0.25 μg of PAS-YNSa8, without added benefit by higher dosing (Fig. 5d).

Differences in the degree of EAE clinical severity and protection provided by IFN therapy were observed between the different experiments despite the identical reagents used. Despite these variations in absolute clinical scores, it was consistently
observed that PAS-YNSa8 outperformed any other IFN-I used in this study (Figs. 5, a and d, and 6).

To test the importance of using a high affinity receptor binding interferon in the treatment of EAE, we performed a similar experiment by injecting the low affinity (original) PASYlabeled IFNα2 (25). Although PAS-IFNα2 might provide transient delay in the onset of clinical symptoms, full disease severity was evident by 21 DPI, despite continued drug injection (Fig. 6). These results support that high affinity IFN-Is are required for effective EAE disease protection, in agreement with clinical findings for human MS (14).

**PAS-YNSa8 Retains a Similar Level of Signaling over the Course of the EAE Experiment**—Repeated injections of Hu-IFN-Is or of the PASYlated proteins into mice may potentially result in a reduced response over time, either through an immune response that neutralizes their effect or through receptor down-regulation. To test the IFN-I response over the course of the EAE treatment, gene induction was monitored at the end of the experiment (21 DPI), which was 1 day after the last PAS-YNSa8 injection. Livers and spleens were collected and the latter were dissociated, stained with different antibodies against surface markers (CD4, CD8, CD11B, and CD19), and subjected to FACS to separate the different immune cell populations. These samples were then used to generate cDNA for qPCR analysis. Gene expression levels for nine IFN-I response genes and four reference genes were analyzed and compared with those for untreated EAE mice (Fig. 7). Nonparametric one-way analysis of variance was performed to determine pairwise significance for EAE vehicle control to that of IFN-treated groups measured until the last day of IFN injection (16 DPI). Three different statistical tests measuring cumulative EAE score, maximum EAE score, and cumulative mouse weight are given. A significant reduction in clinical severity and protection from weight loss was noted only for the Mu-IFNβ twice daily (b.i.d.) injected group.
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[Graph and data analysis]
verifying the level of purity of the sorted cells, and a one to one concordance between cell type and the gene encoding the surface marker used for its purification was demonstrated. Exceptionally, the CD11B<sup>−</sup>-sorted cells encoded both CD4 and CD11B gene products, in line with findings that subpopulations of dendritic cells and macrophages co-express both markers (39, 40).

Next, the immunogenicity of repeated PAS-YNS<sub>8</sub> treatment was evaluated. Serum from mice that had received repeated injections of IFN was extracted at 21 DPI and directly tested for the presence of anti-drug antibodies. Western blot analysis revealed that mice repeatedly dosed with PAS-YNS<sub>8</sub> did not generate antibodies, despite the human origin of the engineered IFN that was administered (Fig. 7b). Surprisingly, however, some mice treated with Mu-IFNβ therapy did raise antibodies to the murine cytokine. Mu-IFNβ is naturally glycosylated, whereas the recombinant Mu-IFN produced in E. coli is not. This may explain why these mice generated an immune response to the injected cytokine. To directly assess the concentration of active nonbound interferon in the serum, the antiviral potency of the serum on human WISH cells was determined and found to be unchanged between mice after 21 days of treatment in comparison with naïve controls (representative plots are shown in Fig. 7c), demonstrating the absence of drug neutralizing activity. Together, our findings demonstrate that the level of PAS-YNS<sub>8</sub> response was not altered during the duration of the experiment, exerting sustained activation of IFN response genes, with no neutralizing anti-drug antibodies being generated.

PAS-YNS<sub>8</sub> Curtails Myeloid Cell Lineage in the Central Nervous System of EAE-induced Mice—Brain and spinal cord infiltrating leukocytes were isolated from individual EAE-induced HyBNAR mice and examined by FACS for changes in immune phenotype following treatment with PAS-YNS<sub>8</sub>. Superagonist treatment resulted in an almost 2-fold reduction of infiltrating (CD11B<sup>+</sup>/CD45<sup>Hi</sup>) macrophages, yet without observing a significant change in the proportion of resident (CD11B<sup>−</sup>/CD45<sup>low</sup>) microglia (Fig. 8a). The decrease in infiltrating macrophages may be accompanied by a reduction in MHCII<sup>+</sup> cells, an indicator of activation of antigen presenting cells, although this latter observation lacked statistical significance (Fig. 8a). In contrast, no differences in the relative fre-
FIGURE 7. **No observed loss of IFN-I responsiveness after repeated PAS-YNS8 injections.** a, 24 h after the last of repeated IFN (or vehicle) injections of EAE-induced mice (21 DPI), tissues were extracted, and the indicated splenocyte cell lineages were FACS-purified and processed for qPCR gene expression analysis for 10 IFN-I response genes and a panel of four reference genes (upper box). Samples were also tested for gene expression of immune cell markers as quality control to verify the FACS sort purity (lower box). The given values are averages from two to three mice for each group tested. b, Western blots were performed to test for immunogenicity of IFN-injected mice to PAS-leptin (as a control), PAS-YNS8, Mu-IFNβ, and YNS8. Panels from left to right show Western blots developed with the following sera. First panel, a mouse after repeat Mu-IFNβ injections; second panel, a commercial anti-His6 antibody; third panel, a mouse after repeat injections with 1 µg; fourth panel, 0.25 µg PAS-YNS8 (active IFN); fifth panel, serum from a naive mouse. c, EAE-induced mice after repeated injections of PAS-YNS8 (0.25 µg q.a.d. from 0 to 20 DPI) had serum collected 24 h after the final IFN injection. Likewise, serum was also collected 24 h after naïve HyBNAR mice were administered with a single dose of PAS-YNS8 for comparative purposes. To indirectly measure IFN serum levels, serial dilutions of blood samples were assayed by vesicular stomatitis virus antiviral assay using human WISH cells (see Fig. 1). All blood samples demonstrated a similar E C50.

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quencies in (CD205+/CD206−) dendritic cells and T-cells (CD4+ or CD8+) were found between experimental groups. We looked into this further by performing a qualitative histological study with cross-sections of spinal cord at 19 DPI from EAE HyBNAR mice treated with either vehicle (PBS), Hu-IFNβ, or PAS-YNS8. Hematoxylin and eosin staining revealed regions of leukocyte infiltration as indicated by clusters of darkly stained nuclei found in the EAE-vehicle control and, to a lesser degree, in the IFNβ-treated sample (Fig. 8b, top panels, white arrows).

We then stained for IBA1 immunoreactivity, a marker of microglia and infiltrating macrophages. A marked up-regulation of IBA1 immunopositive cells was detected for the EAE-induced vehicle control but appeared markedly reduced for both IFNβ- and PAS-YNS8-treated mice (Fig. 8b, middle panels). Finally, using a myelin basic protein antibody, the degree of demyelination (labeled by asterisks) in the IFN-treated EAE-induced mice was reduced in comparison with those with vector control (Fig. 8b, bottom panels).

We next performed semiquantification of the IBA+ immunoreactive myeloid cells. A significant decrease in IBA-positive cells was detected both for IFNβ and PAS-YNS8-treated spinal cord sections in comparison with vehicle-treated EAE mice (Fig. 8c). This result concurs with our FACS findings of a decrease in CD11b+/CD45R1+ myeloid cells. In contrast, consistent with our FACS data, we found no obvious difference in the staining of CD3+ T-cells or CD45R+ B cells as a result of IFN therapy by immunohistochemistry analysis (data not shown). Thus, the FACS and immunohistochemical studies both support that IFN therapy leads to decrease in the number of myeloid cells in the CNS of EAE-induced mice. Based on the FACS data, we suspect that IFN therapy acts to suppress the infiltration of (CD11b+/CD45R1+) macrophages into the CNS.

Increased PD-L1 (CD274) Transcript Expression Correlates with Improved EAE Clinical Outcome—IFN-I therapeutic response in the treatment of MS or in EAE must by default take place by the activation or repression of effector IFN response genes that modulate disease status. It has recently been reported that a novel regulatory T-cell population expressing high levels of PD-L1 (CD274) plays a central role in the IFN-mediated therapeutic activity upon treatment of EAE and MS (41). We have identified this gene as one that is differentially up-regulated in human WISH cells by IFNβ but not by IFNα (31) and thus hypothesized that CD274 may act as a mediator of the IFN effect in this disease. In comparison, we also tested for a relationship between the expression of MX1, as well as MX2, and the disease status. Indeed, MX1 in particular has often been used as a reference marker to test for correlation between gene expression and response to IFNβ therapy in MS. However, although associations have been found, it is of limited predictive value (42–47).

Consequently, FACS-sorted CD4 spleen cells from EAE-induced mice that were either untreated or treated with Mu-IFNβ or with PAS-YNS8 were tested for a relationship between gene expression levels for CD274, MX1, and MX2 and disease severity. The findings are represented in scatter plots with each data point representing a value determined from a single mouse (Fig. 9a). Here, the degree of clinical severity is represented as change in mouse weight in comparison with pre-EAE induction for each animal based on a strong correlation between weight loss and disease scoring (Pearson correlation coefficient r = −0.85; R² = 0.72; Fig. 9b). Weight loss gives a stronger correlation relationship to gene expression than that for measurements of EAE disease score (Fig. 9b), the latter which depends upon the human evaluator. In CD4+ cells, PD-L1 gene expression levels strongly correlate with EAE dis-
ease severity, significantly more than found for either MX1 or MX2 (Fig. 9, a and b). Whereas MX1 and MX2 both demonstrated a trend toward higher gene expression with more potent IFN treatment, the correlation with disease progression was poor. The high quality of gene expression measurements in CD4+ cells was confirmed by the excellent mutual correlation of co-expression of MX1 and MX2 for the different mouse samples (Fig. 9b).

We expanded our study to assess the expression of MX1, MX2, PD-L1, and its receptor PDCD1 (PD-1) in different leukocyte subsets including CD8+, CD11B+, and CD19+ cells, as well as liver (which we assume to not directly relate to EAE disease state). The analyses are summarized in the form of Pearson correlations squared (Fig. 9b). Although for all but CD19+ cells, positive correlations were found between gene expression and the disease state, the most predictive of these were found
DISCUSSION

In this study, we have combined two technologies, a tight receptor-binding type I interferon variant and the novel PASylation technology to generate a long-lived IFN superagonist for potential pharmaceutical application. The PAS amino acid sequence is unstructured and hydrophilic, although uncharged, conferring a large hydrodynamic volume in aqueous solution with the capacity to greatly increase the in vivo half-life of a fused protein by retarding kidney filtration. The PAS tag used in this study comprises 600 residues of Pro, Ala, and Ser with a total mass of $\sim 50$ kDa, which adopts an expanded random coil conformation with an effective size of more than 600 kDa under physiological conditions as previously demonstrated by size exclusion chromatography (25). PASylation thus provides a simple alternative to PEGylation technologies, which suffer from intrinsic technical complexities such as the necessity of chemical in vitro coupling, high cost of goods, inherent polydispersity, and lack of biodegradability.

In the case of the IFN superagonist YNS8, the PAS tag conferred an increased pharmacodynamic half-life of $\sim 10$-fold in mice after intraperitoneal injection (Fig. 2), without inducing a measurable change in biodistribution or biological potency relative to the non-PASylated cytokine (Fig. 3a). If translated to humans, this could conceivably shift a thrice weekly IFN injection regime to a monthly injection of PAS-YNS8 with potentially enhanced clinical efficacy.

Testing the physiological effects of injected human IFNs in animal models is inherently problematic, because interspecies variations in receptor sequence result in suboptimal activation of human IFNs when injected into mice (24). The traditional solution to this approach is to directly perform preclinical studies of human IFNs in primates. To exemplify this, another engineered human IFN-I variant has recently been tested for efficacy in treatment of HIV, utilizing the rhesus macaque SIV model (48). The inherent ethical considerations of working with primates and the extremely high cost of their maintenance make this a problematic experimental option. Here, we adopted an alternative approach using a transgenic mouse strain expressing humanized IFNAR receptors, which respond sensitively to human IFN (24). The HyBNAR mice, which were generated in the purebred C57BL/6 genetic background, are amenable to...
MOG35–55 peptide-induced EAE, a commonly used mouse model to emulate human MS.

In a head to head comparison, PAS-YNSα8 outperformed both Hu-IFNβ and Mu-IFNβ in ameliorating EAE disease symptoms in the HyBNAR mouse model, despite being injected with a 4-fold less frequency and an overall 16-fold lower injection dose (Fig. 5). Administration of low affinity PASylated IFNα2 was not efficacious in treating EAE (Fig. 6), confirming that the tight receptor binding activity of the YNSα8 variant built on the backbone of this IFN converts it into an effective drug for the treatment of this indication. This mirrors clinical trials showing a lack of efficacy for low affinity IFNα in the treatment of MS (14).

Twenty years of clinical data confirm that IFNβ therapy is efficacious in the treatment of MS, albeit with a significant proportion of patients (~35–50%) responding poorly to the drug (49). Currently, there is no accepted biomarker to identify a priori good responders to IFNβ treatment in advance of therapy. This problem extends to other drugs where we still lack reliable personalized medicinal markers to tailor a suitable therapy to treat individual MS patients (50). One major problem for MS research on finding associations between clinical and genomic/transcriptomic data is that there remains a lack of consensus as to what clinical features of MS are suitable to quantify disease severity (51, 52). A further complication is that neuroimmunological studies suggest that MS may not be a homogeneous disease but rather comprises a collection of disease states with related but distinct pathologies (53, 54). The mouse EAE model provides an alternative to study transcriptomic markers that might provide predictive power in assessing IFN therapy in MS. In this model, disease onset is timed and controlled, scoring of disease status is relatively straightforward, and the mice used are inbred; thus differential states of disease severity cannot be attributed to genotypic variation. Using this experimentally controlled EAE model, a clear signal of increased expression of CD274 in CD4+ and CD11B+ spleen-derived cells correlated directly with improved clinical outcome, suggesting its potential role as biomarker. In accordance with our original hypothesis, we further consider that CD274 may not only be acting as a marker of IFN response to in EAE and MS but is by itself an effector molecule that modulates the immune phenotype. This notion is strengthened by a recent study showing that a novel suppressive regulatory T-cell population expressing the transcription factor FoxA1 is instrumental in driving the IFN therapeutic response in EAE and MS (41). These FoxA1-positive T-reg cells were also shown to co-express high levels of CD274 (PD-L1). The number of these FoxA1+ PD-L1 cells was significantly reduced in IFNAR1 knock-out mice (41), indicating that the IFN-I signaling axis plays a major role in delineation of this novel T-Reg cell population. Our gene expression study with CD274 shown herein complements these findings.

In our spinal cord histopathological studies, we observed decreased myeloid cell numbers, perhaps resulting from a decrease of macrophage infiltration into the CNS, a phenotype consistent with studies by others testing the EAE phenotype with genetic deletions of IFNAR1 (55, 56) or IFNβ (57, 58). In particular, a study using cell lineage specific IFNAR1 knock-out mice has identified the myeloid cells as critical for disseminating the IFN-therapeutic potential in EAE (56). How these myeloid cells interact with infiltrating CD4+ subsets in particular will be a subject of future studies, complementing important findings made in this field (41, 59).

CONCLUSIONS

We have used the novel PASylation technology to generate a long-lived human type I IFN superagonist. PASylation of our IFN variant resulted in no loss of receptor binding affinity whatsoever and when injected into a mouse multiple sclerosis model demonstrated superior efficacy to that of IFNβ, without inducing an observable immunogenic footprint. In addition to its increased efficacy in relation to IFNβ, PAS-YNSα8 can potentially act as a replacement therapy for patients defined a priori as good responders to IFNβ treatment but who have developed neutralizing antibodies to the injected interferon. Our preclinical findings provide supportive evidence to vindicate the advance of PAS-YNSα8 to human clinical trials.

Using the HyBNAR transgenic mouse model, we have found a strong linear correlation between increased PD-L1 transcript levels in spleen-derived CD4+ and CD11+ cells and a decrease in EAE disease severity. Thus, we present a model claiming that PD-L1 acts as a predictive maker to determine efficacious IFN therapy in EAE. We extrapolate that this may also hold true for human MS, which should be a matter worth of future study.

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