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(54) Title: MODIFIED INTERFERON-BETA-1 HAVING REDUCED IMMUNOGENICITY

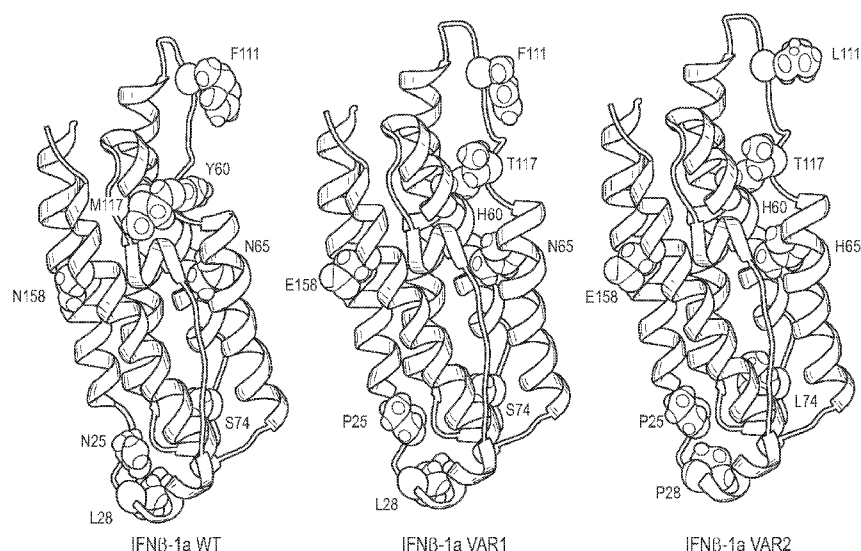


FIG. 2

(57) Abstract: Provided herein are compositions including a modified interferon β -1a polypeptide having interferon β -1a activity and reduced immunogenicity. In some aspects, the modified interferon β -1a polypeptides have the similar, same or greater antiviral biological activity with respect to natural interferon β -1a. Also provided herein are nucleic acid sequences encoding the modified interferon β -1a polypeptide with interferon β -1a activity. The present disclosure provides compositions including a recombinant protein expression cell line including the nucleic acid sequence encoding the modified interferon β -1a polypeptide with interferon β activity and reduced immunogenicity; wherein the cell line includes a plasmid or vector containing the nucleic acid sequence. Also disclosed herein are pharmaceutical formulations including modified interferon- β with reduced immunogenicity for the treatment of multiple sclerosis.

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MODIFIED INTERFERON-BETA-1 HAVING REDUCED IMMUNOGENICITY**CROSS-REFERENCE TO RELATED APPLICATION**

This application depends from and claims priority to Argentina Provisional Application No: AR2358219, titled “Variants of Interferon Beta with Reduced Immunogenicity” and filed July 26, 2023, the entire content of which is incorporated herein by reference.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The instant application contains a Sequence Listing which has been submitted electronically in .xml format and is hereby incorporated by reference in its entirety. The Sequence listing, created July 17, 2024, is named “EPV0053WO.xml” and is 22,764 bytes in size.

TECHNICAL FIELD

The present disclosure generally relates to the development of therapeutic molecules of pharmaceutical interest for application to humans. More particularly, the present disclosure relates to modified interferon Beta-1 (IFN β -1) polypeptides, including IFN β -1a, as well as to compounds and compositions. These modified IFN β -1 polypeptides display proven antiviral biological activity and reduced immunogenicity compared with wild-type IFN β -1. These modified IFN β -1 polypeptides, as well as related compounds and compositions, may be used for human therapy and treatments, including the treatment of multiple sclerosis.

BACKGROUND

Multiple sclerosis (MS) is a chronic inflammatory disease that usually affects young adults between the ages of 20 and 50. Symptoms range from tremors, motor weakness, and reduced mobility to cognitive impairment and depression, among other clinical symptoms and manifestations. Most patients exhibit the relapsing-remitting form of MS (RRMS), which includes an early stage characterized by recurrent neurological episodes, called relapses, typically followed by remission, and then a second phase of progressive accumulation of neurological disability.

MS generally begins when autoreactive T lymphocytes trigger an inflammatory cascade in the central nervous system (CNS), which includes demyelination and axonal loss, culminating in neurodegeneration.

Interferon beta-1 (IFN β -1) is a cytokine from the interferon family, secreted by various immune and non-immune cells, including macrophages, lymphocytes, fibroblasts, and endothelial cells. Interferons have immunomodulatory effects as well as antiviral and antitumor properties.

IFN β -1a is a type 1 interferon naturally produced by fibroblasts and secreted in its mature form of 166 amino acids (SEQ ID No. 1) with N-glycosylation at the asparagine residue at position 80. Its synthesis begins as a 187-amino-acid precursor (SEQ ID No. 9) encoded by a single gene on the short arm of chromosome 9 (SEQ ID No. 12), which, after synthesis and secretion, removes a 21-amino-acid signal peptide (SEQ ID No. 17).

Currently, recombinant proteins for therapeutic use are a routine part of medical practice, used to treat a wide variety of diseases. The administration of recombinant human beta interferon (rhIFN beta) was the first disease-modifying therapy approved by the U.S. Food and Drug Administration (FDA) for the treatment of MS, due to its ability to reduce relapses and delay the onset of disability. The use of IFN beta is not a cure for MS (no known cure exists to date), but it is the first line of treatment because it reduces the damage of neurological episodes by 30% and delays the definitive clinical signs of MS.

There are currently five IFN beta biotherapeutics approved for the treatment of relapsing forms of MS: subcutaneous administration (SC) 1- IFN beta-1b Betaseron® (Bayer HealthCare Pharmaceuticals Inc), 2- Extavia® (Novartis Pharmaceuticals Corp), and 3- IFN beta-1a Rebif® (EMD Serono Inc); and intramuscular administration (IM) 4- IFN beta-1a Avonex® (Biogen Inc) and 5- PEG-interferon SC beta-1a Plegridy® (Biogen Inc). Rebif and Avonex are produced in CHO (eukaryotic) cells, while Betaseron and Extavia are produced in bacterial cells. The amino acid sequences of Avonex and Rebif IFN beta-1a are identical to natural human IFN beta-1a, and both products are glycosylated. In contrast, Betaseron's recombinant IFN beta-1b is a mutated form where cysteine 17 is modified to a serine residue and is a non-glycosylated form of the protein.

During its nearly three decades of use, rhIFN beta has played an important role in understanding the immunomodulatory mechanisms underlying the disease. Despite the approval of other therapies, the use of rhIFN beta persists due to its long-term beneficial effects, such as reduced disability progression and mortality rates and the relative safety for the fetus during pregnancy. Additionally, rhIFN beta has been used as a comparator in two large-scale randomized clinical trials to test innovative MS therapies.

Despite its clinical efficacy, numerous reports have demonstrated the occurrence of immunogenicity events when rhIFN beta is administered to patients. In fact, all products containing rhIFN beta have triggered the formation of neutralizing antibodies (NABs) that negatively impact the clinical efficacy of these drugs. The incidence of this phenomenon varied and was closely related to the product. For example, while between 28% and 47% of patients developed NABs to IFN beta-1b, a smaller proportion of patients developed NABs to IFN beta-1a (12% to 28% for IFN beta-1a Rebif and 2% to 6% for IFN beta-1a Avonex).

Based on their biological characteristics, MS patients who do not respond to IFN beta therapy may be divided into three subgroups: genetic, pharmacological, and pathogenetic non-responders. Pharmacological non-responders show a lack of clinical efficacy due to the presence of serological factors (e.g., anti-IFN beta antibodies) that inhibit the biological activity of IFN beta. Specifically, serological factors interfere with the interaction between IFN beta and its IFNAR receptor, which in turn blocks downstream IFN signaling, the transcription of interferon-stimulated genes (ISGs), and the expression of ISG products. Thus, when the interaction between IFN beta and its receptor is blocked, there can be no biological activity and, consequently, no clinical activity.

The generation of neutralizing antibodies may occur due to various factors, which may be grouped into two main categories: extrinsic factors such as the route of administration, dosage, formulation, presence of aggregates and contaminants, or the presence and type of glycosylations; and intrinsic factors, among which the presence of immunogenic epitopes in the protein stands out. It is well known that the development of neutralizing antibodies is closely related to T helper cells.

Intrinsic factors pose a real challenge since B lymphocyte activation contributes to antibody development. The occurrence of an immunogenicity event from the administration of a recombinant IFN β -1a occurs even when the therapeutic has an amino acid sequence identical to the autologous natural IFN β -1a protein.

B lymphocyte activation may be mediated or not by T lymphocyte collaboration, resulting in T-dependent or T-independent responses, respectively. T-independent responses develop as a result of the activation of a specific group of B lymphocytes, stimulated by certain structural characteristics of some molecules, such as polymeric repeats. Antibodies developed as a result of this activation are primarily low-affinity IgM. In contrast, T-dependent lymphocyte activation is mainly associated with the protein's primary sequence. In this scenario, when the molecule is

endocytosed, processed, and the resulting peptides are presented on the surface of antigen-presenting cells (dendritic cells, macrophages, or B lymphocytes) in the context of Class II Major Histocompatibility Complex (MHC) molecules, some sequences may be recognized by “helper” (Th) or collaborating T lymphocytes (through their surface receptor, called T Cell Receptor or TCR). These specific lymphocytes, once activated, will trigger an immune response leading to B lymphocyte activation and consequent NAb production.

In T-dependent responses, the developed antibodies are IgG, have higher affinity, and are generated more prolonged and sustained over time than those generated without T lymphocyte participation.

T-dependent B lymphocyte activation begins with the interaction of a group of B lymphocytes with specific protein epitopes through their surface antigen receptors (IgM/IgD), constituting the first B lymphocyte activation signal. This signal promotes protein internalization, which will then be processed into small peptide epitopes, ultimately exposed within the “groove” of Class II MHC molecules on the B lymphocyte surface. B cells also co-express the CD40 molecule on their surface. When Th lymphocytes interact through their TCR and the CD40 ligand (CD154) with epitope-MHC class II complexes and CD40 (on the B lymphocyte surface), they trigger the second activation signal. This signal ultimately activates the B lymphocyte, and T lymphocytes produce, among other cytokines, interleukin-4 (IL-4) (in a type 2 Th lymphocyte response) or interferon-gamma (IFN gamma) (type 1 Th lymphocytes), leading to the maturation of the immune response. It should be noted that without the participation of T lymphocytes, which provide the second signal, B lymphocytes undergo programmed cell death (apoptosis). For this reason, attenuating a T lymphocyte-mediated immune response has become the focus of attention in the process known as “de-immunization” of recombinant proteins for therapeutic purposes.

Immunogenicity risk assessment is an important component of biotherapeutic drug development, and part of the overall benefit risk assessment. A robust immunogenicity risk assessment process ensures that the most appropriate candidate molecules advance to the clinical stage, and that clinical immunogenicity is adequately monitored.

As with all therapeutic proteins, one of the main drivers of the risk of developing an immune response to a multi-species therapeutic agent is the primary amino acid sequence and T cell epitopes contained. Any protein that has been designed to change the amino acid sequence, has linker regions that introduce new linear epitopes that are not present in either parent, or has unnatural or modified amino acids that may be at elevated risk of developing an immune response.

Most multi-species therapies contain these elements, and therefore these novel sequences must assess immunogenic risk using available tools such as *in silico* prediction, *in vitro* T cell assays, MHC binding assays, or *ex vivo* models.

Accordingly, there is a need to improve the *in vivo* characteristics of IFN β -1a when administered to a human subject. In this regard, it is highly desirable to provide an IFN β -1a with a reduced potential for immunogenic events to occur in the subject to which it is administered.

SUMMARY

Accordingly, the present disclosure provides modified IFN β -1a polypeptides and related compounds displaying proven biological activity and having reduced immunogenicity compared to wild-type IFN β -1a. The modified IFN β -1a polypeptides find use as a therapeutic in human subjects for a variety of reasons, such as better safety among patient populations, reduced immunogenicity and consistent biological activity. The present disclosure has a potential use in the therapy of multiple sclerosis and other neurodegenerative diseases.

In aspects, the present disclosure provides a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising one or more amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid. In aspects, the modified interferon- β 1a polypeptide having interferon- β 1a activity, comprises an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising at least four amino acid substitutions, the at least four amino acid substitutions at positions 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

In aspects, the present disclosure provides a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising one or more amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid. In aspects, the present disclosure provides a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino

acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising at least four amino acid substitutions, the amino acid substitutions at one or more of positions 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

5 In aspects, the present disclosure provides a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising at least eight amino acid substitutions, the eight amino acid substitutions at one or more of positions 25, 28, 60, 65, 74, 111, 117, and 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine,
10 leucine, threonine, or glutamic acid.

In aspects, the present disclosure provides a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising at least eight amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, and 158, optionally
15 wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

In some aspects, the modified interferon- β 1a polypeptide has amino acid substitution(s) of N25P, L28P, Y60H, N65H, S74L, F111L, M117T, N158E, or a combination thereof. In some aspects, the amino acid substitutions are N25P, Y60H, M117T, and N158E. In some aspects, the
20 polypeptide also has an amino acid substitution of L28P, N65H, S74L, or F111L. In some aspects, the polypeptide also has amino acid substitutions of L28P, N65H, S74L, and F111L.

In some aspects, the modified interferon- β 1a polypeptide has an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3. In some aspects, the modified interferon- β 1a polypeptide has an amino acid sequence of SEQ ID NO: 2. In some aspects, the modified interferon- β 1a
25 polypeptide has an amino acid sequence of SEQ ID NO: 3. In some aspects, the modified interferon- β 1a polypeptide has a reduced immunogenicity as compared to a wild type interferon- β 1a polypeptide of SEQ ID NO:1. In some aspects, the modified interferon- β 1a polypeptide has a relative antiviral activity of about 10% to about 200% compared to a wild type interferon- β 1a polypeptide of SEQ ID NO:1. In some aspects, the modified interferon- β 1a polypeptide has a
30 relative antiviral activity of about 100% to about 140% as compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

In aspects, the present disclosure provides a pharmaceutical composition comprising the modified interferon β -1a polypeptide and a pharmaceutically acceptable excipient. In aspects, the present disclosure provides a method for treating multiple sclerosis in a subject in need thereof, by administering the pharmaceutical composition to the subject.

5 In aspects, the present disclosure provides a nucleotide sequence encoding the modified interferon β -1a polypeptide with interferon β -1a activity. In some aspects, the nucleotide sequence is SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8. In some aspects, the present disclosure provides a plasmid incorporating the nucleotide sequence encoding the modified interferon β -1a polypeptide. In some aspects, the present disclosure provides an expression vector
10 incorporating the nucleotide sequence encoding the modified interferon β -1a polypeptide.

In aspects, the present disclosure provides a cell line for the expression of the modified interferon β -1a polypeptide with interferon β -1a activity, having a nucleotide sequence encoding the modified interferon β -1a polypeptide with interferon β -1a activity. In some aspects, the cell line is selected from CHO-K1, HEK293, NSO, BHK, Sp2/0, CAP or CAP/T. In some aspects,
15 the cell line is CHO-K1.

In aspects, the present disclosure provides a method of preparing the modified interferon β -1a polypeptide with interferon β -1a activity. The method involves cloning a nucleotide sequence into a lentiviral vector. In aspects, the nucleotide sequences is selected from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8. The method also involves
20 transducing a Chinese hamster ovary cell line (CHO-K1) with the vector and expressing the modified interferon β -1a polypeptide. In some aspects, the modified interferon β -1a polypeptide has an amino acid sequence selected from SEQ ID NO: 2 or SEQ ID NO: 3. The method also involves collecting the supernatant and purifying the modified interferon β -1a polypeptide from the supernatant using dye pseudo-affinity chromatography and high-performance liquid
25 chromatography (HPLC). The method also involves concentrating the modified interferon β -1a polypeptide with interferon β -1a activity by diafiltration.

BRIEF DESCRIPTION OF THE DRAWINGS

The present disclosure may be better understood with reference to the following figures.

30 FIG. 1 depicts the EpiMatrix MHC binding cluster immunogenicity scale. IFN β wild type (SEQ ID NO: 1) and the deimmunized variants IFN- β 1a (VAR1) (SEQ ID NO:2) and IFN- β 1a (VAR2) (SEQ ID NO:3) are mapped onto a cluster immunogenicity scale according to their

individual EpiMatrix scores. The EpiMatrix cluster immunogenicity score represents the deviation in putative epitope content from baseline expectation based on a random peptide standard. MHC binding clusters scoring above +10 are considered to be potentially immunogenic, while MHC binding clusters scoring lower than +10 are considered to have less potential to be immunogenic.

5 Some positive control peptides and proteins are also arranged by EpiMatrix score of immunogenicity, from highest (+80) to lowest (−50). The Z Score values assigned by the application of the Interactive Screening and Protein Reengineering Interface (ISPRI) immunoinformatics toolkit, provided by the company EpiVax (Providence, RI), are identified. The Z score is an estimator of immunogenicity from the evaluation of the content of epitopes
10 capable of triggering a T cell-dependent immune response.

FIG. 2 depicts a predicted lower pseudoenergy molecular structures of IFN β wild type (SEQ ID NO:1) and the deimmunized variants IFN- β 1a(VAR1) (SEQ ID NO:2) and IFN- β 1a(VAR2) (SEQ ID NO:3). Peptide backbones are represented by ribbons and residues selected for mutation by Van der Waals spheres. All residues considered for mutation are labeled with a
15 one-letter code to facilitate comparison.

FIGS. 3A-3B depicts a purity evaluation of different modified IFN- β 1a polypeptides. Specifically, FIG. 3A depicts the protein purity of the variants as assessed by SDS-PAGE under reducing conditions, followed by Coomassie blue staining. The purity level of the purified IFN- β -1a and its de-immunized variants as provided herein exceeded 96.5%. FIG. 3B depicts the identity
20 of the observed electrophoretic band, as confirmed by western blotting.

FIGS. 4A-4B depicts circular dichroism UV spectra of different modified IFN- β 1a polypeptides, expressed in molar ellipticity per residue. The spectra were obtained using a Jasco J-1500 spectropolarimeter (Jasco Inc., Easton, USA) at room temperature. FIG. 4A displays results acquired in the range of 250-320 nm (near ultraviolet), while FIG. 4B displays results
25 acquired in the range of 190 nm and 240 nm (far ultraviolet), using a scan speed of 20 nm/min and a response time of 1 second.

FIG. 5 illustrates the functional characterization of the purified IFN β -1a variants through the quantification of their antiviral biological activity. The absorbance data at 280 nm, corresponding to the IFN β -1a activity values (standard) (y-axis), were plotted against the sample
30 dilutions on a logarithmic scale (x-axis). The biological activity (AB) values of each polypeptide were determined by comparison with the standard using the parallel lines test.

FIG. 6 depicts the specific antiviral biological activity of rhIFN β and de-immunized variants in a bar graph. The antiviral activity of the IFN β -1a variants was assessed by their ability to inhibit the cytopathic effect of VSV on WISH cells, normalized to the concentration of each protein. IFN β -1a (VAR2) (SEQ ID NO: 3) showed similar antiviral activity to the wild type IFN β -1a (SEQ ID NO: 1), while IFN β -1a (VAR1) (SEQ ID NO: 2) demonstrated a 40% increase in specific biological activity compared to the original molecule. Assays were conducted in quintuplets, and data is presented as the mean $\pm$ SD of eight independent experiments (* $p < 0.05$).

FIGS. 7A-7F depict the quantification of total antibody titers across various plasma sample dilutions from animals 1-4 (FIG. 7A, FIG. 7C, FIG. 7E) and animals 5-8 (FIG. 7B, FIG. 7D, FIG. 7F) inoculated with IFN β -1a WT (SEQ ID NO: 1), IFN β -1a VAR1 (SEQ ID NO: 2), and IFN β -1a VAR2 (SEQ ID NO: 3), using an indirect ELISA assay.

FIGS. 8A-8C illustrate box-and-whisper plots, illustrating the deimmunized variants exhibit markedly reduced *in vivo* immunogenicity. FIG. 8A demonstrates splenocyte culture from IFN β -1a (WT)-treated mice exhibited robust IFN- γ release, which was expressed as stimulation index (SI). FIG. 8B demonstrates that mice treated with IFN β -1a(WT) developed higher titers of anti-IFN β -1a (WT) BAbs. FIG. 8C demonstrates that mice treated with IFN β -1a (WT) developed higher titers of anti-IFN β -1a (WT) NAbs.

FIG. 9A-9D illustrate the results from flow cytometric analysis using CD69 specific tags to activate memory (FIG. 9B, FIG. 9D) and naïve (FIG. 9A, FIG. 9C) T cells in PBMC samples from patients with multiple sclerosis. Values expressed as percentage of activated T lymphocytes with respect to the negative control (excipients).

FIGS. 10A-10D illustrate the results from flow cytometric analysis using CD69 (FIG. 10A, FIG. 10C) and CD154 (FIG. 10B, FIG. 10D) specific tags to restimulate memory T lymphocytes in PBMC samples from patients with multiple sclerosis. Values expressed as percentage of activated T lymphocytes with respect to the negative control (excipients).

FIGS. 11A-11E illustrate the results from flow cytometric analysis using CD69 specific tags to activate memory and naïve T cells in PBMC samples from multiple sclerosis patients from a cohort of 5 volunteer donors (Donor 7 (FIG. 11A), Donor 8 (FIG. 11B), Donor 9 (FIG. 11C), Donor 10 (FIG. 11D), Donor 11 (FIG. 11E)). Values expressed as percentage of activated T lymphocytes with respect to the negative control (excipients). Results obtained from flow cytometric analysis using CD69 specific tags.

FIGS. 12A-12F illustrate the results from flow cytometric analysis using CD69 (FIG. 12A, FIG. 12C, FIG. 12E) and CD154 (FIG. 12B, FIG. 12D, FIG. 12F) specific tags to restimulate memory T lymphocytes in PBMC from a patient with multiple sclerosis. Values expressed as percentage of activated T lymphocytes with respect to the negative control (excipients).

5

DETAILED DESCRIPTION

The following description of particular aspect(s) is merely exemplary in nature and is in no way intended to limit the scope of the present disclosure, its application, or uses, which may, of course, vary. The present disclosure is described with relation to the non-limiting definitions and terminology included herein. These definitions and terminology are not designed to function as a limitation on the scope or practice of the present disclosure but are presented for illustrative and descriptive purposes only. While the processes and compositions are described as using a specific order of individual steps or specific materials, it is appreciated that steps or materials may be interchangeable such that the description of the present disclosure may include multiple steps or parts arranged in many ways as is readily appreciated by one of skill in the art.

Reference will now be made in detail to various embodiments of the instantly disclosed modified IFN β -1 polypeptides (including modified IFN β -1a and modified IFN β -1b polypeptides) that present biological activity equivalent to recombinant IFN β -1 polypeptides and a reduction in its immunogenicity, nucleic acids that encode such modified IFN β -1 polypeptides, expression cassettes, plasmids, vectors, cells comprising such nucleic acids, and pharmaceutical compositions and formulations comprising the modified IFN β -1 polypeptides. As described these various compositions and formulations may be used in treating one or more diseases or conditions such as but not limited to neurodegenerative diseases, including but not limited to multiple sclerosis (MS).

Although any methods and materials similar or equivalent to those described herein may be used in the practice or testing of the present disclosure, exemplary methods and materials are described. Other features, objects, and advantages of the present disclosure will be apparent from the description and the claims. In the Specification and the appended claims, the singular forms include plural referents unless the context clearly dictates otherwise. All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

Definitions

To further facilitate an understanding of the present disclosure, a number of terms and phrases are defined below. Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. It will be further understood that terms such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the present disclosure, and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

The terminology used herein is for describing particular embodiments/aspects only and is not intended to be limiting.

As used herein, the singular forms “a,” “an,” and “the” are intended to include the plural forms, including “at least one,” unless the content clearly indicates otherwise. “Or” means “and/or.” As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items. It will be further understood that the terms “comprises” and/or “comprising,” or “includes” and/or “including” when used in this specification, specify the presence of stated features, regions, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, regions, integers, steps, operations, elements, components, and/or groups thereof. The term “or a combination thereof” means a combination including at least one of the foregoing elements.

As used herein, the term “biological sample” as refers to any sample of tissue, cells, or secretions from an organism.

As used herein, the term “medical condition” includes, but is not limited to, any condition or disease manifested as one or more physical and/or psychological symptoms for which treatment and/or prevention is desirable, and includes previously and newly identified diseases and other disorders.

As used herein, the term “immune response” refers to the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, granulocytes, and soluble macromolecules produced by the above cells or the liver (including antibodies, cytokines, and complement) that results in selective damage to, destruction of, or elimination from the human body of cancerous cells, metastatic tumor cells, malignant melanoma, invading pathogens, cells or tissues infected with pathogens, or, in cases of autoimmunity or pathological inflammation, normal human cells or tissues.

As used herein, the term “effective amount”, “therapeutically effective amount”, or the like of a composition, including modified interferon- β 1 compounds or compositions of the present disclosure is a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, e.g., an amount that results in the prevention of, or a decrease in, the symptoms associated with a disease that is being treated. The amount of a compound or composition of the present disclosure administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. The compounds and compositions of the present disclosure may also be administered in combination with each other or with one or more additional therapeutic compounds.

As used herein, the term “T cell epitope” means an MHC ligand or protein determinant, 7 to 30 amino acids in length, and capable of specific binding to human leukocyte antigen (HLA) molecules and interacting with specific T cell receptors (TCRs). Generally, T cell epitopes are linear and do not express specific three-dimensional characteristics. T cell epitopes are not affected by the presence of denaturing solvents. The ability to interact with T cell epitopes may be predicted by *in silico* methods (De Groot AS *et al.*, (1997), AIDS Res Hum Retroviruses, 13(7):539-41; Schafer JR *et al.*, (1998), Vaccine, 16(19):1880-4; De Groot AS *et al.*, (2001), Vaccine, 19(31):4385-95; De Groot AR *et al.*, (2003), Vaccine, 21(27-30):4486-504, all of which are herein incorporated by reference in their entirety.

As used herein, the term “T-cell epitope cluster” refers to polypeptide that contains between about 4 to about 40 MHC binding motifs. In particular embodiments, the T-cell epitope cluster contains between about 5 to about 35 MHC binding motifs, between about 8 and about 30 MHC binding motifs; and between about 10 and 20 MHC binding motifs.

As used herein, the term “immune stimulating T-cell epitope polypeptide” refers to a molecule capable of inducing an immune response, e.g., a humoral, T cell-based, or innate immune response.

As used herein, the term “B cell epitope” means a protein determinant capable of specific binding to an antibody. B cell epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-

conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term “subject” as used herein refers to any living organism in which an immune response is elicited. The term subject includes, but is not limited to, humans, nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered.

As used herein, the term “MHC complex” refers to a protein complex capable of binding with a specific repertoire of polypeptides known as HLA ligands and transporting said ligands to the cell surface.

As used herein, the term “MHC Ligand” means a polypeptide capable of binding to one or more specific MHC alleles. The term “HLA ligand” is interchangeable with the term “MHC Ligand”. Cells expressing MHC/Ligand complexes on their surface are referred to as “Antigen Presenting Cells” (APCs).

As used herein, the term “T Cell Receptor” or “TCR” refers to a protein complex expressed by T cells that is capable of engaging a specific repertoire of MHC/Ligand complexes as presented on the surface of APCs.

As used herein, the term “MHC Binding Motif” refers to a pattern of amino acids in a protein sequence that predicts binding to a particular MHC allele.

As used herein, the term “EpiBar™” refers to a 9-mer peptide that is predicted to be reactive to at least four different HLA alleles.

As used herein, the term “Immune Synapse” means the protein complex formed by the simultaneous engagement of a given T cell epitope to both a cell surface MHC complex and TCR.

The term “polypeptide” refers to a polymer of amino acids, and not to a specific length; thus, peptides, oligopeptides and proteins are included within the definition of a polypeptide. As used herein, a polypeptide is said to be “isolated” or “purified” when it is substantially free of cellular material when it is isolated from recombinant and non-recombinant cells, or free of chemical precursors or other chemicals when it is chemically synthesized. A polypeptide (e.g., a modified IFN β -1a polypeptide) of the present disclosure, however, may be joined to, linked to, or inserted into another polypeptide (e.g., a heterologous polypeptide) with which it is not normally

associated in a cell and still be “isolated” or “purified.” When a polypeptide is recombinantly produced, it may also be substantially free of culture medium, for example, culture medium represents less than about 20%, less than about 10%, or less than about 5% of the volume of the polypeptide preparation.

5 The terms “polynucleotide” and “nucleic acid sequence” are used interchangeably to refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues (e.g., peptide nucleic acids) having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides. The term “polynucleotide” is not intended to
10 limit the present invention to polynucleotides comprising DNA. Those of ordinary skill in the art will recognize that polynucleotides, may include ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The polynucleotides as provided herein also encompass all forms of sequences including, but not limited to, single-
15 stranded forms, double-stranded forms, and the like. As used herein, the terms “encoding” or “encoded” when used in the context of a specified polynucleotide mean that the polynucleotide comprises the requisite information to direct translation of the polynucleotide sequence into a specified polypeptide. The information by which a polypeptide is encoded is specified by the use of codons. A polynucleotide encoding a polypeptide may comprise non-translated sequences (e.g.,
20 introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (e.g., as in cDNA).

 As used herein, the term “natural interferon,” “natural human interferon beta-1a” (hIFN β -1a), “wild type interferon,” “native interferon,” or variants thereof refers to a cytokine (e.g., polypeptide, nucleic acid, etc.) as it is found in nature (i.e., wild type), without having been
25 subjected to any kind of artificial modification or mutation.

 As used herein, the term “amino acid substitution” refers to the change of one amino acid in the primary sequence of a natural (i.e., wild type) protein, such as hIFN β -1a, for another amino acid.

 As used herein, the term “modified interferon β -1a,” “human modified interferon- β 1a with
30 reduced immunogenicity,” “modified IFN β -1a,” “modified interferon-beta-1a,” “modified IFN-beta-1a,” “modified interferon-1a,” “modified IFN-1a,” or variants thereof refers to molecules of a modified interferon beta-1a molecule, containing changes to the amino acid or nucleic acid

sequence as compared to the appropriate natural interferon, and in aspects includes at least one glycosylation site. In aspects, the molecules have reduced immunogenicity as compared to natural human interferon.

As used herein, the term “z-score” indicates how many standard deviations an element is from the mean. A z-score may be calculated from the following formula. $z = (X - \mu) / \sigma$ where z is the z-score, X is the value of the element, μ is the population mean, and σ is the standard deviation.

The following abbreviations and/or acronyms are used throughout this application:

	ADA	antibody developed against
10	APC	antigen presenting cells
	DMSO	dimethyl sulfoxide
	DR antibody	antigen D related antibody
	EDTA	ethylenediaminetetraacetic acid
	ELISA	enzyme-linked immunosorbent assay
15	HLA	human leukocyte antigen
	IFN	interferon
	MHC	major histocompatibility complex
	PBMC	peripheral blood mononuclear cell
	RPMI	Roswell Park Memorial Institute medium
20	TCR	T-cell receptor
	Teff	effector T cell
	Th	helper T cell
	TReg	regulatory T cell

25 ***Modified IFN β -1a Polypeptides and Nucleic Acids***

In aspects, the present disclosure provides modified IFN β -1 polypeptides (including modified IFN β -1a and modified IFN β -1b polypeptides) with proven antiviral biological activity and reduced immunogenicity as compared to wild-type IFN β -1, and thus have use in human therapy, including the treatment of neurodegenerative diseases, such as multiple sclerosis. In aspects, a modified IFN β -1 polypeptide of the present disclosure is a modified IFN β -1a polypeptide having IFN β -1a activity and a reduced immunogenicity or a reduced propensity to elicit an immune response as compared to a wild type IFN β -1a polypeptide of SEQ ID NO: 1.

In aspects, the present disclosure provides a modified IFN β -1 polypeptide or nucleic acid encoding the modified IFN β -1 polypeptide having IFN β -1 activity (e.g., anti-viral activity) and reduced immunogenicity. In aspects, the modifications carried out in the natural amino acid sequence of human IFN β -1a for obtaining the modified IFN β -1a of the disclosure are a result of a modification of a gene encoding natural human interferon, such as wild-type IFN β -1a. Further, the modifications are introduced in such a way that they reduce the immunogenicity of the amino acid sequence as compared to natural human interferon, while substantially maintaining or improving its biological activity (such as its antiviral biological activity).

In aspects, the modified IFN β -1a polypeptides and related modified IFN β -1 compounds and compositions of the present disclosure have reduced immunogenicity as compared to natural interferon β -1. Mutations that reduce immunogenicity of a modified IFN β -1a as compared to natural IFN β -1a were identified by Interactive Screening and Protein Reengineering Interface (ISPRI) program analysis, provided by the company EpiVax, Inc. (Providence, Rhode Island), which is used to screen protein sequences for the presence of putative T cell epitopes. Input sequences are parsed into overlapping 9-unit (9-mer) frames where each frame overlaps the last by 8 amino acids. Each of the resulting frames is then scored for predicted binding affinity with respect to a panel of eight common Class II HLA alleles (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301 and DRB1*1501). Raw scores are normalized against the scores of a large sample of randomly generated peptides. The resulting “Z” score is reported. In aspects, any 9-mer frame peptide with an allele-specific EpiMatrix™ Z-score in excess of 1.64, theoretically the top 5% of any given sample, is considered to have a high probability of binding to MHC Class II. In aspects, any 9-mer frame peptide with an allele-specific EpiMatrix™ Z-score in excess of 2.32, theoretically the top 1% of any given sample, is considered to have a high probability of binding MHC Class II and includes most of the published T cell epitopes.

Peptides containing clusters of putative T cell epitopes are more likely to test positive in validating *in vitro* and *in vivo* assays. The results of the initial EpiMatrix™ analysis are further screened for the presence of putative T cell epitope “clusters” using a second proprietary algorithm known as Clustimer™ algorithm. The Clustimer™ algorithm identifies sub-regions contained within any given amino acid sequence that contains a statistically unusually high number of putative T cell epitopes. Typical T-cell epitope “clusters” are from about 9 amino acids in length and, considering their affinity to multiple alleles and across multiple 9-mer frames, may contain

anywhere from about 4 to about 40 putative T cell epitopes. Each epitope cluster identified an aggregate EpiMatrix™ score is calculated by summing the scores of the putative T cell epitopes and subtracting a correcting factor based on the length of the candidate epitope cluster and the expected score of a randomly generated cluster of the same length. EpiMatrix™ cluster scores in excess of +10 are considered significant. In aspects, the modified IFNβ-1 molecules described herein contain one or more modifications (e.g., changes, substitutions, or mutations) within the T cell epitope clusters to reduce their immunogenicity. For example, modified IFNβ-1 mutations for the instantly-disclosed modified IFNβ-1 molecules are selected that not only reduce the immunogenicity of the molecule, but also do not significantly reduce its biological activity, such as its antiviral activity, and/or that do not affect its binding to receptors involved in the interferon's biological activity. In aspects, such modifications for modified IFNβ-1 molecules of the present disclosure are selected that do not disrupt the structure or function of the natural interferon and include substitution of one or more amino acids occupying select positions in the natural human IFNβ-1 for any natural or non-naturally occurring amino acid, but in some aspects optionally for one or more of proline, histidine, leucine, threonine, or glutamic acid.

Many of the most reactive T cell epitope clusters contain a feature referred to as an "EpiBar™". As described previously, an EpiBar™ is a single 9-mer frame that is predicted to be reactive to at least four different HLA alleles. In aspects, the modified IFNβ-1 molecules of the present disclosure may include one or more modifications (e.g., changes, substitutions, or mutations) within the EpiBars® of the natural IFNβ-1. In aspects, said modifications of the modified IFNβ-1 molecules reduce the immunogenicity of the modified IFNβ-1 molecules as compared to the natural IFNβ-1. In aspects, said modifications of the modified IFNβ-1 molecules additionally do not substantially disrupt the structure or function of the natural IFNβ-1 activity. For example, modified IFNβ-1 mutations are selected that do not significantly reduce its biological activity, such as its antiviral activity, and/or that do not affect its binding to receptors involved in the interferon's biological activity. In aspects, such modifications for modified IFNβ-1 molecules of the present disclosure are selected that do not disrupt the structure or function of the natural interferon and include substitution of one or more amino acids occupying select positions in the natural human IFNβ-1 optionally for proline, histidine, leucine, threonine, or glutamic acid.

In aspects, the contribution of each amino acid in these identified cluster regions to HLA binding was evaluated using OptiMatrix tool (part of the EpiVax ISPRI toolkit for deimmunization). OptiMatrix begins with looking at "critical" residues, which contribute most to

MHC binding affinity across multiple 9-mer frames and multiple HLA alleles. The program then iteratively substitutes all 19 alternative amino acids in any given position of a protein sequence (with operator-defined input that may limit the list to naturally conserved variants) and then re-analyzes the predicted immunogenicity of the sequence following that change. To avoid a negative effect on protein structure and consequently in biological activity, a comprehensive search in literature for critical residues was also conducted, which identified amino acids that were not candidates for modification. In aspects, said modifications of the modified IFN β -1 molecules reduce the immunogenicity of the modified IFN β -1 molecules as compared to the natural IFN β -1. In aspects, said modifications of the modified IFN β -1 molecules additionally do not disrupt the structure or function of the natural IFN β -1 activity. For example, modified IFN β -1 mutations are selected that do not significantly reduce its biological activity, such as its antiviral activity, and/or that do not affect its binding to receptors involved in the interferon's biological activity. In aspects, such modifications for modified IFN β -1 molecules of the present disclosure are selected that do not disrupt the structure or function of the natural interferon and include substitution of one or more amino acids occupying select positions in the natural human IFN β -1 optionally for proline, histidine, leucine, threonine, or glutamic acid.

In aspects, a modified IFN β -1 polypeptide comprises the substitution of one or more amino acids in wild-type IFN β -1a (SEQ ID NO: 1). In aspects, the substitutions are of one or more amino acids occupying a position selected from the group consisting of the following positions in the natural IFN β -1a: 25, 28, 60, 65, 74, 111, 117, and/or 158. In aspects, the substitution includes the change of the amino acid from that position to an amino acid that may be any naturally occurring amino acid, any non-naturally occurring amino acid, or in some aspects and amino acid that is proline, histidine, leucine, threonine, or glutamic acid. In some aspects, the substitution reduces the immunogenicity of the modified IFN β -1 polypeptide as compared to the natural human IFN β -1. In aspects, a modified IFN β -1 molecule is a modified IFN β -1a polypeptide. In aspects, a modified IFN β -1 polypeptide is a modified IFN β -1b polypeptide.

In aspects, a modified IFN β -1 polypeptide includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% sequence identity to wild type IFN β -1a (SEQ ID NO: 1). A polypeptide may include one or more amino acid substitutions in any of the positions 25, 28, 60, 65, 74, 111, 117, and/or 158. In aspects, a modified IFN β -1a polypeptide includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% identity to wild type IFN β -1a (SEQ ID NO: 1) and therein includes one or more amino acid substitutions in any of the positions 25, 28, 60, 65,

74, 111, 117, and/or 158, wherein said substitution optionally is or includes the change of the amino acid of said position to proline, histidine, leucine, threonine, or glutamic acid. In aspects, a modified IFN β -1a polypeptide includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% identity to wild type IFN β -1a (SEQ ID NO: 1) and therein includes at least four amino acid substitutions in any of the positions 25, 28, 60, 65, 74, 111, 117, and/or 158. In aspects, a modified IFN β -1a polypeptide includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% homology to wild type IFN β -1a (SEQ ID NO: 1) and therein includes at least four amino acid substitutions in any of the positions 25, 28, 60, 65, 74, 111, 117, and/or 158, optionally wherein said substitution includes the change of the amino acid of said position to proline, histidine, leucine, threonine, or glutamic acid.

In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% identity to wild type interferon- α 2b (SEQ ID NO: 1) and therein includes one or more amino acid substitutions at positions 25, 60, 117, and/or 158, optionally wherein said substitutions include the change of the amino acid of the position to proline, histidine, leucine, threonine, or glutamic acid. In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% identity to wild type IFN β -1a (SEQ ID NO: 1) and therein includes the mutations N25P, Y60H, M117T and N158E. In aspects of the above-described polypeptides, the modified IFN β -1a polypeptides have reduced immunogenicity or a reduced propensity to elicit an immune response as compared to a wild type IFN β -1a polypeptide of SEQ ID NO: 1.

In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% identity to wild type IFN β -1a (SEQ ID NO: 1) and therein includes amino acid substitutions at positions 25, 28, 60, 65, 74, 111, 117, and/or 158, optionally wherein said substitutions include the change of the amino acid of the position to proline, histidine, leucine, threonine, or glutamic acid. In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity includes an amino acid sequence with at least 60%, 70%, 80%, 90%, or 95% identity to wild type IFN β -1a (SEQ ID NO: 1) and therein includes the mutations N25P, L28P, Y60H, N65H, S74L, F111L, M117T, and/or N158E. In aspects of the above-described polypeptides, the modified IFN β -1a polypeptides have reduced immunogenicity or a reduced propensity to elicit an immune response as compared to a wild type IFN β -1a polypeptide of SEQ ID NO: 1.

In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity is SEQ ID NO: 2 or SEQ ID NO: 3. In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity includes an amino acid sequence of SEQ ID NO: 2 (IFN β -1a(VAR1)) form. In aspects, a modified IFN β -1a polypeptide having IFN β -1a activity includes an amino acid sequence of SEQ ID NO: 3 (IFN β -1a(VAR2)). In aspects of the above-described polypeptides, the modified IFN β -1a polypeptides have reduced immunogenicity or a reduced propensity to elicit an immune response as compared to a wild type IFN β -1a polypeptide of SEQ ID NO: 1.

In aspects, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity of about 5% to about 200% as compared to a wild type IFN β -1a polypeptide of SEQ ID NO: 1. In aspects, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity of about 10% to about 175% as compared to a wild type IFN β -1a polypeptide of SEQ ID NO: 1. In aspects, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity of about 20% to about 150% as compared to a wild type interferon- α 2b polypeptide of SEQ ID NO: 1. In aspects, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity equal to or greater than a wild type interferon- α 2b polypeptide of SEQ ID NO: 1. In aspects, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity of about 100% to about 150% as compared to a wild type interferon- α 2b polypeptide of SEQ ID NO: 1. In aspects, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity of about 100% to about 140% as compared to a wild type interferon- α 2b polypeptide of SEQ ID NO: 1. Optionally, the instantly-disclosed modified IFN β -1a polypeptides having IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides, have a relative antiviral activity as compared to a wild type interferon- α 2b polypeptide of SEQ ID NO: 1 of about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, or about 150%.

In aspects, the present disclosure provides a polynucleotide or nucleic acid (e.g., DNA, including cDNA or RNA, including mRNA) encoding a modified IFN β -1a polypeptide having

IFN β -1a activity, such as the above-described modified IFN β -1a polypeptides. For example, in aspects, the present disclosure provides a nucleic acid encoding for one or more modified IFN β -1a polypeptides of: SEQ ID NO: 2 or SEQ ID NO: 3. In aspects, a nucleic acid encoding for one or more one or more modified IFN β -1a polypeptides includes one or more nucleic acid sequences of: SEQ ID NO: 5 or SEQ ID NO: 6. In aspects, a nucleic acid encoding a modified IFN β -1a polypeptide includes a nucleic acid sequence of SEQ ID NO: 5, encoding (IFN β -1a(VAR1)). In aspects, a nucleic acid encoding a modified IFN β -1a polypeptide includes a nucleic acid sequence of SEQ ID NO: 6, (IFN β -1a(VAR2)).

In aspects, the present disclosure provides a vector or plasmid comprising a nucleic acid of the present disclosure encoding one or more modified IFN β -1a polypeptides of the present disclosure. In aspects, the vector or plasmid includes a nucleic acid (e.g., DNA or RNA) encoding a modified IFN β -1a polypeptide, having a sequence comprising, consisting of, or consisting essentially of one or more of SEQ. ID NO: 7 (IFN β -1a(VAR1)) or SEQ ID NO: 8 (IFN β -1a(VAR2)). In aspects, the present disclosure is directed to a cell comprising a vector or plasmid of the present disclosure. It will be appreciated that the nucleic acid sequence of the vector or plasmid may have slight variations based on codon use optimization to effect expression of the IFN β -1a polypeptide in the cell.

In aspects, a modified IFN β -1a polypeptide as described herein is joined to or linked to (e.g., fused in-frame, chemically-linked, or otherwise bound) a heterologous polypeptide. With respect to the one or more modified IFN β -1a polypeptides of the instant disclosure, the term “heterologous polypeptide” is intended to mean that the one or more modified IFN β -1a polypeptides of the instant disclosure are not included naturally in the heterologous polypeptide. In aspects, one or more of the instantly-modified IFN β -1a polypeptides may be added to the C-terminus (with or without the use of linkers, as is known in the art), and/or added to the N-terminus (with or without the use of linkers, as is known in the art) of the heterologous polypeptide.

The present disclosure also provides chimeric or fusion polypeptides (which in aspects may be isolated, synthetic, or recombinant) wherein one or more of the instantly disclosed modified IFN β -1a polypeptides is a part thereof. In aspects, the one or more modified IFN β -1a polypeptides of the present disclosure may be joined or linked to (e.g., fused in-frame, chemically-linked, or otherwise bound) a small molecule, drug, or drug fragment, for example, but not limited to, a drug or drug fragment that is binds with high affinity to defined receptors.

As used herein, two polypeptides (or a region of the polypeptides) are substantially homologous or identical when the amino acid sequences have a certain percentage or more identity, e.g., at least about 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity, optionally at least about 70-75%, optionally at least about 80-85%, optionally equal to or greater than about 90%, and optionally equal to or greater than 95% or more homologous or identical. Percent homology may be determined as is known in the art. For example, to determine the percent homology or identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps may be introduced in the sequence of one polypeptide or nucleic acid molecule for optimal alignment with the other polypeptide or nucleic acid molecule). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid “identity” is equivalent to amino acid “homology”). As is known in the art, the percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. Sequence homology for polypeptides is typically measured using sequence analysis software.

In aspects, the present disclosure also encompasses polypeptides (e.g., modified IFN β -1a polypeptides and modified IFN β -1a compositions as disclosed herein) having a lower degree of identity but having sufficient similarity so as to perform one or more of the same functions performed by a polypeptide encoded by a nucleic acid molecule as provided herein. Similarity is determined by conserved amino acid substitution. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Conservative substitutions are likely to be phenotypically silent. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, Met, and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Trp, and Tyr. Guidance concerning which amino acid changes are likely to be phenotypically silent are found (Bowie, JU, et al., (1990), Science, 247(4948):130610, which is herein incorporated by reference in its entirety). For example, amino acid sequences having the function of an interferon may be identified by

performing a protein-protein BLAST (blastp) search of the non-redundant protein sequences (nr) database using the amino acid sequences of these proteins as query. The search may be conducted on the National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov>) using default parameters.

5 Fragments and variants of the disclosed modified IFN β -1a polypeptides and polynucleotides are also encompassed by the present disclosure. "Fragment" is intended to mean a portion of the polypeptide or polynucleotide. Fragments of a polypeptide or a nucleotide sequence as disclosed herein may encode polypeptide fragments that retain the biological activity of the polypeptides of the instant disclosure, and hence have retain IFN β -1a activity (e.g., antiviral
10 biological activity) with reduced immunogenicity as compared to wild-type IFN β -1a. In aspects, the present disclosure also encompasses fragments of the variants of the polypeptides and polynucleotides described herein.

 In aspects, a variant polypeptide (e.g., a variant of a modified IFN β -1a polypeptide of the present disclosure) may differ in amino acid sequence by one or more substitutions, deletions,
15 insertions, inversions, fusions, and truncations or a combination of any of these.

 Variant polypeptides may be fully functional (e.g., retain IFN β -1a activity, such as antiviral biological activity) or may lack function in one or more activities. Fully functional variants typically contain only conservative variation or variation in non-critical residues or in non-critical regions. Functional variants may also contain substitution of similar amino acids that
20 result in no change or an insignificant change in function (e.g., retain antiviral biological activity with reduced immunogenicity). Alternatively, such substitutions may positively or negatively affect function to some degree. Non-functional variants typically contain one or more non-conservative amino acid substitutions, deletions, insertions, inversions, or truncation or a substitution, insertion, inversion, or deletion in a critical residue or critical region. In aspects, a
25 modified IFN β -1a polypeptide of the instant disclosure may differ in amino acid sequence by one or more substitutions, deletions, insertions, inversions, fusions, and truncations or a combination of any of these, provided said variants retain biological activity (e.g., IFN β -1a activity, such an antiviral activity) and have reduced immunogenicity (as compared to wild-type IFN β -1a).

 In aspects, fully functional variants of modified IFN β -1a do not contain mutations at one
30 or more critical residues or regions. In aspects, said one or more critical residues of modified IFN β -1a that should not be mutated include: residues involved in or essential for biological activity, residues of functional hotspots that are heavily conserved between various wild type

interferon alleles (such as between species), residues implicated in binding to the interferon's natural receptor, residues involved in structural interactions that are important to the structural integrity of the natural interferon, residues engaged in disulfide bonds of the natural interferon (e.g., intramolecular disulfide bonds that occur in the natural interferon upon proper folding in its natural environment in vivo), and/or residues that are the site of glycosylation in the natural, wild type interferon (including N-glycosylation sites and O-glycosylation sites).

In aspects, the instantly-disclosed modified IFN β -1a polypeptides, including fully functional variants of disclosed modified IFN β -1a, do not contain mutations at one or more critical residues or regions, wherein said one or more critical residues or regions are selected from the group comprising: residues of functional hotspots, residues that are heavily conserved between various wild type interferon alleles (such as between species), residues engaged in disulfide bonds of the natural interferon (e.g., intramolecular disulfide bonds that occur in the natural interferon upon proper folding in its natural environment in vivo), and/or residues that are the site of glycosylation in the natural, wild type interferon (including N-glycosylation sites and O-glycosylation sites).

The polypeptides of the instant disclosure may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants and fragments of the instantly-disclosed polypeptides may be prepared by mutations in the DNA. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel et al. (1987) *Methods in Enzymol.* 154:367-382; U.S. Pat. No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff et al. (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be optimal. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe and Tyr. Guidance concerning which amino acid changes are likely to be

phenotypically silent are found (Bowie J U et al., (1990), Science, 247(4948):130610, which is herein incorporated by reference in its entirety). For the purposes of the present disclosure, polypeptides may include, for example, modified forms of naturally occurring amino acids such as D-stereoisomers, non-naturally occurring amino acids, amino acid analogs, and mimetics.

5 It should be clarified that the modifications/substitutions presented by the deimmunized variants of IFN β -1 correspond to those amino acids not involved in the biological structure or function of cytokine. That is, these mutations may be performed on any of the following interferon variants: IFN β -1a, IFN β -1b, or any other variant of IFN β -1.

The manner of producing the modified IFN β -1a polypeptides of the present disclosure will
10 vary widely, depending upon the nature of the various elements comprising the molecule. For example, the modified IFN β -1a polypeptides may be purified from cells that have been altered to express it (recombinant), or synthesized using known protein synthesis methods. The synthetic procedures may be selected so as to be simple, provide for high yields, and allow for a highly purified stable product. For example, polypeptides of the instant disclosure may be produced
15 either from a nucleic acid disclosed herein, or by the use of standard molecular biology techniques, such as recombinant techniques, mutagenesis, or other known means in the art. An isolated polypeptide may be purified from cells that naturally express it, purified from cells that have been altered to express it (recombinant), or synthesized using known protein synthesis techniques. In aspects, a polypeptide of the instant disclosure is produced by recombinant DNA or RNA
20 techniques. In aspects, a polypeptide of the instant disclosure may be produced by expression of a recombinant nucleic acid of the instant disclosure in an appropriate host cell. For example, a nucleic acid molecule encoding the polypeptide is cloned into an expression cassette or expression vector, the expression cassette or expression vector introduced into a host cell and the polypeptide expressed in the host cell. The polypeptide may then be isolated from the cells by an appropriate
25 purification scheme using standard protein purification techniques. Alternatively a polypeptide may be produced by a combination of *ex vivo* procedures, such as protease digestion and purification. Further, polypeptides of the instant disclosure may be produced using site-directed mutagenesis techniques, or other mutagenesis techniques known in the art (see e.g., James A. Brannigan and Anthony J. Wilkinson., 2002, Protein engineering 20 years on. Nature
30 Reviews Molecular Cell Biology 3, 964-970; Turanli-Yildiz B. et al., 2012, Protein Engineering Methods and Applications, intechopen.com, which are herein incorporated by reference in their entirety).

In aspects, the present disclosure is also directed to a method of synthesizing modified IFN β -1a polypeptides. The instantly-disclosed modified the modified IFN β -1a polypeptides with the improved properties (e.g., with reduced immunogenicity) may be created through genetic modification in one of a variety of ways that are described herein. The term “the modified IFN β -1a” as used herein, may refer to the group of instantly disclosed modified the modified IFN β -1a polypeptides having an intentionally altered amino acid sequence, i.e., a “non-wild type” amino acid sequence, or to a microbial organism (depending upon placement of either term as an adjective) having a genome that has been intentionally altered as to (at least) the specific, modified IFN β -1a molecules described herein, or both. Such alterations may be accomplished via recombinant technology, wherein one or more genes are transferred from a second, different microbial organism into a target microbial organism. Recombinant technology may be accomplished using fully synthetic DNA that is transferred to the target microbial organism using conventional methods. Such alterations may also be accomplished via engineered technology, wherein the nucleic acids within the target microbial organism are altered, generally via site-directed mutagenesis, resulting in the conversion of at least one nucleic acid to a different nucleic acid and therefore modification of one or more enzymes. Combinations of any of the above methods and those described throughout the application may also be employed. Thus, it will be understood that the instantly disclosed modified IFN β -1a molecule may be produced either *in vivo*, i.e., by a genetically modified microorganism, or *in vitro*.

In aspects, the present disclosure provides a method for generating said amino acid substitutions to reduce immunogenicity. Said method optionally includes the generation of point mutations in the nucleotide sequence of the gene encoding the human natural interferon (e.g., natural IFN β -1a), by means of a site-directed mutagenesis technique in said gene. The method includes the following steps: 1) cloning a gene encoding natural human interferon (e.g IFN β -1a) in a suitable plasmid; 2) generating mutations required for producing the modified IFN β -1a of the present disclosure using a site-directed mutagenesis technique; and 3) cloning the modified gene from step 2, into a suitable expression vector. In aspects, the expression vector is capable of carrying the gene of the present disclosure and further containing the necessary elements for expressing the gene of interest in eukaryotic cells.

In aspects, the site-directed mutagenesis technique of the present disclosure involves the use of oligonucleotides specifically designed to that end. This technique includes two stages. In the first stage, two PCR reactions are carried out separately using oligonucleotides that hybridize

to the terminal ends of the fragment cloned into a suitable vector, and oligonucleotides carrying a point mutation corresponding to an amino acid substitution that reduces immunogenicity (as described here) that hybridize to the internal region of the gene where the mutation is to be introduced. A reaction mixture is obtained in tube a using a reverse external oligonucleotide and the direct oligonucleotide mut a. Another reaction mixture is obtained in tube b with a direct external oligonucleotide and the reverse oligonucleotide mut b. PCR products from both reactions are purified by agarose gel electrophoresis and used as a template for the second stage. This second stage includes a second PCR reaction using direct and reverse external oligonucleotides. The first three cycles are carried out without the addition of primers to allow hybridization and elongation of the complete product and finally these are added for the amplification.

In aspects, to obtain more than one amino acid substitution sites that reduce immunogenicity within a modified IFN β -1a of the instant disclosure, said modified IFN β -1a is constructed sequentially as follows: first, a modified IFN β -1a with amino acid substitution site is generated, using a site-directed mutagenesis technique, and then said modified IFN β -1a is used as a starting template for generating a new amino acid substitution site.

In aspects, the present disclosure also provides for nucleic acids (e.g., DNA, RNA, vectors, viruses, or hybrids thereof, all of which may be isolated, synthetic, or recombinant) that encode in whole or in part one or more modified IFN β -1a polypeptides of the present disclosure and/or chimeric or fusion polypeptide compositions of the present disclosure. In aspects, the nucleic acid further includes, or is contained within, an expression cassette, a plasmid, and expression vector, or recombinant virus, wherein optionally the nucleic acid, or the expression cassette, plasmid, expression vector, or recombinant virus is contained within a cell, optionally a human cell or a non-human cell, and optionally the cell is transformed with the nucleic acid, or the expression cassette, plasmid, expression vector, or recombinant virus. In aspects, cells are transduced, transfected, or otherwise engineered to contain within one or more of e.g., polypeptides (modified IFN β -1a polypeptides) of the present disclosure; isolated, synthetic, or recombinant nucleic acids, expression cassettes, plasmids, expression vectors, or recombinant viruses as disclosed herein; and/or isolated, synthetic, or recombinant chimeric or fusion polypeptide compositions as disclosed herein. In aspects, the cell may be a mammalian cell, bacterial cell, insect cell, or yeast cell. In aspects, the nucleic acid molecules of the present disclosure may be inserted into vectors and used, for example, as expression vectors or gene therapy vectors. Gene therapy vectors may be delivered to a subject by, e.g., intravenous injection, local administration (U.S. Pat. No.

5,328,470) or by stereotactic injection (Chen S H et al., (1994), Proc Natl Acad Sci USA, 91(8):3054-7, which are herein incorporated by reference in their entirety). The pharmaceutical preparation of the gene therapy vector may include the gene therapy vector in an acceptable diluent, or may include a slow release matrix in which the gene delivery vehicle is imbedded.

5 Alternatively, where the complete gene delivery vector may be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation may include one or more cells that produce the gene delivery system. Such pharmaceutical compositions may be included in a container, pack, or dispenser together with instructions for administration. In aspects, the present disclosure is directed to a cell comprising a vector of the present disclosure. In aspects, the cell
10 may be a mammalian cell, bacterial cell, insect cell, or yeast cell.

For polynucleotides, a “variant” includes a deletion and/or addition of one or more nucleotides at one or more internal sites within the polynucleotide sequences of the instant disclosure and/or a substitution of one or more nucleotides at one or more sites in the polynucleotide sequences of the instant disclosure. One of skill in the art will recognize that
15 variants of the polynucleotides of this disclosure will be constructed such that the open reading frame is maintained. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides as provided herein. Naturally occurring allelic variants such as these may be identified with the use of well-known molecular biology techniques, as, for example, with
20 polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis but which still encode a polynucleotide having the desired activity as provided herein (i.e., encoding a polypeptide that possesses the desired biological activity, that is, antipathogenic activity, antifungal activity, antialgal activity, and/or
25 enzymatic activity against chitin and/or polyglucuronic acid as described herein). Generally, variants of a particular polynucleotide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters described elsewhere herein.

30 Variants of a particular polynucleotide of the present disclosure (i.e., the reference polynucleotide) may also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference

polynucleotide. Percent sequence identity between any two polypeptides may be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides of the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

The polynucleotides provided herein (whether RNA, DNA, expression cassettes, vectors, viruses or hybrids thereof) that encode in whole or in part one or more polypeptides of the present disclosure may be isolated from a variety of sources, genetically engineered, amplified, synthetically produced, and/or expressed/generated recombinantly. Recombinant polypeptides generated from these nucleic acids may be individually isolated or cloned and tested for a desired activity. Any recombinant expression system may be used, including e.g. in vitro, bacterial, fungal, mammalian, yeast, insect or plant cell expression systems. In aspects polynucleotides provided herein are synthesized in vitro by well-known chemical synthesis techniques (as described in, e.g., Adams (1983) *J. Am. Chem. Soc.* 105:661; Belousov (1997) *Nucleic Acids Res.* 25:3440-3444; Frenkel (1995) *Free Radic. Biol. Med.* 19:373-380; Blommers (1994) *Biochemistry* 33:7886-7896; Narang (1979) *Meth. Enzymol.* 68:90; Brown (1979) *Meth. Enzymol.* 68:109; Beaucage (1981) *Tetra. Lett.* 22:1859; U.S. Pat. No. 4,458,066, all of which are herein incorporated by reference in their entirety). Further, techniques for the manipulation of polynucleotides provided herein, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature (see, e.g., Sambrook, ed., *Molecular Cloning: A Laboratory Manual* (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); *Current Protocols In Molecular Biology*, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); *Laboratory Techniques In Biochemistry And Molecular Biology: Hybridization With Nucleic Acid Probes*, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993), all of which are herein incorporated by reference in their entirety).

In aspects, the present disclosure is directed to a characterized cell line comprising the nucleic acid that encodes for a modified IFN β -1a as provided herein. In aspects, said cell line is suitable for the production of a modified IFN β -1a as provided herein. In some aspects, a cell line suitable for the production of a modified interferon- α 2 as provided herein is selected from the set of CHO-K1, HEK293, NS0, BHK, Sp2/0, CAP, and CAP/T. In aspects, the present disclosure is

also directed to a method for obtaining a eukaryotic cell line for producing a modified IFN β -1a as provided herein by transformation or transfection of a cell line containing said gene encoding a modified IFN β -1a as provided herein, inserted in a suitable expression vector. Optionally, the eukaryotic cell line is a CHO.K1 cell line. In aspects, the present disclosure is directed to a method for producing a modified IFN β -1a as provided herein, said method including the steps of a) culturing said transformed or transfected eukaryotic cell line with an expression vector containing the gene encoding a modified IFN β -1a polypeptide as disclosed herein, and b) isolating the expressed and secreted modified IFN β -1a polypeptide from the culture medium.

In aspects, modified IFN β -1a compounds or compositions of the present disclosure (including one or more modified IFN β -1a polypeptides, polynucleotides, microorganism that expresses one or more polypeptides or polynucleotides, expression cassettes, plasmids, expression vectors, chimeric or fusion polypeptides, recombinant viruses and/or pharmaceutical compositions of the present disclosure) may be purified to homogeneity or partially purified. It is understood, however, that preparations in which the modified IFN β -1a compositions are not purified to homogeneity are useful. The critical feature is that the preparation allows for the desired function of the modified IFN β -1a, even in the presence of considerable amounts of other components. Thus, the present disclosure encompasses various degrees of purity. In one embodiment, the language “substantially free of cellular material” includes preparations of the modified interferon- α 2 having less than about 30% (by dry weight) other proteins (e.g., contaminating protein), less than about 20% other proteins, less than about 10% other proteins, less than about 5% other proteins, less than about 4% other proteins, less than about 3% other proteins, less than about 2% other proteins, less than about 1% other proteins, or any value or range there between.

In aspects, a modified IFN β -1a compound or composition of the present disclosure is recombinantly produced, wherein said modified IFN β -1a composition may also be substantially free of culture medium, for example, culture medium represents less than about 20%, less than about 10%, or less than about 5% of the volume of the modified IFN β -1a polypeptide, nucleic acid, or chimeric or fusion polypeptide preparation. The language “substantially free of chemical precursors or other chemicals” includes preparations of the polypeptide, nucleic acid, or chimeric or fusion polypeptide in which it is separated from chemical precursors or other chemicals that are involved in the synthesis of the modified IFN β -1a. The language “substantially free of chemical precursors or other chemicals” may include, for example, preparations of modified

IFN β -1a polypeptide, nucleic acid, or chimeric or fusion polypeptide having less than about 30% (by dry weight) chemical precursors or other chemicals, less than about 20% chemical precursors or other chemicals, less than about 10% chemical precursors or other chemicals, less than about 5% chemical precursors or other chemicals, less than about 4% chemical precursors or other chemicals, less than about 3% chemical precursors or other chemicals, less than about 2% chemical precursors or other chemicals, or less than about 1% chemical precursors or other chemicals.

In aspects, a modified IFN β -1a compound or composition of the present disclosure may be produced by standard recombinant DNA or RNA techniques as are known in the art. For example, DNA or RNA fragments coding for the different polypeptide sequences may be ligated together in-frame in accordance with conventional techniques. In another embodiment, the fusion gene may be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, polymerase chain reaction (PCR) amplification of nucleic acid fragments may be carried out using anchor primers which give rise to complementary overhangs between two consecutive nucleic acid fragments which may subsequently be annealed and re-amplified to generate a chimeric nucleic acid sequence (Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, (2ND, 1992), FM Asubel et al. (eds), Green Publication Associates, New York, N.Y. (Publ), ISBN: 9780471566355, which is herein incorporated by reference in its entirety). Further, one or more polypeptides (e.g., modified IFN β -1a polypeptide) of the present disclosure may be inserted into a heterologous polypeptide or inserted into a non-naturally occurring position of a polypeptide through recombinant techniques, synthetic polymerization techniques, mutagenesis, or other standard techniques known in the art. For example, protein engineering by mutagenesis may be performed using site-directed mutagenesis techniques, or other mutagenesis techniques known in the art (see e.g., James A. Brannigan and Anthony J. Wilkinson., 2002, Protein engineering 20 years on. Nature Reviews Molecular Cell Biology 3, 964-970; Turanli-Yildiz B. et al., 2012, Protein Engineering Methods and Applications, intechopen.com, which are herein incorporated by reference in their entirety).

Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g., a GST protein). A nucleic acid molecule encoding a modified IFN β -1a of the invention may be cloned into such an expression vector such that the fusion moiety is linked in-

frame to the at least one modified IFN β -1a. Such linking of the fusion moiety may be done, for example, to improve protein purification yields.

Pharmaceutical Compositions and Formulations

5 In aspects, one or more modified IFN β -1a polypeptides, chimeric polypeptides, polynucleotides, microorganism that expresses one or more polypeptides or polynucleotides, expression cassettes, plasmids, expression vectors, and/or recombinant viruses of the present disclosure (hereafter referred to as “modified interferon β -1a compounds or compositions of the present disclosure” or the like) may be comprised in a pharmaceutical composition or formulation.

10 In aspects, pharmaceutical compositions or formulations generally include a modified interferon β -1a compound or composition of the present disclosure and a pharmaceutically-acceptable carrier and/or excipient. In aspects, said pharmaceutical compositions are suitable for administration.

Pharmaceutically-acceptable carriers and/or excipients are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions for administering the instantly disclosed modified interferon β -1a compositions (see, e.g., Remington’s Pharmaceutical Sciences, (18th ed., 1990), Mack Publishing Co., Easton, PA Publ)). In aspects, the pharmaceutical compositions are generally formulated as sterile, substantially isotonic, and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

20 The terms “pharmaceutically-acceptable,” “physiologically-tolerable,” and grammatical variations thereof, as they refer to compositions, carriers, excipients, and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a subject without the production of undesirable physiological effects to a degree that would prohibit administration of the composition. For example, “pharmaceutically-acceptable excipient” means, 25 for example, an excipient that is useful in preparing a pharmaceutical composition that is generally safe, non-toxic, and desirable, and includes excipients that are acceptable for veterinary use as well as for human pharmaceutical use. Such excipients may be solid, liquid, semisolid, or, in the case of an aerosol composition, gaseous. A person of ordinary skill in the art would be able to 30 determine the appropriate timing, sequence and dosages of administration for modified interferon β -1a compositions of the present disclosure.

In aspects, exemplary carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and compounds for pharmaceutically active substances is well known in the art. Except insofar as any conventional
5 media or compound is incompatible with the modified interferon β -1a compounds or compositions of the present disclosure and as previously described above, use thereof in the compositions is contemplated. Supplementary active compounds may also be incorporated into the compositions.

In aspects, a modified interferon β -1a compound or composition of the present disclosure is formulated to be compatible with its intended route of administration. The modified interferon β -
10 1a compounds or compositions of the present disclosure may be administered by parenteral, topical, intravenous, oral, subcutaneous, intra-arterial, intradermal, transdermal, rectal, intracranial, intrathecal, intraperitoneal, intranasal; vaginally; intramuscular route or as inhalants. In aspects, modified interferon β -1a compounds or compositions of the present disclosure may be injected directly into a particular tissue. In other aspects, intramuscular injection or intravenous
15 infusion may be used for administration of modified interferon β -1a compounds or compositions of the present disclosure. In some methods, modified interferon β -1a compounds or compositions of the present disclosure are administered as a sustained release composition or device, such as but not limited to a Medipad™ device.

In aspects, modified interferon β -1a compounds or compositions of the present disclosure
20 may optionally be administered in combination with other agents that are at least partly effective in treating various medical conditions as described herein. For example, modified interferon β -1a pharmaceutical compositions or formulations of the present disclosure may also be administered in conjunction with other agents that stimulate antiviral activity of the immune system, improve pharmacokinetic parameters of the composition, enhance and/or compliment the natural biological
25 activity of interferon β -1a, and/or reduce immunogenicity of the composition.

In aspects, solutions or suspensions used for parenteral, intradermal, or subcutaneous application may include, but are not limited to, a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial compounds such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic
30 acid or sodium bisulfite; chelating compounds such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and compounds for the adjustment of tonicity such as sodium chloride or dextrose. The pH may be adjusted with acids or bases, such as hydrochloric

acid or sodium hydroxide. Examples of excipients may include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, water, ethanol, DMSO, glycol, propylene, dried skim milk, and the like. The composition may also contain pH buffering reagents, and wetting or emulsifying agents. In aspects, the parenteral preparation may be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

In aspects, pharmaceutical compositions or formulations suitable for injectable use include sterile aqueous solutions (where water-soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition is sterile and should be fluid to the extent that easy syringeability exists. It is stable under the conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacteria and fungi. In aspects, modified interferon β -1a formulations may include aggregates, fragments, breakdown products and post-translational modifications, to the extent these impurities have reduced immunogenicity and high relative antiviral activity that is similar to pure modified interferon β -1a. The carrier may be a solvent or dispersion medium containing, e.g., water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity may be maintained, e.g., by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms may be achieved by various antibacterial and antifungal compounds, e.g., parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In some aspects, isotonic compounds, e.g., sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride, may be included in the composition. Prolonged absorption of the injectable compositions may be brought about by including in the composition a compound that delays absorption, e.g., aluminum monostearate and gelatin.

In aspects, sterile injectable solutions may be prepared by incorporating the modified interferon β -1a compounds or compositions of the present disclosure in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the binding agent into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that

yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. Further, modified interferon β -1a compounds or compositions of the present disclosure may be administered in the form of a depot injection or implant preparation that may be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

In aspects, solutions or suspensions of pharmaceutical compositions or formulations are maintained at a pH at which the modified interferon β -1a polypeptide is in its natural structural conformation. In aspects, said pH is maintained below pH 10. In aspects, said pH is maintained below pH 7. In aspects, said pH is maintained between pH 3-10. In aspects, said pH is maintained between pH 4-9. In aspects, said pH is maintained between pH 5-8. In aspects, said pH is maintained between pH 6-7.5. In aspects, a buffer is provided to maintain the pH at a desired level. In aspects, said buffer is a phosphate buffer. In aspects, said buffer is an acetate buffer.

In aspects, solutions or suspensions of pharmaceutical compositions or formulations include surface adsorption inhibitors. In aspects, said surface adsorption inhibitors are provided that inhibit the adsorption of components of the pharmaceutical compositions or formulations by surfaces that enclose the compositions or formulations (such as ampoules, syringes, or vials made of glass or plastic). In some embodiments, said surface adsorption inhibitors are provided that inhibit the adsorption of one or more modified interferon β -1a polypeptides by glass surfaces that enclose the compositions or formulations. In aspects, the pharmaceutical compositions or formulations are enclosed in ampoules, syringes, or vials made of borosilicate glass, and the pharmaceutical compositions or formulations include a surface adsorption inhibitor (e.g., a surface adsorption inhibitor that inhibits the adsorption of one or more modified interferon β -1a polypeptides by the borosilicate glass surface). In aspects, said surface adsorption inhibitor is Polysorbate 80. In aspects, said surface adsorption inhibitor is albumin.

In aspects, solutions or suspensions of pharmaceutical compositions or formulations include degradation inhibitors. In aspects, degradation inhibitors are provided that inhibit the degradation of a modified interferon β -1a polypeptide. In aspects, degradation inhibitors are provided that inhibit the oxidative degradation of a modified interferon β -1a polypeptide. In aspects, degradation inhibitors are provided that inhibit the oxidative degradation of a modified interferon β -1a polypeptide, wherein said degradation inhibitor is benzyl alcohol.

In aspects, pharmaceutical compositions or formulations include a sterile powder for the extemporaneous preparation of sterile injectable solutions or dispersion, wherein said sterile

powder comprises: a dry powder formulation of one or more modified interferon β -1a polypeptides, a bulking agent, and a surface adsorption inhibitor. In aspects, said bulking agent is glycine. In aspects, said surface adsorption inhibitor is albumin. In aspects, said sterile powder further comprises one or more antimicrobial preservatives. In aspects, said one or more antimicrobial preservatives are selected from the group comprised of: m-cresol, benzyl alcohol, and phenol. In aspects, said sterile powder further comprises sodium phosphate dibasic and sodium phosphate monobasic. In aspects, said sterile powder is provided as a tablet-like solid that is whole, in pieces, and/or in a loose powder. In aspects, said dry powder formulation of one or more modified interferon β -1a polypeptides is a lyophilized powder. In aspects, said one or more modified interferon β -1a polypeptides are provided that have a desired specific activity. In aspects, said sterile powder is stored at a cold temperature prior to administration to a subject. In aspects, said sterile powder is stored at a temperature in the range of 2°C-8°C prior to administration to a subject. In aspects, prior to administration to a subject, said sterile powder is reconstituted with a diluent to provide a sterile solution. In aspects, said reconstitution is accomplished by dissolving the sterile powder in the diluent (e.g., by stirring, swirling, inverting, shaking, vortexing, or other means known and understood in the art) to produce the sterile solution. In aspects, said diluent comprises one or more components selected from the group comprised of: sterile water, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, EDTA, polysorbate 80, and m-cresol. In aspects, said resuspension is performed in a single-use vial, ampoule, or syringe. In aspects, said sterile solution provides one or more modified interferon β -1a polypeptides at a desired concentration. In aspects, said desired concentration of modified interferon β -1a polypeptide is 1-100 million IU/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 10-50 million IU/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 1-10 million IU/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is decreased for a maintenance dose during maintenance treatment of a condition in a subject. In aspects, said sterile solution is stored at a cold temperature prior to administration to a subject. In aspects, said sterile solution is stored at a temperature in the range of 2°C-8°C prior to administration to a subject.

In aspects, pharmaceutical compositions or formulations include solutions or suspensions comprising one or more modified interferon β -1a polypeptides and one or more components, wherein said components are selected from the group comprised of: sterile water, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, EDTA, one or more surface adsorption

inhibitors (e.g., polysorbate 80), one or more antimicrobial preservatives (e.g., m- cresol), one or more bulking agents, and one or more degradation inhibitors. In aspects, said solution or suspension comprises: one or more modified interferon β -1a polypeptides, sterile water, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, EDTA, polysorbate 80, and m-cresol. In aspects, said one or more modified interferon β -1a polypeptides are provided that have a desired specific activity. In aspects, said solution or suspension provides said one or more modified interferon β -1a polypeptides at a desired concentration. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 1-100 million IU/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 10-50 million IU/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 1-10 million IU/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is decreased for a maintenance dose during maintenance treatment of a condition in a subject. In aspects, said solution or suspension is stored at a cold temperature prior to administration to a subject. In aspects, said solution or suspension is stored at a temperature in the range of 2°C-8°C prior to administration to a subject.

In aspects, solutions or suspensions of pharmaceutical compositions or formulations comprise: one or more modified interferon β -1a polypeptides, a salt, and a buffer. In aspects, said buffer is provided to maintain the pH at a desired level. In aspects, said buffer is phosphate buffer and said salt is sodium chloride.

In aspects, pharmaceutical compositions or formulations include a sterile powder for the extemporaneous preparation of sterile injectable solutions or dispersion, wherein said sterile powder comprises a dry powder formulation of one or more modified interferon β -1a polypeptides. In aspects, said sterile powder further comprises one or more components selected from the group comprised of: dibasic sodium phosphate anhydrous, monobasic sodium phosphate dihydrate, sucrose, and polysorbate 80. In aspects, said sterile powder is provided as a tablet-like solid that is whole, in pieces, and/or in a loose powder. In aspects, said dry powder formulation of one or more modified interferon β -1a polypeptides is a lyophilized powder. In aspects, said one or more modified interferon β -1a polypeptides are provided that have a desired specific activity. In aspects, said sterile powders are stored at a cold temperature prior to administration to a subject. In aspects, said sterile powders stored at a temperature in the range of 2°C-8°C prior to administration to a subject. In aspects, said sterile powders are stored at room temperature prior to resuspension. In aspects, said sterile powders stored at a temperature in the range of 15°C-30°C prior to

resuspension. In aspects, prior to administration to a subject, said sterile powder is reconstituted with a diluent to provide a sterile solution. In aspects, said reconstitution is accomplished by dissolving the sterile powder in the diluent (e.g., by stirring, swirling, inverting, shaking, vortexing, or other means known and understood in the art) to produce the sterile solution. In aspects, said diluent comprises sterile water. In aspects, said resuspension is performed in a single-use vial, ampoule, or syringe. In aspects, said resuspension is performed in a dual-chamber cartridge, wherein a first chamber contains said sterile powder and a second chamber contains said diluent, and wherein, prior to injection, the components of the two chambers are combined to produce a sterile solution. In aspects, said dual chamber cartridge is used to inject said sterile solution into a subject via an injection apparatus that is a part of the dual-chamber cartridge. In aspects, said sterile solution provides said one or more modified interferon β -1a polypeptides at a desired concentration. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 50-500 mcg/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 100-300 mcg/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 100-2000 mcg/mL. In aspects, said desired concentration of a modified interferon β -1a polypeptide is 400-1200 mcg/mL. In aspects, said sterile solution is stored at a cold temperature prior to administration to a subject. In aspects, said sterile solution is stored at a temperature in the range of 2°C-8°C prior to administration to a subject.

In aspects, pharmaceutical compositions or formulations of a modified interferon β -1a compound or composition of the present disclosure are co-administered with one or more other pharmaceutical compositions or formulations. In aspects, said one or more other pharmaceutical compositions or formulations are selected from the group consisting of: ribavirin (e.g., REBETOL®), PegIntron®, and INTRON-A®.

In aspects, oral compositions generally include an inert diluent or an edible carrier and may be enclosed in gelatin capsules or compressed into tablets. In aspects, for the purpose of oral therapeutic administration, the binding agent may be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions may also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding compounds, and/or adjuvant materials may be included as part of the composition. In aspects, the tablets, pills, capsules, troches and the like may contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient

such as starch or lactose, a disintegrating compound such as alginic acid, Primogel or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening compound such as sucrose or saccharin; or a flavoring compound such as peppermint, methyl salicylate or orange flavoring.

5 For administration by inhalation, modified interferon β -1a compounds or compositions of the present disclosure may be delivered in the form of an aerosol spray from pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

In aspects, systemic administration of modified interferon β -1a compounds or compositions of the present disclosure may also be by transmucosal or transdermal means. For
10 transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, e.g., for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration may be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the modified interferon β -1a compounds or compositions of the
15 present disclosure may be formulated into ointments, salves, gels, or creams and applied either topically or through transdermal patch technology as generally known in the art.

In aspects, modified interferon β -1a compounds or compositions of the present disclosure may also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

20 In aspects, modified interferon β -1a compounds or compositions of the present disclosure are prepared with carriers that protect the modified interferon β -1a compositions against rapid elimination from the body, such as a controlled-release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers may be used, such as, for example, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen,
25 polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials may also be obtained commercially, e.g., from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) may also be used as pharmaceutically-acceptable carriers. These may be prepared according to methods known to
30 those skilled in the art (U.S. Pat. No. 4,522,811, which is herein incorporated by reference in its entirety). In aspects, the modified interferon β -1a compounds or compositions of the present

disclosure may be implanted within or linked to a biopolymer solid support that allows for the slow release of the modified interferon β -1a compositions to the desired site.

In aspects, it may be advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. “Dosage unit form” as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of binding agent calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the instant disclosure are dictated by and directly dependent on the unique characteristics of the binding agent and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such modified interferon β -1a compounds or compositions of the present disclosure for the treatment of a subject.

Methods of Use

The modified IFN β -1a compounds or compositions of the present disclosure (including one or more modified IFN β -1a polypeptides, polynucleotides, microorganism that expresses one or more polypeptides or polynucleotides, expression cassettes, plasmids, expression vectors, chimeric or fusion polypeptides, recombinant viruses and/or pharmaceutical compositions of the present disclosure) find use in protecting/treating against neurodegenerative diseases, including, but not limited to, multiple sclerosis and chronic inflammatory demyelinating polyneuropathy; autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis; inflammatory diseases, including, but not limited to, idiopathic pulmonary fibrosis, dermatomyositis, and polymyositis; cancer; and viral diseases, such as hepatitis C and hepatitis B.

In aspects, the present disclosure provides the use of a modified IFN β -1a compounds or compositions of the present disclosure, such as disclosed herein, for manufacturing a medicament for the treatment of neurodegenerative diseases, including, but not limited to, multiple sclerosis and chronic inflammatory demyelinating polyneuropathy; autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis; inflammatory diseases, including, but not limited to, idiopathic pulmonary fibrosis, dermatomyositis, and polymyositis; cancer; and viral diseases, such as hepatitis C and hepatitis B.

In aspects, the present disclosure is directed to methods of preventing or treating one or more medical conditions in a subject comprising administering one or more modified IFN β -1a compounds or compositions of the present disclosure, and preventing or treating the medical

condition in a subject by said step of administering said one or more modified IFN β -1a compounds or compositions of the present disclosure. The medical condition can be, for example against neurodegenerative diseases, including, but not limited to, multiple sclerosis and chronic inflammatory demyelinating polyneuropathy; autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis; inflammatory diseases, including, but not limited to, idiopathic pulmonary fibrosis, dermatomyositis, and polymyositis; cancer; and viral diseases, such as hepatitis C and hepatitis B. In aspects, the modified IFN β -1a compounds or compositions of the present disclosure can be used with in conjunction with other proteins or compounds used for treating a subject with the medical condition in order to reduce adverse events or enhance the efficacy of the co-administered compound.

In a particular aspect, the present disclosure is directed to, for example, methods of treating multiple sclerosis, said method comprising administering one or more modified IFN β -1a compounds or compositions of the present disclosure, and preventing or treating IFN β -1a in a subject by said step of administering said one or more modified IFN β -1a compounds or compositions of the present disclosure. The modified IFN β -1a compounds or compositions of the present disclosure can be used with in conjunction with other proteins or compounds used for treating a subject with multiple sclerosis in order to reduce adverse events or enhance the efficacy of the co-administered compound. In aspects, the modified IFN β -1a compounds or compositions of the present disclosure demonstrate high relative antiviral activity with reduced immunogenicity in multiple sclerosis treatment.

In a particular aspect, the present disclosure is directed to, for example, methods of treating chronic inflammatory demyelinating polyneuropathy, said method comprising administering one or more modified IFN β -1a compounds or compositions of the present disclosure, and preventing or treating chronic inflammatory demyelinating polyneuropathy in a subject by said step of administering said one or more modified IFN β -1a compounds or compositions of the present disclosure. The modified IFN β -1a compounds or compositions of the present disclosure can be used with in conjunction with other proteins or compounds used for treating a subject with chronic inflammatory demyelinating polyneuropathy in order to reduce adverse events or enhance the efficacy of the co-administered compound. In aspects, the modified IFN β -1a compounds or compositions of the present disclosure demonstrate high relative antiviral activity with reduced immunogenicity in chronic inflammatory demyelinating polyneuropathy treatment.

In aspects of the above-described methods, said modified IFN β -1a compounds or compositions of the present disclosure are co-administered with one or more other pharmaceutical compositions or formulations. In aspects, said one or more other pharmaceutical compositions or formulations are selected from the group consisting of: AVONEX®, REBIF®, glatiramer acetate
5 ofatumumab, alemtuzumab, natalizumab, ocrelizumab, cladribine, dimethyl fumarate, diroximel fumarate, monomethyl fumarate, fingolimod, siponimod, ozanimod, ponesimod, and teriflunomide.

The methods described herein can be performed, e.g., by utilizing pre-packaged kits comprising at least one pharmaceutical formulation or composition for treatment and/or
10 prevention of a disease as described herein (including a melanoma or viral infection and/or related diseases), which can be conveniently used, e.g., in clinical settings to treat subjects exhibiting symptoms or family history of a medical condition described herein. In one embodiment, the kit further comprises instructions for use of the at least one modified interferon- α 2 composition of the instant disclosure to treat subjects exhibiting symptoms or family history of a medical
15 condition described herein.

EXAMPLES

The examples that follow are not to be construed as limiting the scope of the disclosure or claims in any manner. In light of the present disclosure, numerous aspects within the scope of the claims will be apparent to those of ordinary skill in the art.

Example 1: In-silico Identification of Immunogenic Regions of Therapeutics

T cells specifically recognize epitopes presented by antigen presenting cells (APCs) in the context of MHC (Major Histocompatibility Complex) Class II molecules. These T-helper epitopes can be represented as linear sequences comprising 7 to 30 contiguous amino acids that fit into the
25 MHC Class II binding groove. A number of computer algorithms have been developed and used for detecting Class II epitopes within protein molecules of various origins (De Groot AS et al., (1997), AIDS Res Hum Retroviruses,13(7):539-41; Schafer JR et al., (1998), Vaccine,16(19):1880-4; De Groot AS et al., (2001), Vaccine, 19(31):4385-95; De Groot AS et al., (2003), Vaccine, 21(27-30):4486-504). These “in silico” predictions of T-helper epitopes have
30 been successfully applied to the design of vaccines and the de-immunization of therapeutic proteins, i.e. antibody-based drugs, Fc fusion proteins, anticoagulants, blood factors, bone

morphogenetic proteins, engineered protein scaffolds, enzymes, growth factors, hormones, interferons, interleukins, and thrombolytics (Dimitrov DS, (2012), Methods Mol Biol, 899:1-26).

The EpiMatrix™ system (EpiVax, Providence, Rhode Island) is a set of predictive algorithms encoded into computer programs useful for predicting class I and class II HLA ligands and T cell epitopes. The EpiMatrix™ system uses 20 x 9 coefficient matrices in order to model the interaction between specific amino acids (20) and binding positions within the HLA molecule(9). In order to identify putative T cell epitopes resident within any given input protein, the EpiMatrix™ System first parses the input protein into a set of overlapping 9-mer frames where each frame overlaps the last by eight amino acids. Each frame is then scored for predicted affinity to one or more common alleles of the human HLA molecule; typically DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, and DRB1*1501 (Mack et al., (2013), TISS Antig, 81(4):194–203). Briefly, for any given 9-mer peptide specific amino acid codes (one for each of 20 naturally occurring amino acids) and relative binding positions (1-9) are used to select coefficients from the predictive matrix. Individual coefficients are derived using a proprietary method similar to, but not identical to, the pocket profile method first developed by Sturniolo (Sturniolo T et al., 1999, Nat Biotechnol, 17(6):555-61). Individual coefficients are then summed to produce a raw score. EpiMatrix™ raw scores are then normalized with respect to a score distribution derived from a very large set of randomly generated peptide sequences. The resulting “Z” scores are normally distributed and directly comparable across alleles.

EpiMatrix™ peptide scoring.

It was determined that any peptide scoring above 1.64 on the EpiMatrix™ “Z” scale (approximately the top 5% of any given peptide set) has a significant chance of binding to the MHC molecule for which it was predicted and are designated a “hit.” Peptides scoring above 2.32 on the scale (the top 1%) are extremely likely to bind; most published T cell epitopes fall within this range of scores. Previous studies have also demonstrated that EpiMatrix™ accurately predicts published MHC ligands and T cell epitopes.

Identification of T cell Epitope Clusters.

Potential T cell epitopes are not randomly distributed throughout protein sequences but instead tend to “cluster.” T cell epitope “clusters” range from 9 to roughly 30 amino acids in length and, considering their affinity to multiple alleles and across multiple frames, contain anywhere from 4 to 40 binding motifs. Following epitope mapping, the result set produced by the EpiMatrix™ algorithm was screened for the presence of T cell epitope clusters and EpiBars™ by

using a proprietary algorithm known as Clustimer™. Briefly, the EpiMatrix™ scores of each 9-mer peptide analyzed are aggregated and checked against a statistically derived threshold value. High scoring 9 mers are then extended one amino acid at a time. The scores of the extended sequences are then re-aggregated and compared to a revised threshold value. The process is repeated until the proposed extension no longer improves the overall score of the cluster. Regions of high immunogenic potential, defined as having a score above 10 (including multiple ‘hits’ against many different HLA DR alleles), were identified as T cell epitope clusters. They contain significant numbers of putative T cell epitopes and EpiBars™ indicating a high potential for MHC binding and T cell reactivity.

10 *Prediction of Amino Acids Implicated in HLA Binding.*

The contribution of each amino acid in these regions to HLA binding was evaluated using OptiMatrix tool (part of the EpiVax ISPRI toolkit for deimmunization). OptiMatrix begins with looking at “critical” residues, which contribute most to MHC binding affinity across multiple 9-mer frames and multiple HLA alleles. The program then iteratively substitutes all 19 alternative amino acids in any given position of a protein sequence (with operator-defined input that may limit the list to naturally conserved variants) and then re-analyzes the predicted immunogenicity of the sequence, following that change. To avoid a negative effect on protein structure and consequently in biological activity a comprehensive search in literature for critical residues was also conducted, which identified amino acids that were not candidates for modification.

20 *In silico immunogenicity prediction and deimmunized proteins design.*

Peptide binding to HLA molecules is the first step required for a T cell response. In fact, the strength of peptide binding to MHC molecules is particularly important in determining protein immunogenicity. (Lazarski CA et al, (2005) Immunity. 23: 29–40). To analyze the potential immunogenicity of wild-type IFNβ-1a (SEQ ID NO: 1), the complete amino acid sequence was screened using EpiMatrix. As shown in FIG. 1, wild-type human IFNβ-1a demonstrates a very high potential for immunogenicity, having a Z-score of 101.81.

Further analysis using the ClustiMer algorithms allowed for the identification of putative 9-mer MHC binding peptides and their combination into cluster regions. A total of four clusters were defined, spanning the following residues of IFNβ-1a (SEQ ID NO: 1): 5–32, 57–80, 108–132, 145–158. Two out of the four predicted MHC binding clusters overlapped with previously reported T cell epitopes.

Then, using OptiMatrix, amino acid residues that disrupted or reduced MHC II binding affinity were identified. Among the changes suggested by OptiMatrix, modifications that were not identified as critical for biological activity or receptor binding were selected. These results were considered along with the ClustiMer MHC binding cluster predictions.

5 Based on this comparison, eight sites for modification of IFNβ-1a (SEQ ID NO: 1) were selected, which correspond to the following positons in the amino acid sequence: 25, 28, 60, 65, 74, 111, 117, and 158. OptiMatrix iteratively substituted all 19 alternative amino acids at the given positions in the amino acid sequence protein sequence and then reanalyzed the predicted immunogenicity of the sequence.

10 Based on the *in silico* analysis, the following mutations were selected for the IFNβ-1a variants: N25P; L28P; Y60H; N65H; S74L; F111L; M117T; N158E.

Two variants of IFNβ were developed: IFNβ(VAR1) (SEQ ID NO:2), engineered with four mutations (N25P, Y60H, M117T, N158E) selected for their substantial impact on protein immunogenicity; and IFNβ-1a(VAR2) (SEQ ID NO: 3), which includes these four mutations
15 along with four supplementary modifications (L28P; N65H; S74L; and F111L) aimed at further modulating immunogenicity. Immunogenicity scores for each of the variants was calculated using EpiMatrix, as described above. As shown in FIG. 1, the EpiMatrix immunogenicity global score for each variant is markedly reduced in comparison with the original molecule. Table 1 summarizes the IFNβ variants.

20 **Table 1: IFNβ-1a Variants**

Variant Name	SEQ ID NO	Modification Sites	Amino Acid Sequence (mutations in bold)
IFNβ-1a	1	None	MSYNLLGFLQRSSNFQCQKLLWQLNGRLEYCLKDRMNFDI PEEIKQLQQFQKEDAALTIYEMLQNIFAIFRQDSSSTGWNETI VENLLANVYHQINHLKTVLEEKLEKEDFTRGKLMSSLHLKR YYGRILHYLKAKEYSHCAWTIVRVEILRNIFYFINRLTGYLR N
IFNβ-1a VAR1	2	25, 60, 117, 158	MSYNLLGFLQRSSNFQCQKLLWQLPGRLEYCLKDRMNFDI PEEIKQLQQFQKEDAALTI H EMLQNIFAIFRQDSSSTGWNETI VENLLANVYHQINHLKTVLEEKLEKEDFTRGKL T SSLHLKR YYGRILHYLKAKEYSHCAWTIVRVEILRNIFY F IERLTGYLRN
IFNβ-1a VAR2	3	25, 28, 60, 65, 74, 111, 117 158	MSYNLLGFLQRSSNFQCQKLLWQLPGRPEYCLKDRMNFDI PEEIKQLQQFQKEDAALTI H EMLQ H IFAIFRQD L SSTGWNET IVENLLANVYHQINHLKTVLEEKLEKED T TRGKL T SSLHLK

			RYYGRIHLHYLKAKEYSHCAWTIVRVEILRNIFYFIERLTGYLR
			N

Subsequently, the stability of each variant was assessed by calculating $\Delta\Delta G$ using dg_monomer4 software within the Rosetta5 Bioinformatics package. This computational tool estimates the difference in pseudo-energies between the wild-type and mutant structures defined by Rosetta. Specifically, 50 models each of the wild-type and mutant structures were generated, and $\Delta\Delta G$ was determined as the difference between the mean energies of the top three highest-scoring wild-type structures and the top three highest-scoring mutant structures. Rosetta conventionally interprets negative $\Delta\Delta G$ values as indicating increased stability, calculated as $\Delta\Delta G = \text{mutant energy} - \text{wild-type energy}$. For the variants analyzed, the $\Delta\Delta G$ values were $\Delta\Delta G \text{ IFN}\beta \text{ VAR 1} = 18.569$ and $\Delta\Delta G \text{ IFN}\beta \text{ VAR 2} = 3.044$.

Despite incorporating the highest number of mutations, IFN β -1a(VAR2) (SEQ ID NO:3) exhibited the least impact on protein stability ($\Delta\Delta G = 3.044$) compared to IFN β -1a(VAR1) (SEQ ID NO:2) ($\Delta\Delta G = 18.569$). These findings align with predictions from FoldX, which similarly indicated greater stability for IFN β -1a(VAR2) over IFN β -1a(VAR1) (data not shown). Molecular structures of IFN β -1a(WT) and the de-immunized variants are depicted in FIG. 2, highlighting residues considered for mutation represented as Van der Waals spheres on their peptide ribbon structures. Residues selected for mutation are labeled using one-letter codes to facilitate structural comparison.

Example 2: Production, purification, and characterization of IFN β -1a de-immunized variants

Wild-type IFN β -1a (SEQ ID NO:1), IFN β -1a(VAR1) (SEQ ID NO:2), and IFN β -1a (VAR2) (SEQ ID NO:3) were expressed in CHO-K1 cells using nucleotide sequences containing the coding sequence and signal peptide. The nucleotide sequences encoding the precursor forms (SEQ ID NO:12, SEQ ID NO: 13 and SEQ ID NO: 14) that include the signal peptide coding sequence (SEQ ID NO: 18) and coding sequences for mature IFN β -1a (SEQ ID NO:9), mature IFN β -1a(VAR1) (SEQ ID NO: 10) and mature IFN β -1a (VAR2) (SEQ ID NO:11). To achieve better expression of these nucleotide sequences, they were optimized for codon usage specific for the different de-immunized IFN β -1a variants IFN β -1a(VAR1) (SEQ ID NO:15) and IFN β -1a (VAR2) (SEQ ID NO:16). The nucleotide sequences were synthesized artificially.

Cell culture

Chinese hamster ovary (CHO-K1) cells were grown in the basal culture medium as previously described (Kratje R B, Wagner R, (1992), Biotechnol. Bioeng. 39: 233-242)

supplemented with 5% (v/v) fetal calf serum (FCS) (PAA, Argentina) and supplemented with 5% (v/v) fetal calf serum (FCS) (PAA, Argentina). Madine Darby bovine kidney (MDBK) cells were grown in a minimum essential medium (MEM; Gibco, USA) supplemented with 10% (v/v) FCS. Bioassays were performed using MEM supplemented with 2% (v/v) FCS (assay medium).

5 *Vector Construction. Production and purification of IFN β -1a variants.*

To produce a sufficient amount of protein for further analysis, a third-generation lentiviral strategy was carried out which included the packaging construct (pMDLg/pRRE), VSV-G expressing construct (pMD.G), and the Rev. expressing construct (pRSV-Rev) (Addgene, USA; Plasmid numbers #12251, #12259, #12253, respectively)

10 The IFN β -1a de-immunized variants were synthetically generated by Gene Universal™ (USA) and cloned into a self-inactivating (SIN) promoter containing the expression vector. The sequences of all of the newly designed de-immunized constructs were confirmed by DNA sequencing.

LV particles production and titration.

15 Research-grade HIV-based lentiviral particles containing IFN β -1a variant transgenes were manufactured following the protocol suggested by Naldini et al. (1996, Science, 272: 263-267) and Dull et al. (1998, J. Virol. 72: 8463-71). Following the standard lipofection, adherent HEK293T cells (1.2×10^7 cells per well) were simultaneously co-transfected with four plasmids: the packaging construct (pMDLg/pRRE) (Dull et al. (1998), J. Virol. 72: 8463-71), the VSV-G-
20 expressing construct (pMD.G) (Dull et al. (1998), J. Virol. 72: 8463-71), the Rev-expressing construct (pRSV-Rev) (Naldini et al. (1996), Science, 272: 263-267), and the self-inactivating (SIN) promoter-containing LV.

Lentiviral particles (LVPs) containing supernatants were collected 72 h after transfection. Simultaneously, EGFP-containing LVPs were also assembled. IFN β -1a-containing LVPs titers
25 were estimated based on the titer obtained from EGFP transduced cells by flow cytometry. The titer of LVPs was used to calculate the necessary supernatant dilutions to transduce cells at a similar multiplicity of infection (MOI).

Lentiviral transduction

Transductions were carried out by incubating 6.0×10^4 cells per well seeded onto 6-well
30 plates (Greiner) with LVPs at a final MOI of sixty. Twenty-four hours post-transduction, media were replaced with fresh media. To eliminate the remaining wild-type cells, 96 h post-transduction a selective pressure process was started by replacing supernatants with fresh growth mediums

containing 10 µg/ml puromycin (Invivogen, USA). The selective medium was changed every 3–4 days until controlled cell death occurred.

IFNβ-1a Variants Production and Purification

Transduced cells were grown for IFNβ-1a production until confluence was obtained in 5 500 cm² triple flasks using a growth medium. The medium was then changed to basal medium supplemented with 0.5% (v/v) FCS (production medium). Every 48 or 72 h, the conditioned medium was harvested and replaced with a fresh production medium. Harvests were clarified by centrifugation and then stored at –20 °C until protein purification. This procedure was repeated between 8 and 10 times. Every harvest was assayed for IFNβ-1a concentration by a non-10 commercial (in-house) ELISA sandwich assay.

Example 3: Protein Purification and Analysis

IFNβ-1a deimmunized variants were synthesized and cloned into a third-generation lentiviral vector as described herein, followed by production in CHO cells. Cell culture 15 supernatants were initially screened for antiviral activity, demonstrating robust production levels of each IFNβ-1a protein (data not shown).

Purification of the secreted IFNβ-1a variants from individual harvests involved a three-step purification process: Blue Sepharose chromatography, followed by RP-HPLC-C4 chromatography, and protein concentration with simultaneous buffer exchange via diafiltration.

20 IFNβ-1a variants were purified by Blue Sepharose chromatography followed by RP-HPLC-C4 chromatography. Culture supernatants were passed through a column of Blue Sepharose 6 Fast Flow (GE Healthcare, Buckinghamshire, UK), previously equilibrated with Buffer A (20 mM sodium phosphate, 0.15 M NaCl, pH 7.2). The flow through was collected and the column was washed with Buffer A (35 mL) and 35 mL of Buffer B (20 mM sodium phosphate, 25 2 M NaCl, pH 7.2). IFN was eluted with Buffer B containing 50% ethylene glycol.

After elution, the eluate was sequentially loaded onto C4 Reverse phase-high performance liquid chromatography (RP-HPLC). All IFNβ-1a protein variants were eluted at 1 mL/min using a linear gradient of Buffer A (0% acetonitrile (ACN), 0.1% TFA, pH 2.0) into Buffer B (90% ACN, 0.1% TFA, pH 2.0). Purified proteins were then concentrated and diafiltrated against NaAc 30 100 mM, and Mannitol 300 mM (pH 3.8).

The wild type IFNβ-1a (SEQ ID NO:1) and the mutated forms IFNβ-1a (VAR 1)(SEQ ID NO:2) and IFNβ-1a(VAR2) (SEQ ID NO:3) were obtained in purified form.

SDS-PAGE and western blotting

The protein purity was analyzed by SDS-PAGE under reducing conditions followed by Coomassie blue staining. The concentration of purified IFN β -1a and its de-immunized variants was analyzed by spectrophotometry considering a molar absorptivity index of 1.45.

5 SDS-PAGE analysis was performed according to the standard method using 15% (w/v) polyacrylamide resolving gels and 5% (w/v) stacking gels. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (BioRad). Blots were blocked for 1 h with 5% (w/v) non-fat milk in Tris-buffered saline (TBS) and then probed with rabbit anti-IFN β -1a polyclonal antibodies. After 1 h, blots were incubated with the same peroxidase-conjugated described in the
10 ELISA assay. Immunoreactive bands were visualized using an ECLTM Chemiluminescent Western Blotting Analysis System (GE Healthcare). Washes between steps were performed with TBS containing 0.05% (v/v) Tween 20 (TBS-T). Dilutions were prepared in TBS-T containing 0.5% (w/v) nonfat milk.

Electrophoretic migration comparison by SDS-PAGE, as depicted in FIG. 3A, revealed
15 similar banding patterns for IFN β -1a (WT) and IFN β -1a (VAR1), characterized by a dominant band at approximately 22 kDa, consistent with the original molecular size. In contrast, IFN β -1a (VAR2) exhibited a slightly delayed migration compared to the other variants. Identity verification of these bands was confirmed via western blot analysis, which demonstrated the presence of a single band with the molecular size expected for all rhIFN β variants (FIG. 3B).

20 *UV Near and Far Circular Dichroism (CD)*

To investigate the structural impact of the introduced mutations in the IFN β -1a sequence on the de-immunized variants, the UV-far and UV-near circular dichroism (CD) spectra were analyzed to assess changes in secondary and tertiary structure composition, respectively.

CD spectra were recorded in the ranges of 250–320 nm (near-UV) and 190–240 nm (far-
25 UV) using a Jasco J-1500 spectropolarimeter (Jasco Inc., Easton, USA) at room temperature, with a scan speed of 20 nm/min and a response time of 1s. IFN β , IFN β (VAR1), and IFN β (VAR2) protein samples were prepared at a concentration of 0.33 mg/ml in a cuvette with a 0.1 cm path length. Each spectrum represents the average of three scans, corrected by subtracting the buffer signal. CD data were expressed as molar ellipticity, $[\theta]$, in degrees cm²/dmol, calculated based on
30 a mean residue weight of 116.76 Da per amino acid residue. Spectra were further corrected by subtracting the excipient signal.

CD spectra deconvolution was performed to dissect the contributions from different secondary structures within the molecule. Using reference spectra from proteins with known structures, various empirical algorithms were applied, based on the linear independence and additivity of different components to produce the net spectrum. The CD spectra deconvolution in this study was conducted using the SELCOM software, accessed via the DichroWeb online server. Predictions utilized reference database 7, comprising 48 proteins.

As shown in FIG. 4A, the far-UV CD spectra of all three variants display characteristics consistent with alpha-helix structures, evidenced by negative bands at 222 nm and 208 nm and a positive band at 193 nm. However, the spectra of IFN β -1a (VAR1) and IFN β -1a (VAR2) differ from that of IFN β -1a (WT). The Manhattan distances between IFN β -1a (WT) and IFN β -1a (VAR1) and between IFN β -1a (WT) and IFN β -1a (VAR2) were ten times greater than the distance between IFN β -1a (VAR1) and IFN β -1a (VAR2), indicating significant differences in secondary structure content between the wild-type and de-immunized variants. A similar pattern was observed in the analysis of the UV-near spectra, as shown in FIG. 4B. The slight differences observed in the original IFN β -1a spectrum are attributable to background noise arising from the low protein concentration.

Example 4: Determination of the antiviral biological activity of the different variants of IFN β -1a de-immunized.

The antiviral biological activity of IFN β -1a and its de-immunized variants was assessed based on their ability to inhibit the cytopathic effect induced by vesicular stomatitis virus (VSV, Indiana strain) on WISH cells (ATCC, USA). The experimental procedure was as follows:

2.5×10^4 WISH cells were cultured overnight at 37 °C. After incubation, the culture supernatants were removed, and 1:2 serial dilutions of either the WHO International Standard for IFN β -1a (NIBSC 00/572) ranging from 20 U/ml to 0.16 U/ml or the IFN β -1a variants were added to the WISH cell cultures in assay medium. Following a 6-hour incubation period, the supernatants were removed, and the cells were infected with VSV at a ratio of 1.6 PFU/cell. The viral treatment was terminated once the cytopathic effect was clearly observed in the control cell culture, which was not treated with IFN β -1a.

To visualize the cytopathic effect, the cells were stained using a crystal violet solution (0.75% w/v in 40% v/v methanol) for 15 minutes. Excess stain was washed off with water, and

the stained cells were subsequently treated with acetic acid (20% v/v). The intensity of the staining was measured at 540 nm. Each experiment was conducted in quintuplicate.

The antiviral biological activity of the IFN β -1a variants obtained in Example 2 was quantified by evaluating their ability to inhibit viral propagation or replication. This was measured by determining the ability of the interferon variants to protect susceptible cells from the cytopathic effects of a lytic virus over a range of cytokine concentrations.

The biological antiviral activities of the parent IFN β -1a and the mutated variants IFN β -1a(VAR1) and IFN β -1a(VAR2) were specifically determined by their ability to inhibit the cytopathic effect caused by VSV on WISH cells. 2.5×10^5 WISH cells were grown overnight at 37 °C, and the culture supernatants were removed. Serial 1:2 dilutions of the WHO International Standard for rhIFN β (NIBSC 00/572) ranging from 20 U/ml to 0.16 U/ml, along with serial dilutions of the IFN β -1a variants, were added to the assay medium. Following a 6-hour incubation, the supernatants were removed, and the cells were infected with VSV at a ratio of 1.6 PFU/cell. Once the cytopathic effect was evident in the control cells (without IFN), the cells were stained with a crystal violet solution (0.75% w/v in 40% v/v methanol) for 15 minutes. After washing with water, the stained cells were treated with acetic acid (20% v/v), and the signal intensity was measured at 540 nm. Determinations were performed in quintuplicate.

The functional characterization of the purified IFN β -1a variants involved quantifying their specific antiviral biological activity. Absorbance data at 540 nm were plotted against the IFN β -1a activity values (standard) and sample dilutions on a logarithmic scale. The biological activity (AB) values for each polypeptide were calculated by comparison with the standard using the parallel lines test (FIG. 5). The specific biological activity (ABE) values of each protein were determined by calculating the ratio between the AB and the protein concentration in the samples.

Eight independent experiments were conducted, and the results were statistically analyzed. The percentage biological activity value was determined by calculating the ratio between the mean ABEs of each de-immunized variant and the mean ABEs of IFN β -1a WT (33.93 ± 4.00 UI/ng). The relative antiviral activity results (%) for the IFN β -1a variants are shown in Table 2. The specific antiviral activity of IFN β -1a VAR1 shows a statistically significant 40% higher than the natural variant, while IFN β -1a VAR2 presents a similar activity to natural IFN β -1a, as shown in FIG. 6.

Table 2

Polypeptide	Relative Antiviral Activity (%)
-------------	---------------------------------

IFNβ-1a VAR 1 (SEQ ID NO:2)	140.05
IFNβ-1a VAR 2 (SEQ ID NO:3)	123.66

Example 5: IFNβ-1a(VAR1) and IFNβ-1a(VAR2) exhibit a markedly reduced in vivo immunogenicity

Mutant HLA-DRB1*03:01 mice humanized for both HLA-A2 and HLA-DR3, and deleted for H-2 class I and II molecules (β2m^{-/-} H-2Db^{-/-} IAβ^{-/-} IAα^{-/-} IEβ^{-/-}), were provided by the Pasteur Institute and developed by François Lemonnier. Transgenic mice aged 6–10 weeks were housed in pathogen-free conditions at the animal facilities of the Faculty of Veterinary Sciences, Universidad Nacional del Litoral (UNL). All animal handling procedures and protocols were approved by the UNL Research Ethics Committee, code 2021-21CE-C.

Groups of seven mice were immunized three times via intraperitoneal (i.p.) injection with PBS or 25 μg of IFNβ-1a (wild-type), IFNβ-1a(VAR1), or IFNβ-1a(VAR2) emulsified in complete Freund's adjuvant (CFA) at a final volume of 200 μl. Blood and spleen samples were collected seven days post-immunization, and plasma and spleen cells were isolated for further studies.

Detection of Total and Neutralizing Antibodies to IFNβ-1a

Total antibody titers (BAbs) against IFNβ-1a, VAR1, and VAR2 were determined by indirect ELISA. 96-well plates were coated with 1 μg/ml IFNβ protein, blocked with 1% BSA in PBS, and incubated with serial dilutions of plasma samples. After incubation with peroxidase-conjugated anti-mouse Igs, titers were defined as the dilution exceeding the limit (OD for the negative control) plus three standard deviations.

Neutralizing antibody titers (NAbs) were determined by antiviral bioassays. These results are depicted in Table 3. Serial dilutions of mouse plasma were pre-incubated with a constant concentration of IFNβ-1a variants, then added to WISH cell cultures. The reduction in antiviral activity was measured by the degree of cell lysis. Titers were expressed as the serum dilution that reduced the initial IFN antiviral activity by 50% (IC50).

Table 3:

	IFN beta WT	IFN beta VAR1	IFN beta VAR2
Animal 1	79.53	0	0
Animal 2	61.36	0	0
Animal 3	56.54	0	0
Animal 4	154.87	0	83.57

Animal 5	106.15	79.53	0
Animal 6	300.12	0	0
Animal 7			0
Animal 8			61.76

As shown in FIG. 7, mice treated with IFN β -1a(WT) developed higher titers of anti-IFN β -1a BAbs. A significant reduction in BAbs and NAbs titers was observed in animals treated with the de-immunized variants, consistent with the reduced T cell responses. Statistical analysis using the Kruskal-Wallis test revealed significant differences between the medians of the total anti-IFN β -1a antibody titers, with a p-value < 0.05, confirming these differences at a 95% confidence level.

Plasma from mice inoculated with IFN β -1a(WT) showed high neutralizing capacity for IFN β -1a antiviral activity in vitro. In contrast, plasma from mice treated with the de-immunized variants exhibited negligible NAb activity.

10 *Spleen T Cell Stimulation and Cytokine Assay*

Spleen cells were seeded in 96-well plates and evaluated under four conditions: negative control (culture media), positive control (Phaseolus vulgaris lectins), excipient control, and IFN β -1a variants. After 72 hours, culture supernatants were harvested, and mouse IFN- γ and IL-4 levels were assayed by sandwich ELISA. The Stimulation Index (SI) was defined as the ratio of cytokine concentration from protein-challenged samples to excipient-treated samples.

As illustrated in FIGS. 8A-8C, splenocyte samples treated with IFN β -1a(WT) demonstrated robust IFN- γ stimulation index (SI). In contrast, T cell immune responses were barely detectable in mice treated with the de-immunized IFN β -1a variants. Importantly, mIL-4 was not detected in the splenocyte culture supernatants (data not shown), suggesting that IFN β -1a primarily induces T cell polarization towards the Th1 profile.

Given the close link between anti-drug T cell immune responses and antibody formation, we evaluated the development of binding antibodies (BAbs) in the plasma of IFN β -treated mice. Consistent with the T cell responses observed in mice treated with IFN β -1a(WT), high titers of anti-IFN β -1a(WT) BAbs were detected. This highlights the usefulness of this in vivo experimental platform as a screening tool for predicting potential undesired immune responses to this biologic. Additionally, in correlation with the in vitro T cell assay results, a significant reduction in BAbs titers was observed in animals treated with the de-immunized IFN β -1a variants.

To assess the neutralizing capacity of the antibodies, we evaluated the inhibitory effect of the antiviral activity of antibodies present in plasma samples from mice treated with IFN β -1a

variants on WISH cells. As shown in FIG. 8C, plasma samples from mice inoculated with IFN β -1a(WT) exhibited high neutralizing capacity against IFN β -1a antiviral activity in vitro. In contrast, plasma samples from mice treated with the de-immunized variants displayed near-null neutralizing antibody (NAb) activity.

5

Example 6: Ex vivo immunogenicity assays of the different variants of IFN β -1a de-immunized in samples of human peripheral blood mononuclear cells (PMBC).

An immunogenicity study was conducted using IFN β -1a variants developed in Example 2. This study employed an ex vivo assay with samples of human peripheral blood mononuclear cells (PBMCs) from patients with multiple sclerosis.

10

Samples from two voluntary donors were incubated with IFN β -1a WT and T lymphocyte activating agents (positive controls). After 5 hours, samples were stained with antibodies conjugated to specific fluorochromes to analyze T-lymphocyte activation markers (CD69, CD137, CD154) via flow cytometry. As shown in FIG. 9, IFN β -1a WT specifically activated naïve and memory T cells in PBMC samples from both donors. The values are expressed as a percentage of activated T lymphocytes relative to the negative control (excipients).

15

Activated memory T lymphocytes were isolated and restimulated with IFN β -1a WT, IFN β -1a VAR 1, and IFN β -1a VAR 2. FIGS. 10-11 indicate that IFN β -1a WT induced a higher level of lymphocyte activation compared to the de-immunized variants. This was observed for T lymphocyte activation markers CD69 and CD154. Notably, IFN β -1a(VAR2) exhibited lower lymphocyte activation than IFN β -1a VAR 1, correlating with the *in silico* analysis results.

20

The cohort was expanded to include five donors, and initial stimulations were performed with all three proteins. FIG. 11 summarizes lymphocyte activation based on the CD69 marker. Except for donor 10, PBMC samples from multiple sclerosis patients showed greater lymphocyte activation with IFN β -1a WT compared to the de-immunized variants. These results demonstrate that mutations in the IFN β -1a WT sequence reduce immunogenicity.

25

Specific memory T lymphocytes from donor 8, activated by the three proteins, were isolated and restimulated. FIG. 12 demonstrates that restimulation with IFN β -1a WT resulted in a higher percentage of activation compared to the de-immunized proteins. The restimulation results for IFN β -1a VAR 1 and IFN β -1a VAR 2 showed a similar pattern to FIG. 10, demonstrating the effectiveness of the de-immunization strategy.

30

These findings underscore the reduced immunogenicity of the de-immunized IFN β -1a variants and validate the de-immunization approach disclosed herein

FURTHER EXEMPLARY ASPECTS

5 In a first exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising one or more amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or
10 glutamic acid.

In a second exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising at least four amino acid substitutions, the one or more amino acid substitutions
15 at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

In a third exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising one or more amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or
20 glutamic acid.

25 In a fourth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising at least four amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution
30 comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

In a fifth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising at least eight amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, and 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

In a sixth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising at least eight amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, and 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

In a seventh exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the amino acid substitutions are N25P, L28P, Y60H, N65H, S74L, F111L, M117T, N158E, or a combination thereof.

In an eighth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the amino acid substitutions comprise N25P, Y60H, M117T, and N158E.

In a ninth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, further comprising an amino acid substitution of L28P, N65H, S74L, or F111L.

In a tenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the amino acid substitutions comprise L28P, N65H, S74L, and F111L.

In an eleventh exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the amino acid sequence is SEQ ID NO: 2 or SEQ ID NO: 3.

In a twelfth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide,, wherein the modified

interferon- β 1a polypeptide has a reduced immunogenicity as compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

In a thirteenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the modified
5 interferon- β 1a polypeptide has a relative antiviral activity of about 10% to about 200% compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

In a fourteenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the modified
10 interferon- β 1a polypeptide has a relative antiviral activity of about 100% to about 140% as compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

In a fifteenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the amino acid
sequence is SEQ ID NO: 2.

In a sixteenth exemplary aspect, alone or in combination with any other provided herein,
15 the present disclosure relates to a modified interferon- β 1a polypeptide, wherein the amino acid sequence is SEQ ID NO: 3.

In a seventeenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a pharmaceutical composition comprising modified interferon β -1a polypeptide and a pharmaceutically acceptable excipient.

20 In an eighteenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a method for treating multiple sclerosis in a subject in need thereof, the method comprising administering the pharmaceutical composition to the subject.

In a nineteenth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a nucleotide sequence encoding the modified interferon β -1a
25 polypeptide with interferon beta-1a activity.

In a twentieth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a nucleotide sequence selected from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

In a twenty-first exemplary aspect, alone or in combination with any other provided herein,
30 the present disclosure relates to a plasmid comprising a nucleotide sequence. The nucleotide sequence is optionally selected from the set comprised of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

In a twenty-second exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to an expression vector comprising a nucleotide sequence encoding the modified interferon beta-1a polypeptide. The nucleotide sequence is optionally selected from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, or SEQ ID NO: 8.

5 In a twenty-third exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a cell line for the expression of the modified interferon β -1a polypeptide with interferon beta-1a activity, comprising a nucleotide sequence encoding the modified interferon beta-1a polypeptide with interferon beta-1a activity. The nucleotide sequence is optionally selected from the set comprised of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7
10 and SEQ ID NO: 8.

In a twenty-fourth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a cell-line selected from a list comprised of CHO-K1, HEK293, NSO, BHK, Sp2/0, CAP and CAP/T.

15 In a twenty-fifth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a CHO-K1 cell line.

In a twenty-sixth exemplary aspect, alone or in combination with any other provided herein, the present disclosure relates to a method of preparing the modified interferon β -1a polypeptide with interferon beta-1a activity, the method comprising cloning a nucleotide sequence selected from the group comprised of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID
20 NO: 8 into a lentiviral vector; transducing a Chinese hamster ovary cell line (CHO-K1) with the vector; expressing the modified interferon β -1a polypeptide, the modified interferon β -1a polypeptide having an amino acid sequence selected from SEQ ID NO: 2 or SEQ ID NO: 3; collecting supernatant; purifying the modified interferon β -1a polypeptide from the supernatant using dye pseudo-affinity chromatography and high-performance liquid chromatography (HPLC);
25 and concentrating the modified interferon β -1a polypeptide with interferon beta-1a activity selected from the group comprised of SEQ ID NO: 2 and SEQ ID NO: 3 by diafiltration.

EQUIVALENTS

While the instant disclosure has been described in connection with the specific aspects thereof, it will be understood that it is capable of further modification. Furthermore, this
30 application is intended to cover any variations, uses, or adaptations of the invention, including such departures from the present disclosure as come within known or customary practice in the art to which the disclosure pertains, and as fall within the scope of the appended claims.

Various modifications of the present invention, in addition to those shown and described herein, will be apparent to those skilled in the art of the above description. Such modifications are also intended to fall within the scope of the appended claims.

5 It is appreciated that all reagents are obtainable by sources known in the art unless otherwise specified. Methods of nucleotide amplification, cell transfection, and protein expression and purification are similarly within the level of skill in the art.

10 Patents, publications, and applications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents, publications, and applications are incorporated herein by reference to the same extent as if each individual patent, publication, or application was specifically and individually incorporated herein by reference. The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims, including all equivalents thereof, are intended to define the scope of the invention.

CLAIMS

1. A modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising one or more amino acid substitutions, the one or more amino acid substitutions at one or more of
5 position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.
2. A modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising
10 at least four amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.
3. A modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide
15 comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising one or more amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.
- 20 4. A modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising at least four amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, or 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.
25
5. A modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 80% identity to SEQ ID NO: 1 and comprising at least eight amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, and 158, optionally wherein the substitution comprises
30 changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

6. A modified interferon- β 1a polypeptide having interferon- β 1a activity, the polypeptide comprising an amino acid sequence having at least 90% identity to SEQ ID NO: 1 and comprising at least eight amino acid substitutions, the one or more amino acid substitutions at one or more of position 25, 28, 60, 65, 74, 111, 117, and 158, optionally wherein the substitution comprises changing the amino acid at the position to proline, histidine, leucine, threonine, or glutamic acid.

7. The modified interferon- β 1a polypeptide of any of claims 1-6, wherein the amino acid substitutions are N25P, L28P, Y60H, N65H, S74L, F111L, M117T, N158E, or a combination thereof.

8. The modified interferon- β 1a polypeptide of claim 7, wherein the amino acid substitutions comprise N25P, Y60H, M117T, and N158E.

9. The modified interferon- β 1a polypeptide of claim 8, further comprising an amino acid substitution of L28P, N65H, S74L, or F111L.

10. The modified interferon- β 1a polypeptide of claim 9, wherein the amino acid substitutions comprise L28P, N65H, S74L, and F111L.

11. The modified interferon- β 1a polypeptide of any of claims 1-6, wherein the amino acid sequence is SEQ ID NO: 2 or SEQ ID NO: 3.

12. The modified interferon- β 1a polypeptide of any of claims 1-6, wherein the modified interferon- β 1a polypeptide has a reduced immunogenicity as compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

13. The modified interferon- β 1a polypeptide of any of claims 1-6, wherein the modified interferon- β 1a polypeptide has a relative antiviral activity of about 10% to about 200% compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

14. The modified interferon- β 1a polypeptide of any of claims 1-6, wherein the modified interferon- β 1a polypeptide has a relative antiviral activity of about 100% to about 140% as compared to a wild type interferon- β 1a polypeptide of SEQ ID NO: 1.

5 15. The modified interferon- β 1a polypeptide of claim 1, wherein the amino acid sequence is SEQ ID NO: 2.

16. The modified interferon- β 1a polypeptide of claim 1, wherein the amino acid sequence is SEQ ID NO: 3.

10

17. A pharmaceutical composition comprising the modified interferon β -1a polypeptide of any of claims 1-16 and a pharmaceutically acceptable excipient.

15 18. A method for treating multiple sclerosis in a subject in need thereof, the method comprising administering the pharmaceutical composition of claim 17 to the subject.

19. A nucleotide sequence encoding the modified interferon β -1a polypeptide with interferon beta-1a activity of any one of claims 1-6.

20 20. The nucleotide sequence of claim 19, wherein the nucleotide sequence is selected from SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

21. A plasmid comprising the nucleotide sequence of claim 19, wherein the nucleotide sequence is selected from the set comprised of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and
25 SEQ ID NO: 8.

22. An expression vector comprising a nucleotide sequence encoding the modified interferon beta-1a polypeptide of claim 1, wherein the nucleotide sequence is SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

30

23. A cell line for the expression of the modified interferon β -1a polypeptide with interferon beta-1a activity of any one of claims 1-6, comprising a nucleotide sequence encoding the modified

interferon beta-1a polypeptide with interferon beta-1a activity comprised of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

5 24. The cell line of claim 23, wherein the cell line is selected from a list comprised of CHO-K1, HEK293, NSO, BHK, Sp2/0, CAP and CAP/T.

25. The cell line of claim 26 wherein the cell line is CHO-K1.

10 26. A method of preparing the modified interferon β -1a polypeptide with interferon beta-1a activity of claim 1, the method comprising:

cloning a nucleotide sequence selected from the group comprised of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8 into a lentiviral vector;

transducing a Chinese hamster ovary cell line (CHO-K1) with the vector;

15 expressing the modified interferon β -1a polypeptide, the modified interferon β -1a polypeptide having an amino acid sequence selected from SEQ ID NO: 2 or SEQ ID NO: 3;

collecting supernatant;

purifying the modified interferon β -1a polypeptide from the supernatant using dye pseudo-affinity chromatography and high-performance liquid chromatography (HPLC);

20 and

concentrating the modified interferon β -1a polypeptide with interferon beta-1a activity selected from the group comprised of SEQ ID NO: 2 and SEQ ID NO: 3 by diafiltration.

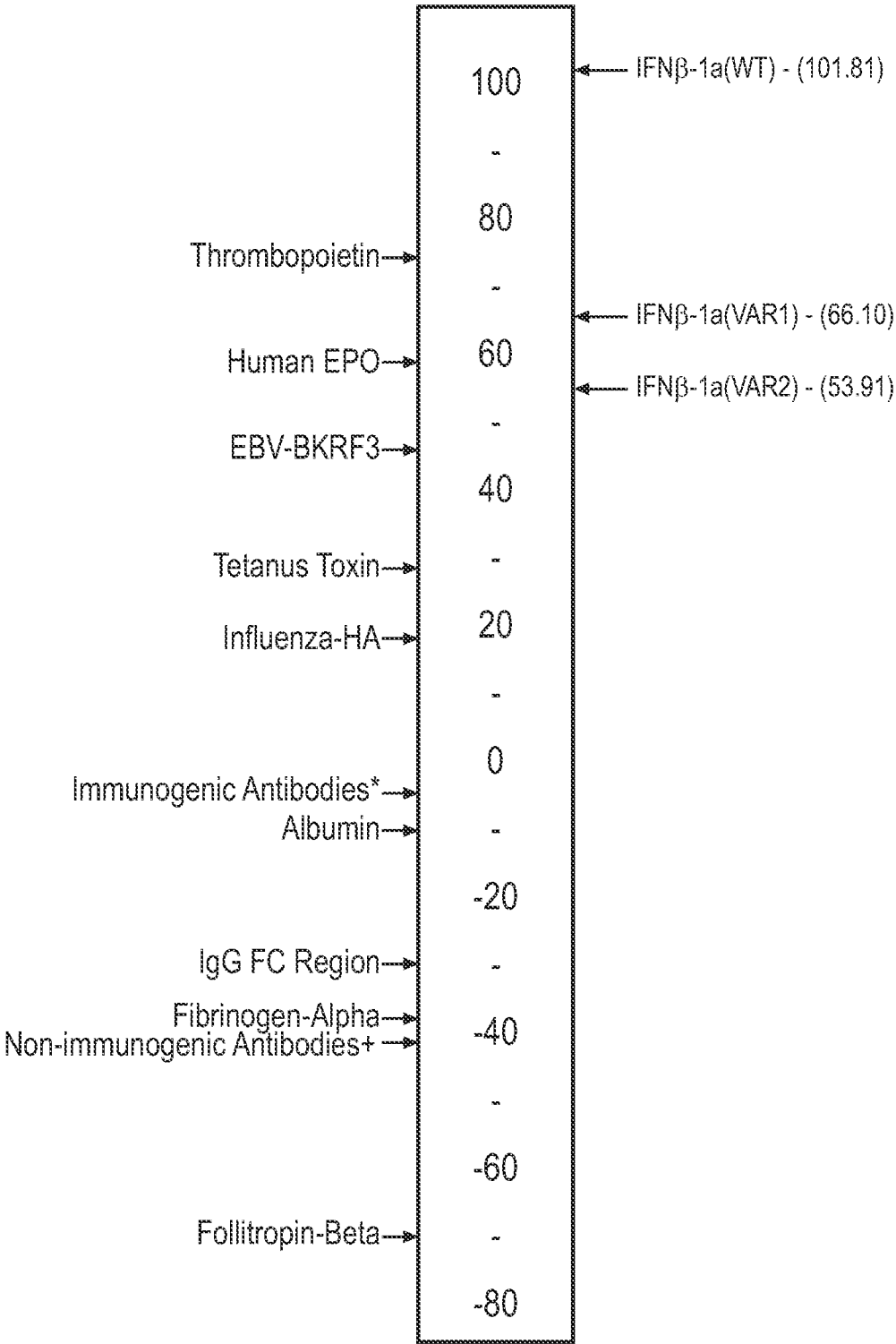


FIG. 1

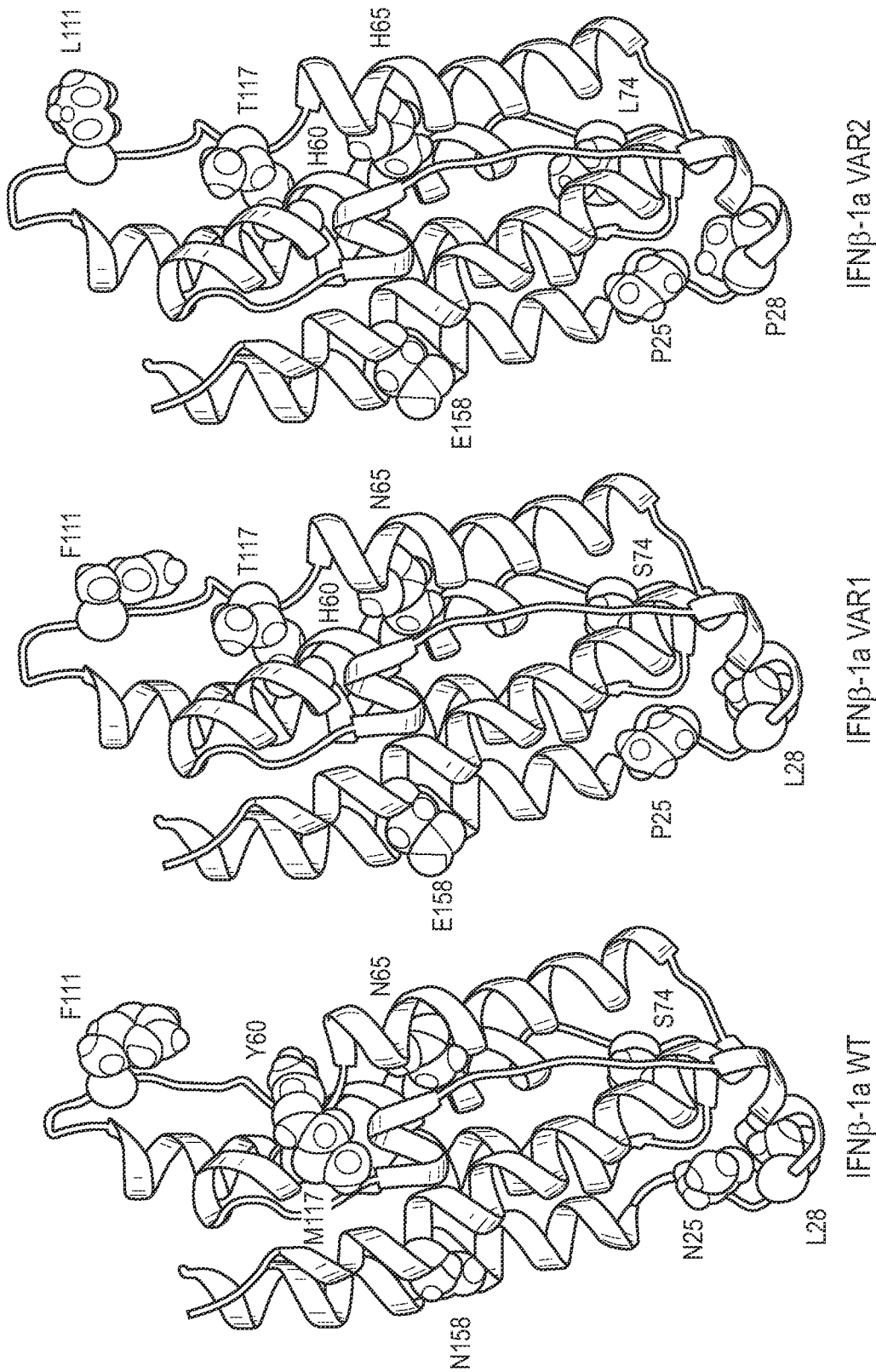


FIG. 2

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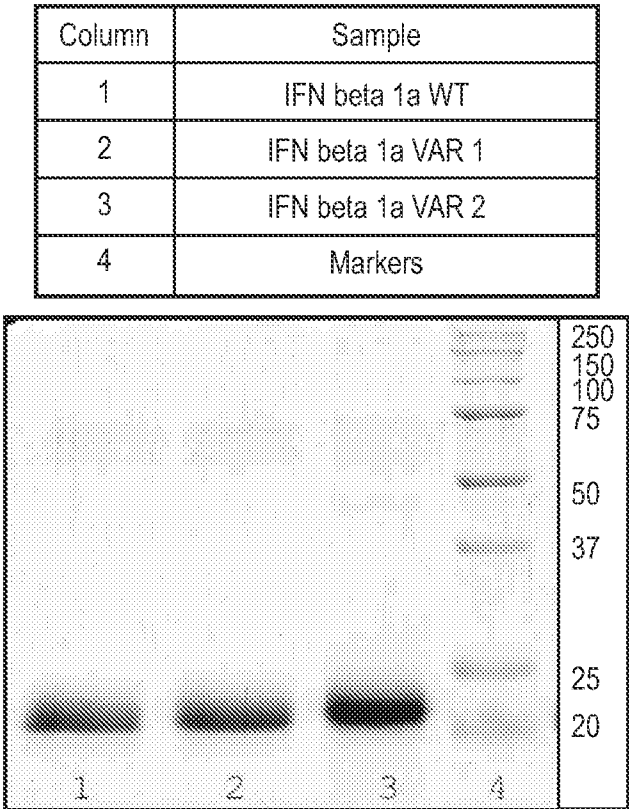


FIG. 3A

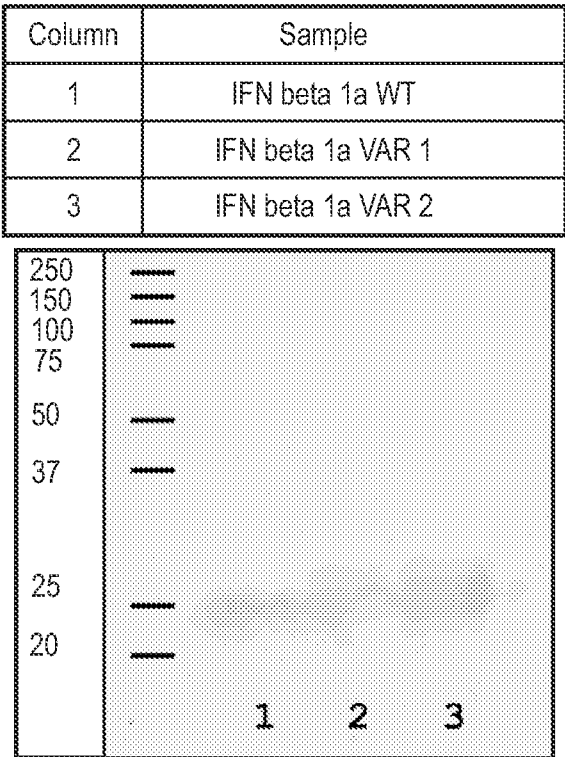
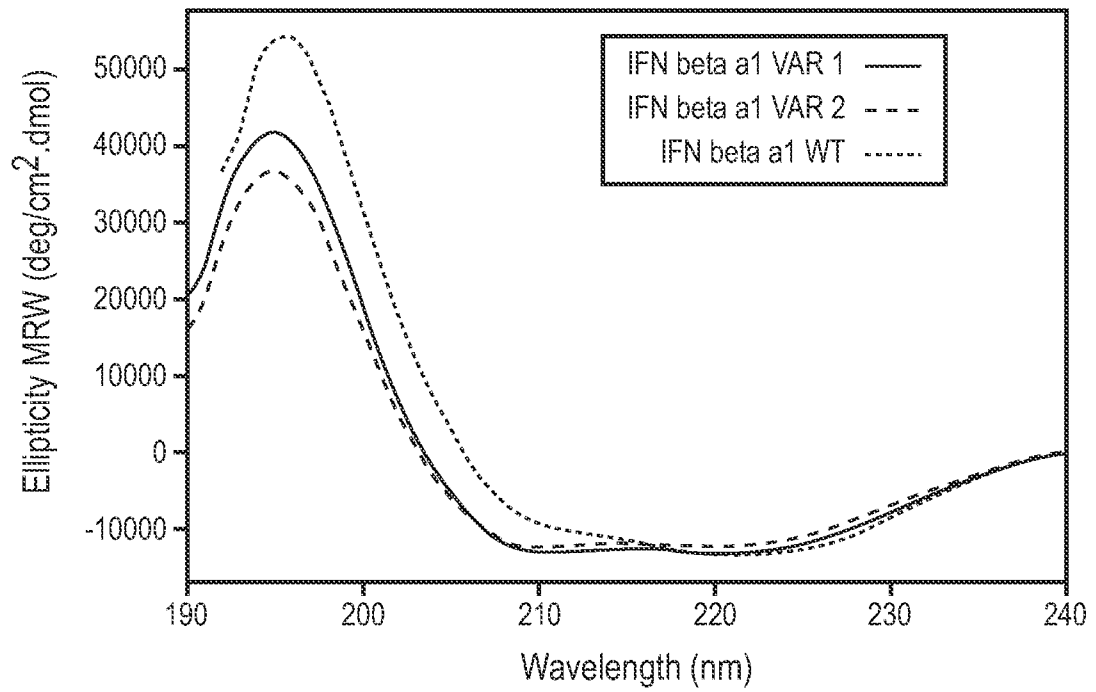
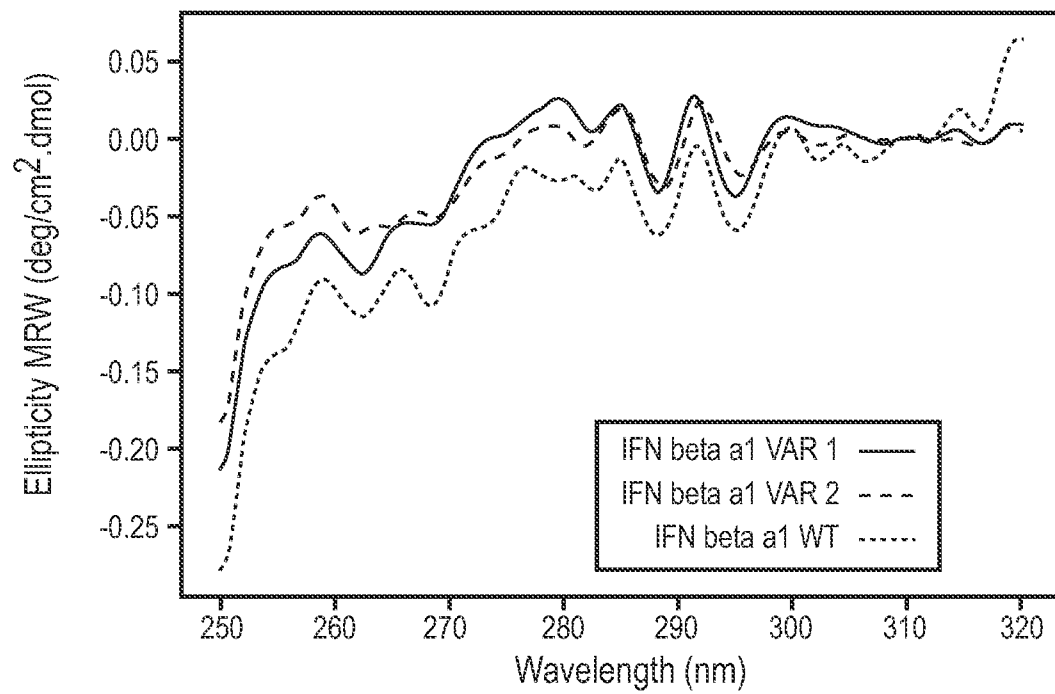


FIG. 3B

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**FIG. 4A****FIG. 4B**

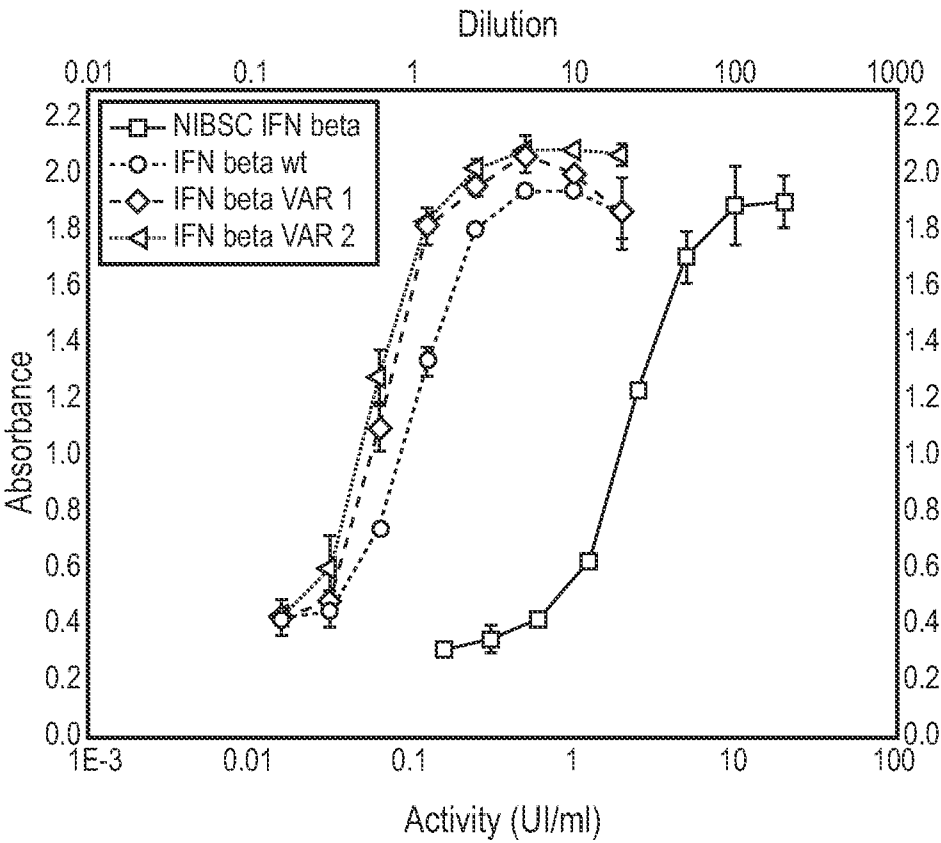


FIG. 5

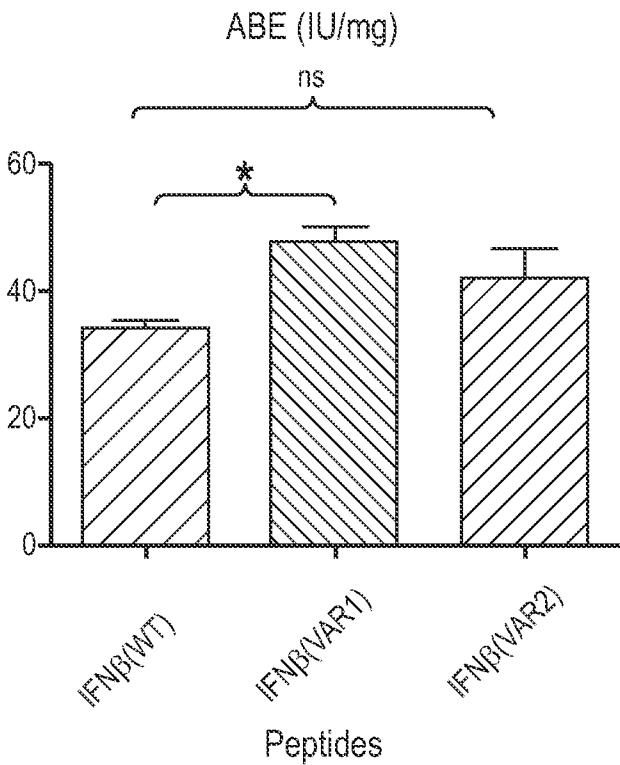
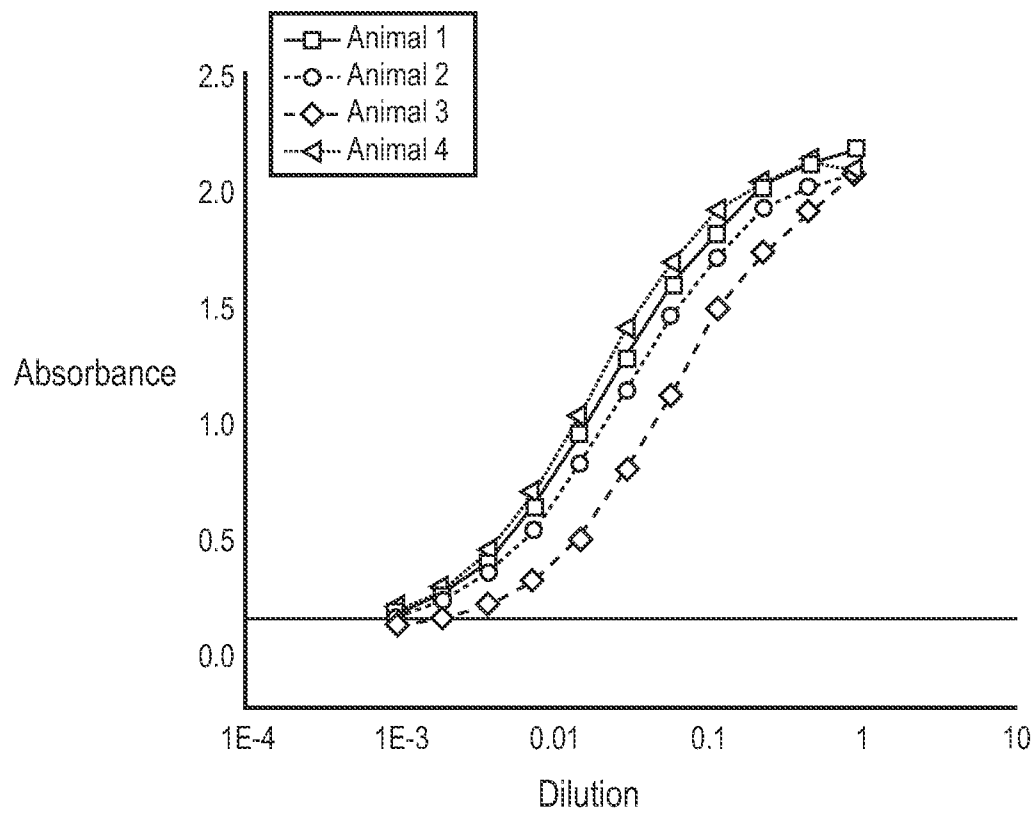
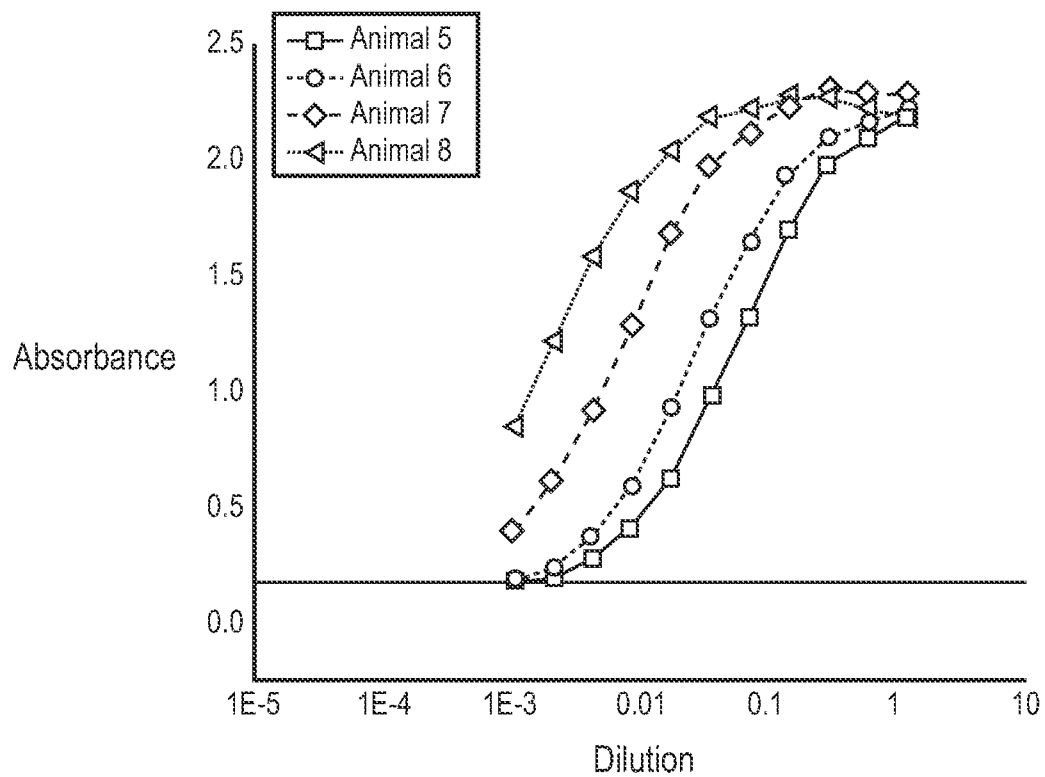


FIG. 6

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**FIG. 7A****FIG. 7B**

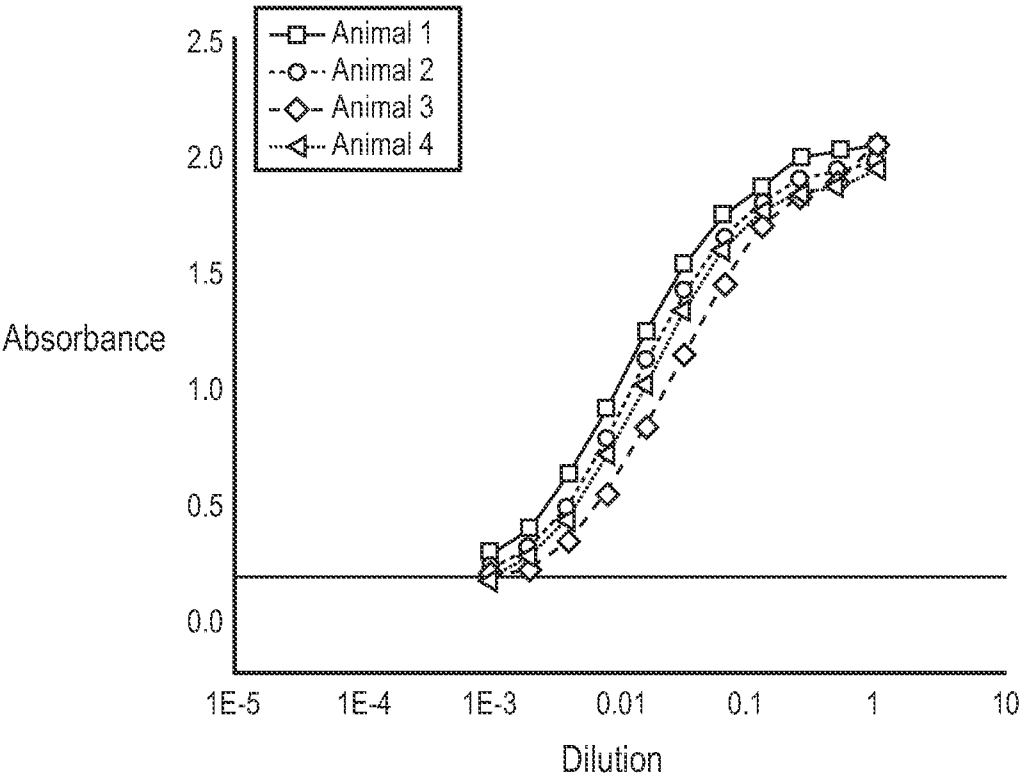


FIG. 7C

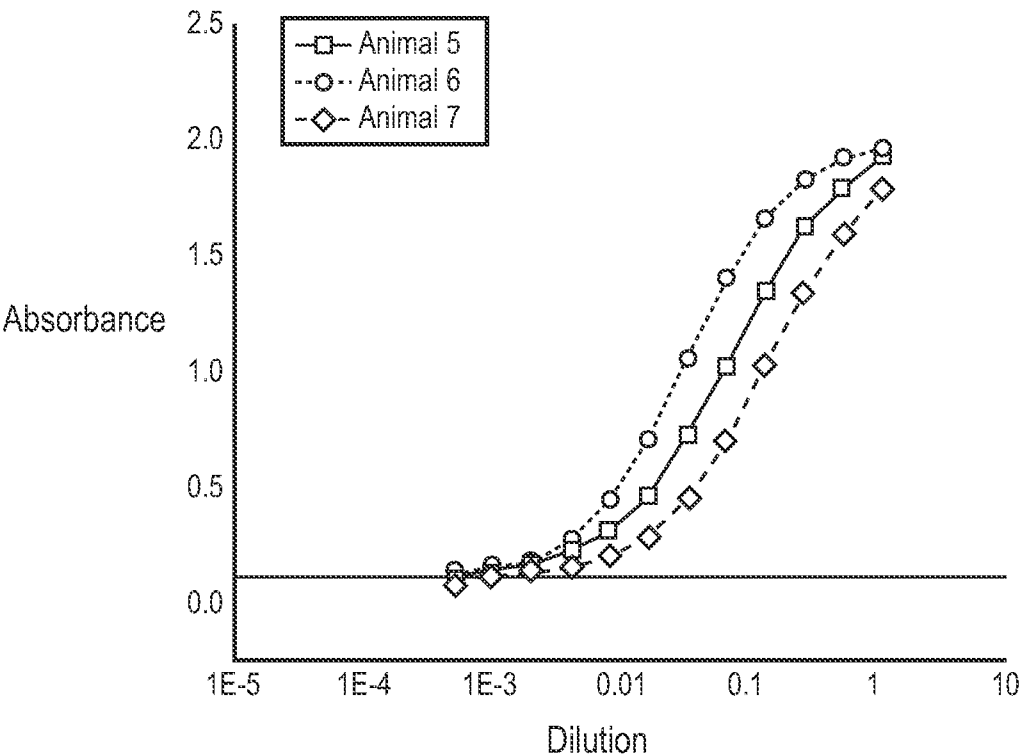
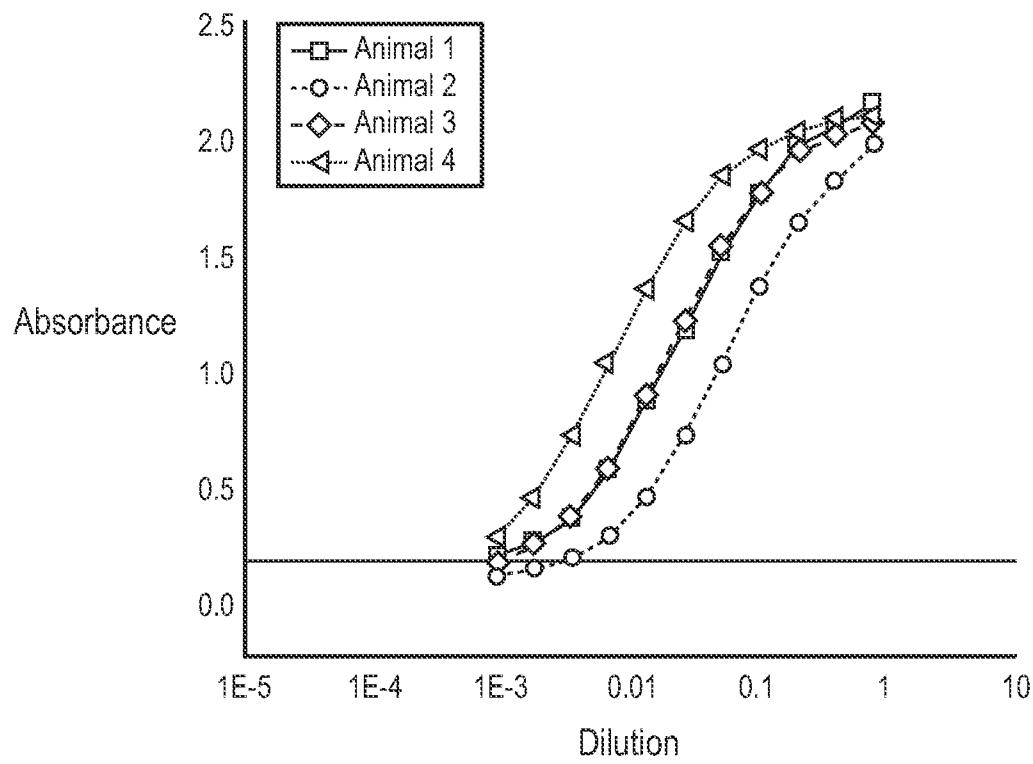
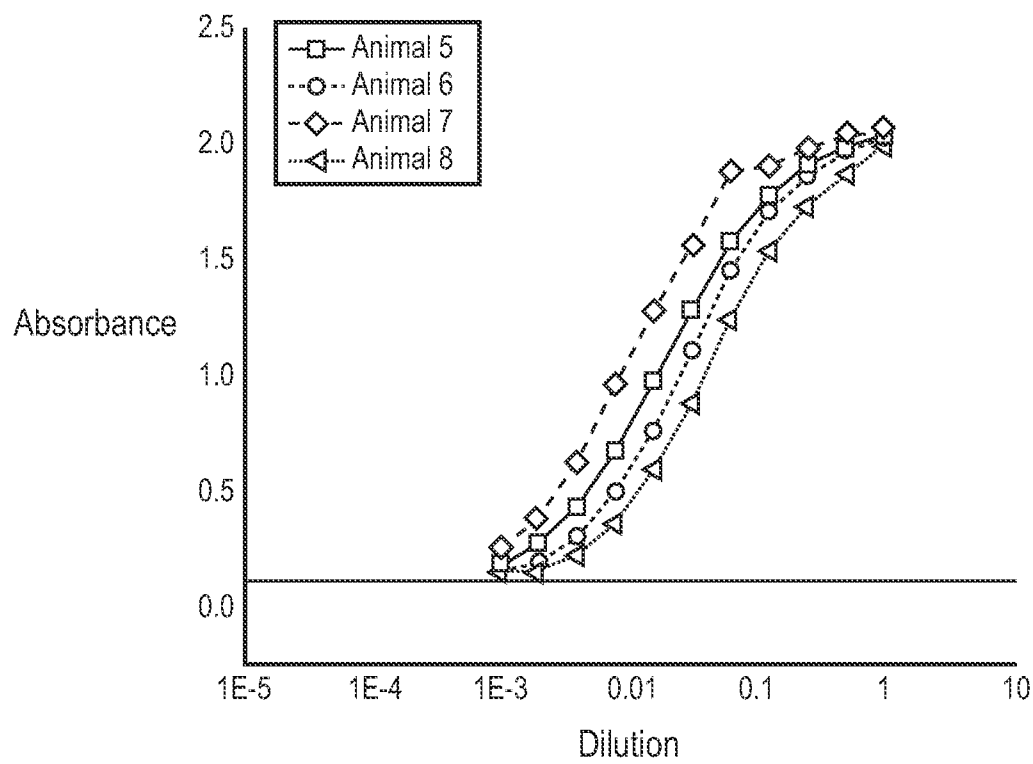
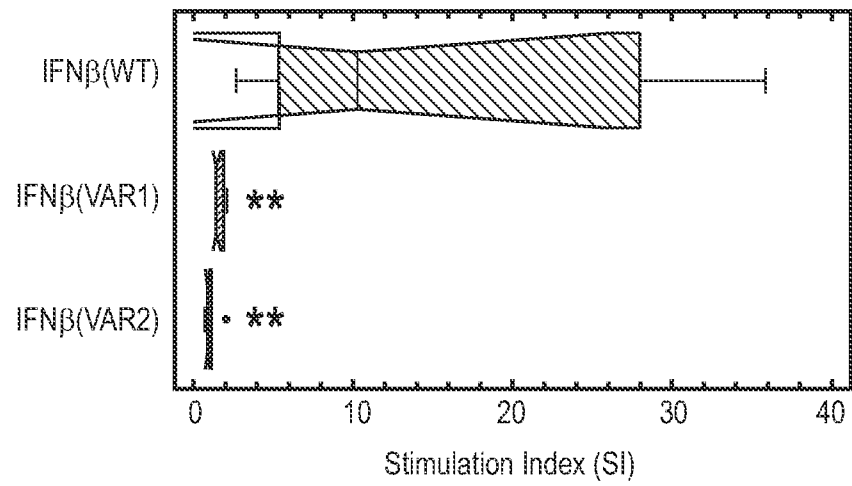
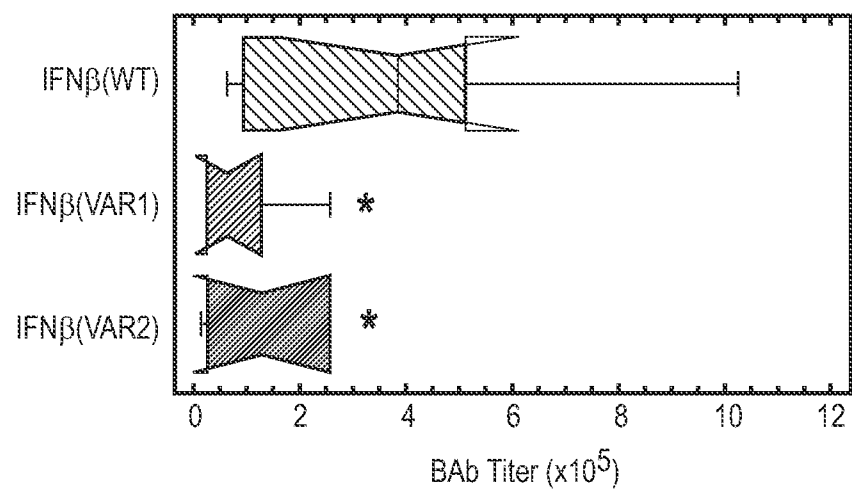
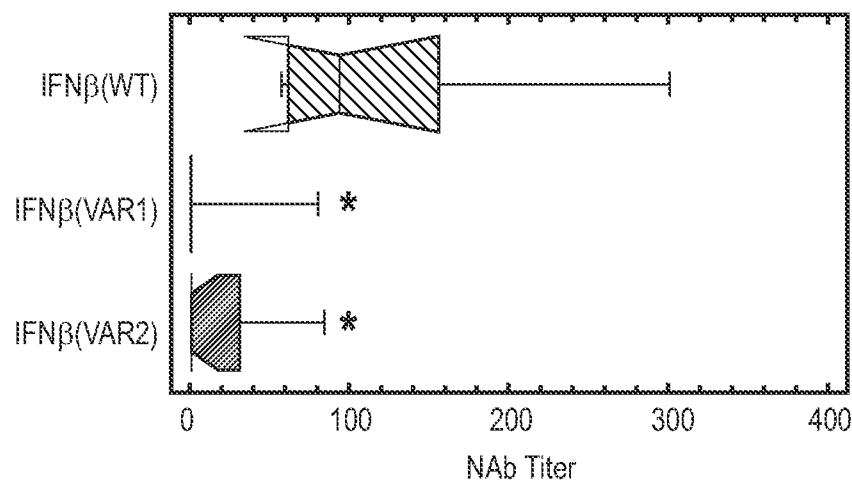


FIG. 7D

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**FIG. 7E****FIG. 7F**

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**FIG. 8A****FIG. 8B****FIG. 8C**

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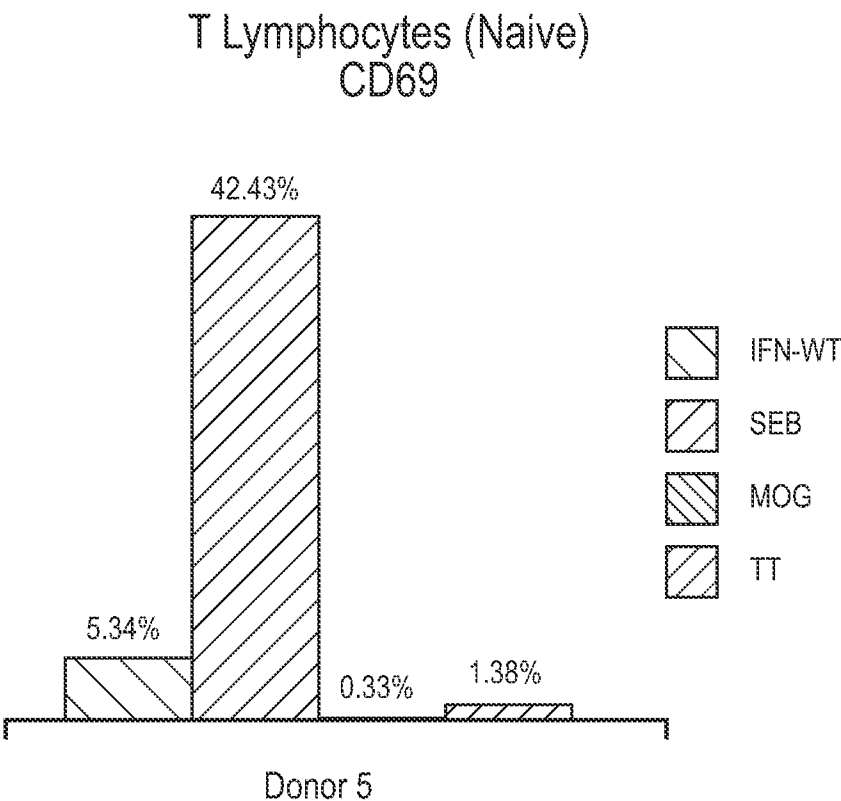


FIG. 9A

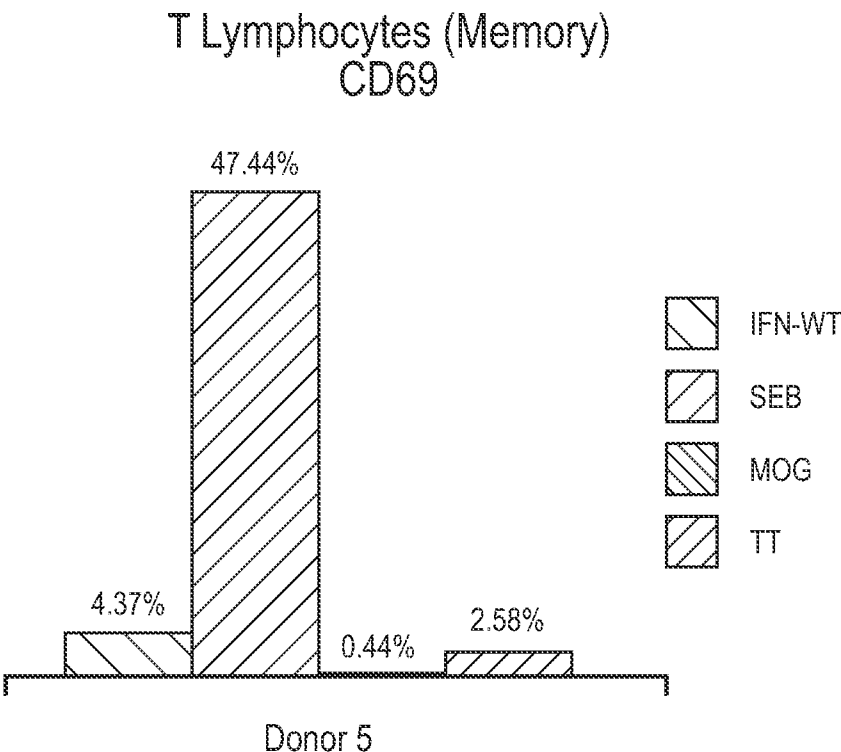
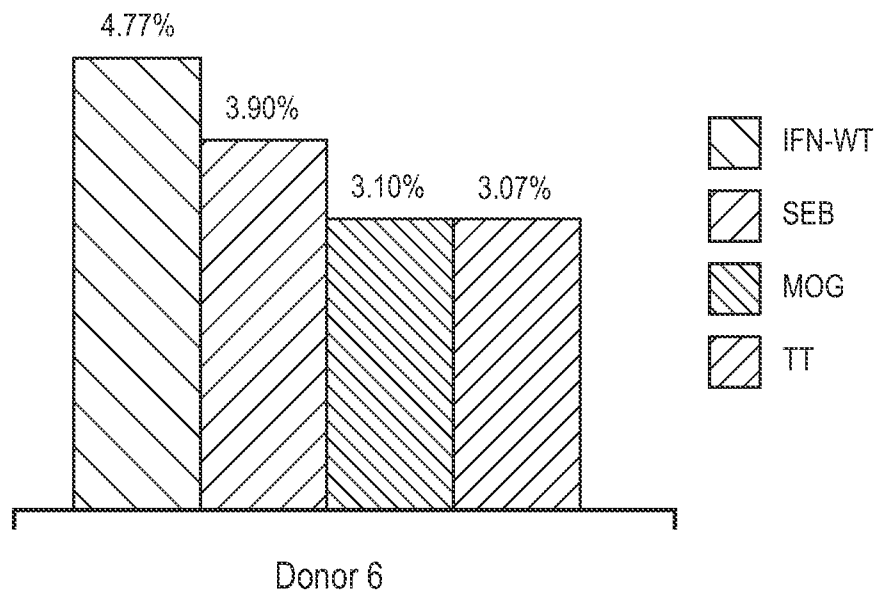
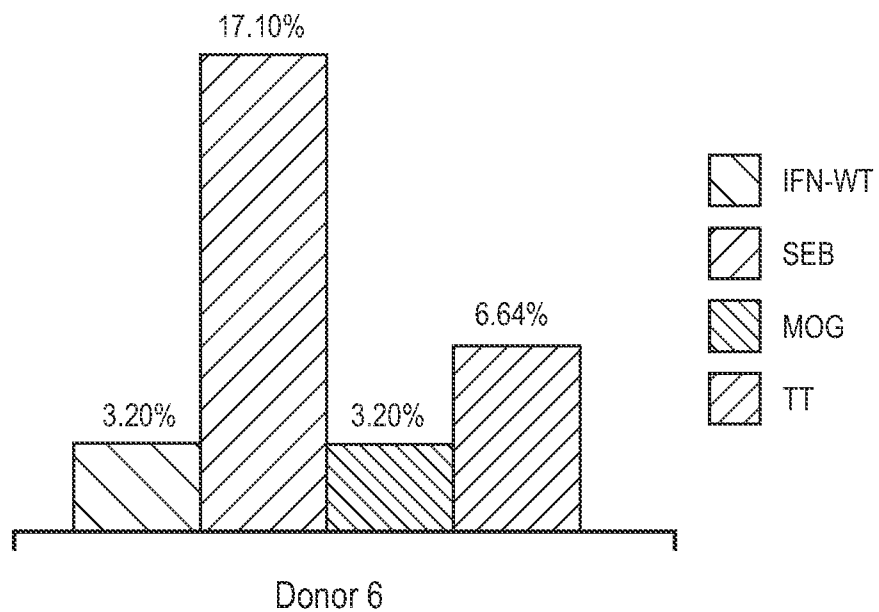


FIG. 9B

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T Lymphocytes (Naive)
CD69**FIG. 9C**T Lymphocytes (Memory)
CD69**FIG. 9D**

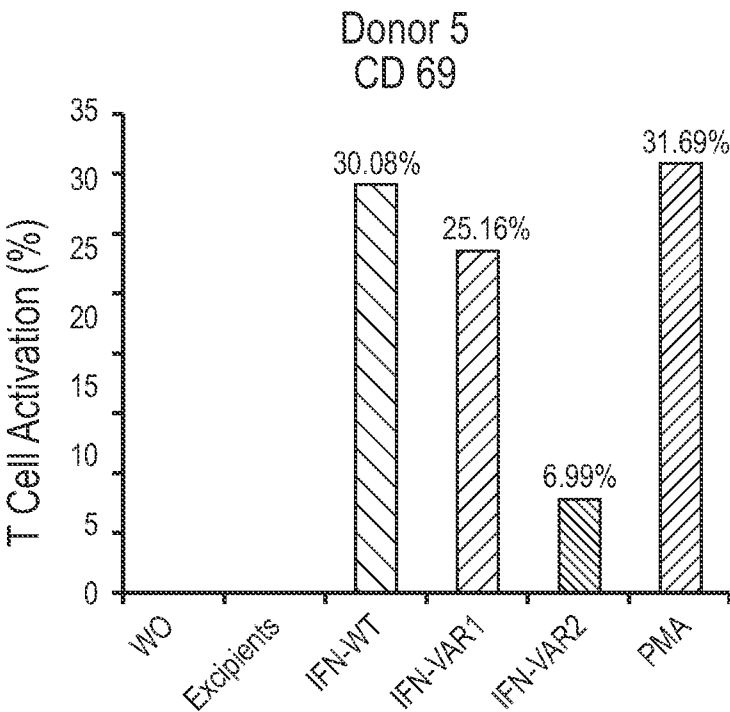


FIG. 10A

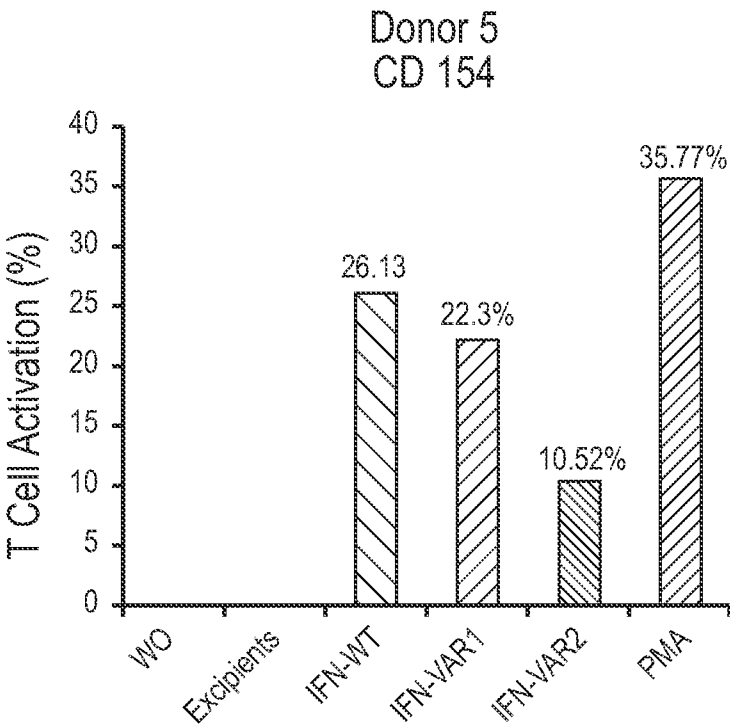


FIG. 10B

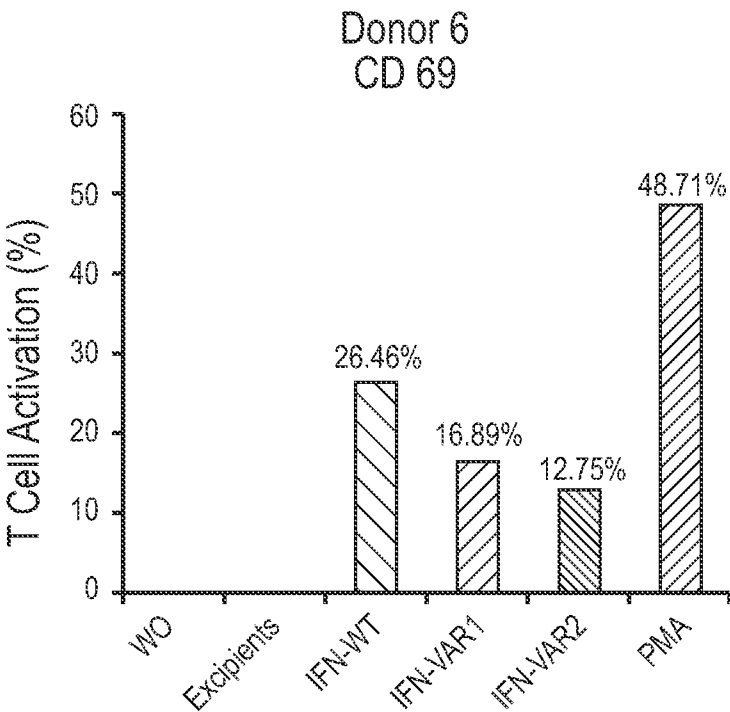


FIG. 10C

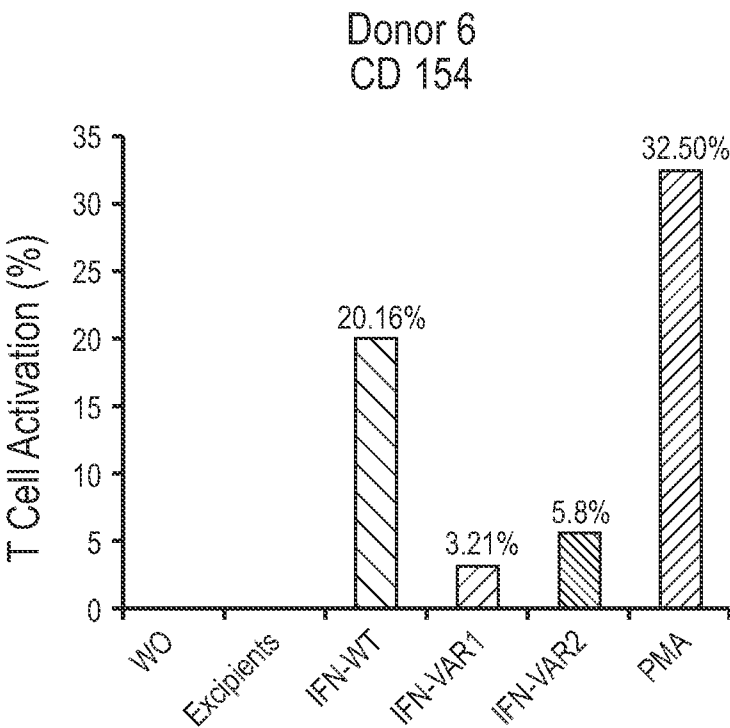


FIG. 10D

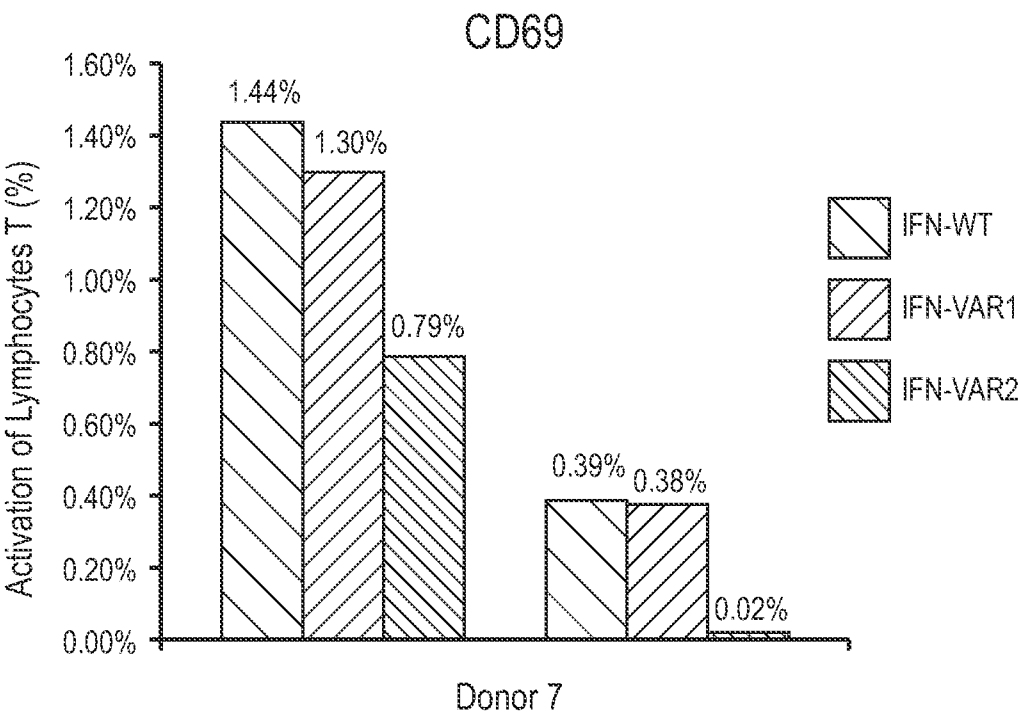


FIG. 11A

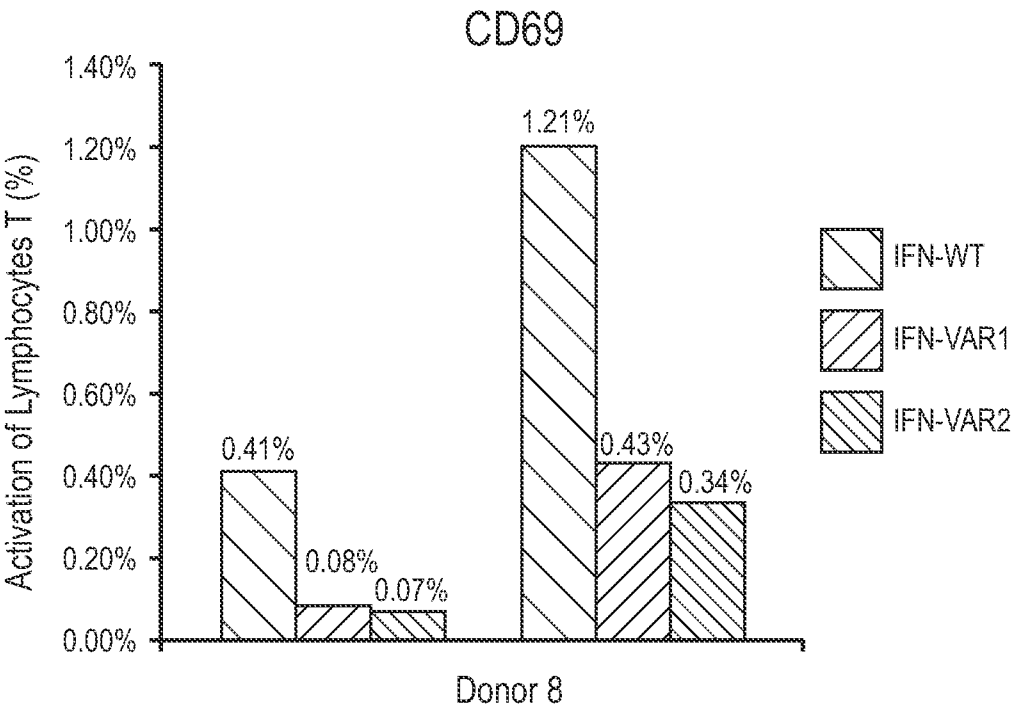


FIG. 11B

FIG. 11C

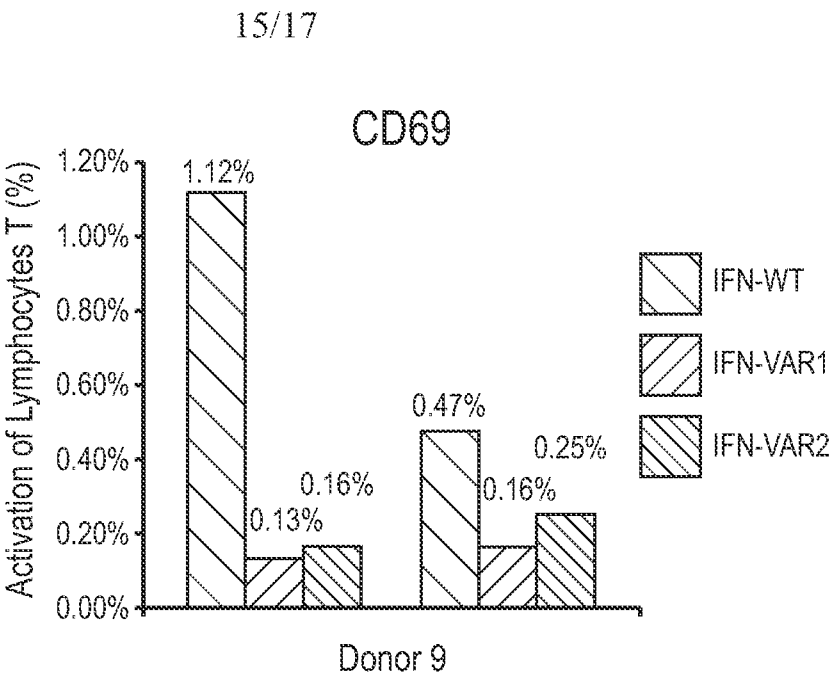


FIG. 11D

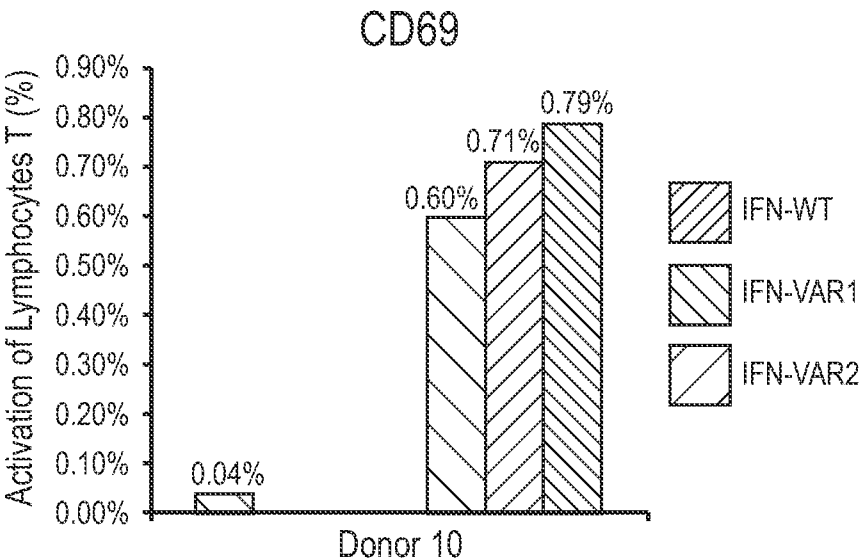


FIG. 11E

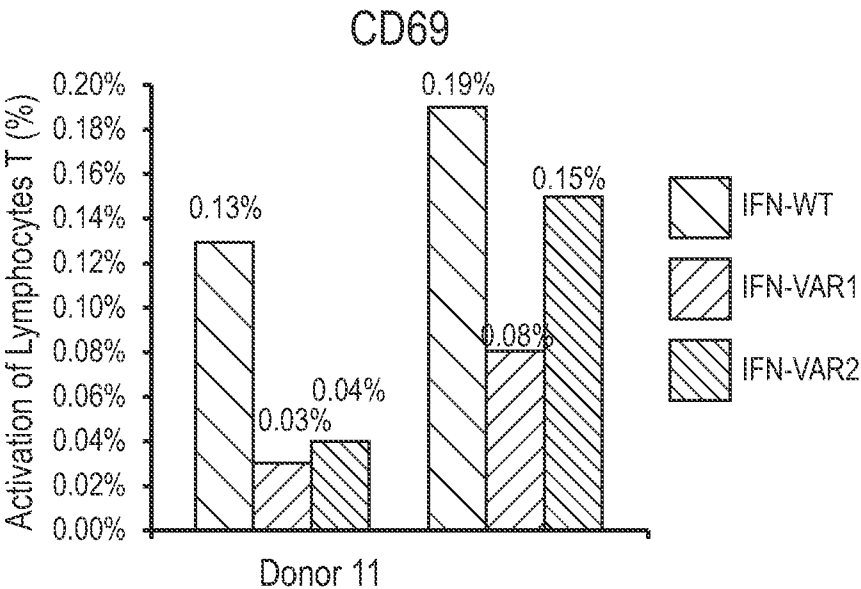


FIG. 12A

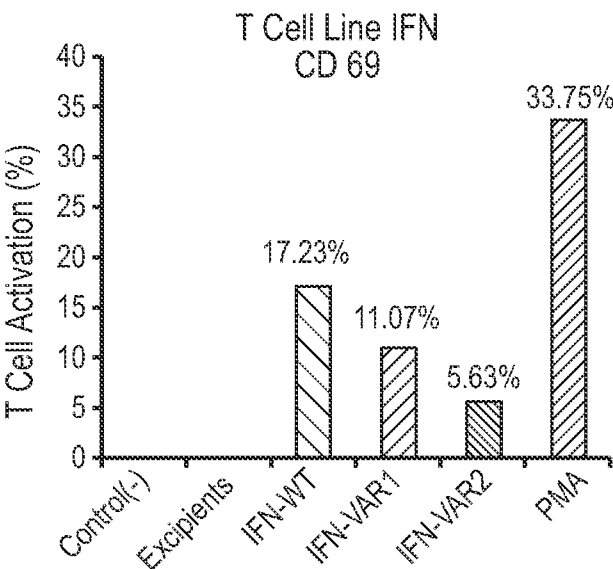


FIG. 12B

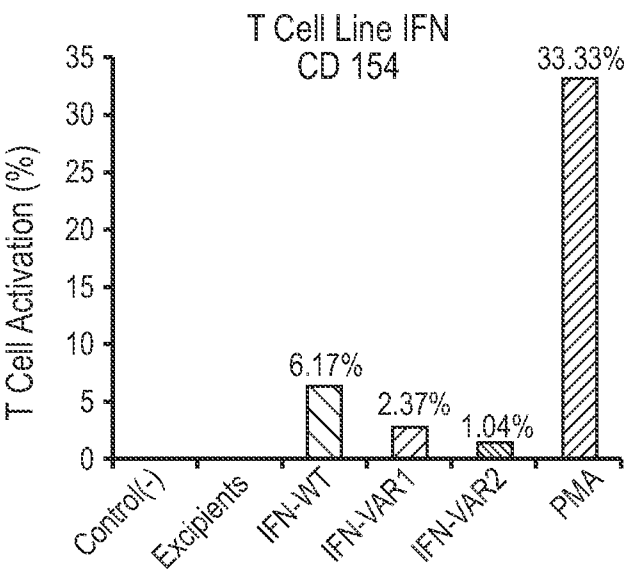


FIG. 12C

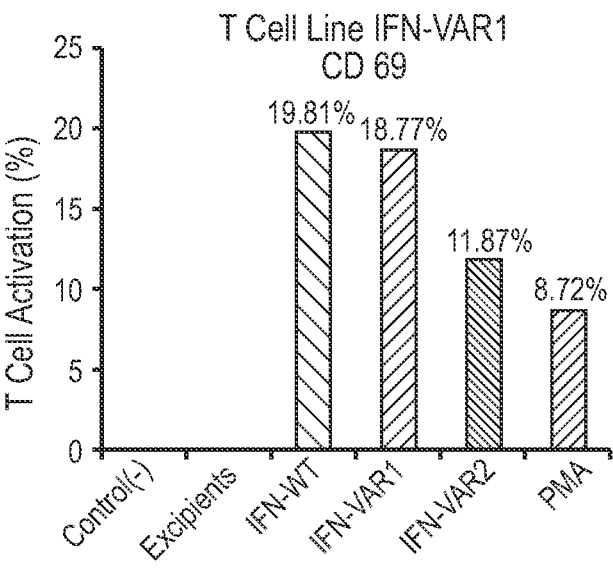


FIG. 12D

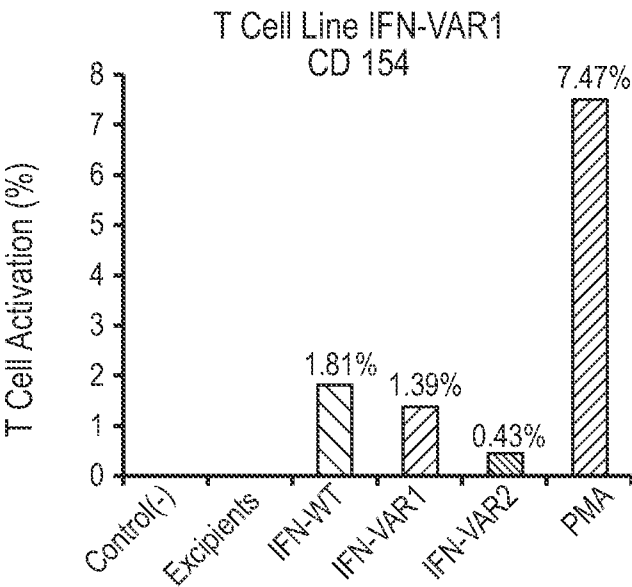


FIG. 12E

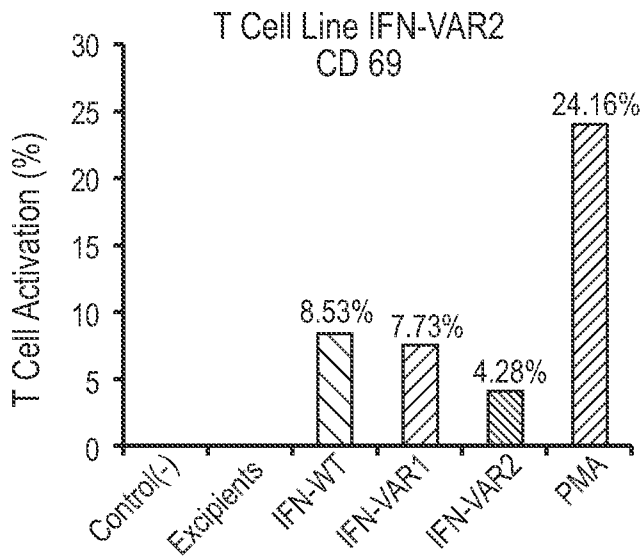
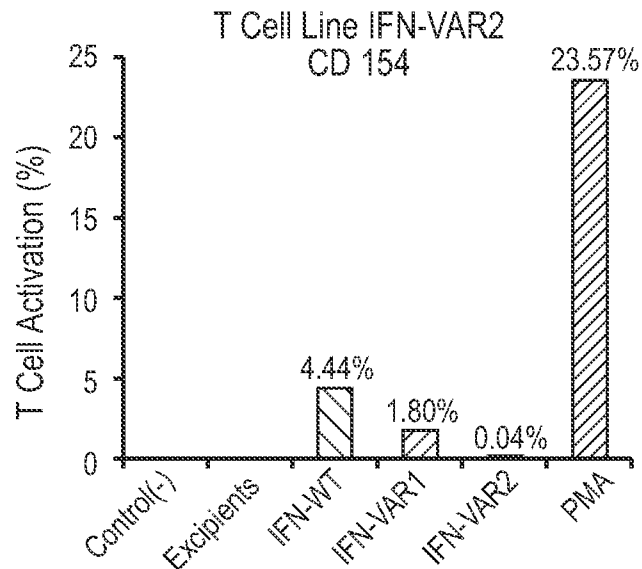


FIG. 12F



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/039763

A. CLASSIFICATION OF SUBJECT MATTERIPC: **C07K 14/565** (2023.01); **A61K 38/21** (2023.01)CPC: **C07K14/565; A61K38/215**; A61P25/28; A61P29/00; A61P31/14; A61P35/00; A61P43/00; C07K2319/00; C12N2740/10041; Y10S930/142

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	US 2009/0311216 A1 (JOHNSON-JACKSON DEBORAH et al.) 17 December 2009 (17.12.2009) abstract; paragraphs [0018], [0043], [0050] and [0070]; claim 29; fig. 1	1-6, 12 and 19 13-14 7
Y A	US 2008/0003202 A1 (GUYON THIERRY et al.) 03 January 2008 (03.01.2008) paragraphs [0015], [0159]-[0160], [0167] and [0188]	13-14 7
A	US 2009/0087411 A1 (FARES FUAD et al.) 02 April 2009 (02.04.2009) abstract; paragraphs [0069] and [0120]-[0121]	7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

30 September 2024 (30.09.2024)

Date of mailing of the international search report

11 December 2024 (11.12.2024)

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/039763

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **17-18**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, claims 1-16, 19-26, SEQ ID NO: 1 w/ N25P (modified interferon-Beta1a polypeptide), are directed to a modified interferon-Beta1a polypeptide having interferon-Beta1a activity.

The polypeptide of Claims 1-7 (in-part), 12-14, and 19 are believed to encompass the first named invention of Groups I+ and are the claims that will be searched to the extent that they comprise a modified interferon-Beta1a polypeptide encompassing SEQ ID NO: 1 w/ N25P (first exemplary modified interferon-Beta1a polypeptide). This first named invention of Group I+ has been selected to encompass the first species of the genus found in claim 1 based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines.

Applicant is invited to elect additional modified interferon-Beta1a polypeptide(s) to be searched. Additional modified interferon-Beta1a polypeptide (s) will be searched upon the payment of additional fees. Applicants must specify the searchable claims that encompass any additionally elected modified interferon-Beta1a polypeptide (s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 w/ N25P, Y60H, M117T, and N158E (modified interferon-Beta1a polypeptide).

No technical features are shared between the amino acid sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a modified interferon-beta-1a polypeptide having interferon-beta-1a activity; these shared technical features are previously disclosed by US 2003/0021765 A1 to Pepinsky et al. (hereinafter "Pepinsky").

Pepinsky discloses a modified interferon-beta-1a polypeptide having interferon-beta-1a activity (an interferon-beta-1a composition with increased activity relative to interferon-beta-1b and that also has the salutary properties of pegylated proteins in general with no effective loss in activity as compared to interferon-beta-1a forms that are not conjugated, wherein the interferon-beta-1a is a mutant with an alanine substitution; paragraphs [0008], [0026]).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/039763

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Pepinsky reference, unity of invention is lacking.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-7 (each in-part), 12-14, 19; SEQ ID NO: 1 w/ N25P (modified interferon-Beta1a polypeptide)**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	RICOTTI. "Development of IFNbeta-1a versions with reduced immunogenicity and full in vitro biological activity for the treatment of multiple sclerosis" 1-7. Clinical Immunology. Web. 01 December 2023; <entire document=""> <doi: 10.1016="" j.clim.2023.109831=""></doi:></entire> Entire Document	1-7, 12-14, and 19