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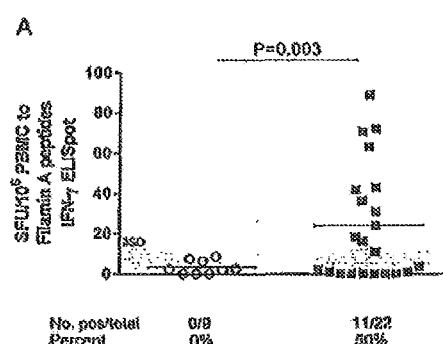
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(54) Title: AUTOANTIGENS FOR DIAGNOSIS OF RHEUMATOID ARTHRITIS

FIG . 5A T Cell Response



(57) **Abstract:** Disclosed herein are methods of diagnosing Rheumatoid arthritis in a subject comprising determining whether the subject is immunologically reactive with N-acetylglucosamine-6-sulfatase and/or filamin-A, wherein immunological reactivity of the subject to one or more of N-acetylglucosamine-6-sulfatase or filamin-A, as compared to an appropriate control, indicates the subject has rheumatoid arthritis. Examples of specific assays and kits for use with the methods are also disclosed.

AUTOANTIGENS FOR DIAGNOSIS OF RHEUMATOID ARTHRITIS

TECHNICAL FIELD

[0001] The present invention relates to the field of diagnostics using humoral and cellular autoimmune responses to disease-related autoantigens. In particular, it relates to autoantigenic biomarkers for rheumatoid arthritis.

CROSS-REFERENCE TO RELATED APPLICATION

[0002] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Nos. 62/160,320, filed May 12, 2015 and 62/162,295 filed May 15, 2015, the contents of which are incorporated herein by reference in their entireties.

GOVERNMENTAL SUPPORT

[0003] This invention was made with government support under Grant Nos. P41 GM104603, S10 RR020946, S10 OD010724 and R01 AI110175 awarded by the National Institutes of Health (NIH) and Grant No. HHSN268201000031C awarded by the NIH National Heart, Lung, and Blood Institute (NHLBI). The government has certain rights in the invention.

BACKGROUND

[0004] Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown cause that affects primary joints. Human leukocyte antigen – antigen D related (HLA-DR) molecules play a central role in autoimmune diseases, as first reported by McDevitt in 1974, (1) HLA-DR molecules are highly expressed in synovial tissue (ST), the target of the immune response in chronic inflammatory forms of arthritis, including rheumatoid arthritis (RA) and Lyme arthritis (LA) (2, 3). The greatest known genetic risk factors for these diseases are certain HLA-DR alleles (4). HLA-DR molecules present foreign or self-peptides to CD4+ T cells, leading to their activation (5). After activation, T cells can supply the necessary help to B cells to produce sustained, high-titer antibodies against the same antigen. In RA, 10 HLA-DR alleles, including the HLA-DRB1*0101 and 0401 alleles (6, 7) convey the greatest risk. These alleles code for a similar sequence of amino acids, called the RA shared epitope, which line the P4 pocket of the HLA-DR molecule. Anti-citrullinated protein antibodies (ACPA), which are specific for RA, are significantly more frequent in patients who have the shared epitope (8-13).

[0005] In both RA and LA, synovial tissue shows marked synovial hypertrophy, vascular proliferation, mononuclear cell infiltration, and intense expression of HLA-DR molecules on synoviocytes and infiltrating cells (2, 3). The tissue is bathed in synovial fluid (SF), which contains a rich mixture of

inflammatory cells, including large numbers of dendritic cells and macrophages; the latter also have increased expression of HLA-DR molecules (28, 29). However, the repertoire of specific self-peptides presented by HLA-DR molecules in synovial tissues in RA is unknown. Accordingly there is an unmet need of identifying autoantigens presented by HLA-DR and pathogenic T-cell epitopes in synovial tissues, synovial fluid as well as peripheral blood mononuclear cells of RA patients.

SUMMARY

[0006] Aspects of the invention relate to a method of diagnosing rheumatoid arthritis in a subject comprising determining whether the subject is immunologically reactive with one or more of filamin-A and N-acetylglucosamine-6-sulfatase, wherein immunological reactivity of the subject to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, as compared to an appropriate control, indicates the subject has rheumatoid arthritis.

[0007] In one embodiment, the determining is by evaluating a biological sample obtained from the subject for immunological reactivity with the one or more of filamin-A and N-acetylglucosamine-6-sulfatase.

[0008] In one embodiment of the foregoing aspects, determining immunological reactivity is by detecting the presence of T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, comprising the steps: a) stimulating peripheral blood mononuclear cells (PBMC) of the subject or the synovial fluid mononuclear cells (SFMC) of the subject *in vitro* with one or more of filamin-A and N-acetylglucosamine-6-sulfatase, whole protein or polypeptide fragments; b) measuring T cell proliferation *in vitro* or secretion of IFN- γ into cell culture supernatants; and c) identifying the subject as having T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when T cell proliferation or secretion of IFN- γ is measured as significantly increased over that of an appropriate control.

[0009] In one embodiment, step b) is measuring T cell proliferation *in vitro*, and the subject is identified as having T cell reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when T cell proliferation is measured as significantly increased over that of an appropriate control. In one embodiment, step b) is measuring T cell secretion of IFN- γ into cell culture supernatants, and the subject is identified as having T cell reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when secretion of IFN- γ is measured as significantly increased over that of an appropriate control.

[0010] In one embodiment, determining immunological reactivity comprises determining if the subject has a B-cell response to one or more of filamin-A and N-acetylglucosamine-6-sulfatase resulting in the production of autoantibodies that specifically recognize the one or more filamin-A and N-

acetylglucosamine-6-sulfatase, by contacting the sample with filamin-A protein or a polypeptide fragment thereof, and/or N-acetylglucosamine-6-sulfatase protein or a polypeptide fragment thereof, under conditions that allow an immunocomplex of the antibody and the filamin-A or N-acetylglucosamine-6-sulfatase form, and detecting the presence or absence of an immunocomplex, wherein the presence of an immunocomplex indicates the subject presents a B-cell response to filamin-A and/or N-acetylglucosamine-6-sulfatase and wherein the absence of an immunocomplex indicates the subject fails to present a B-cell response to filamin-A and/or N-acetylglucosamine-6-sulfatase.

[0011] In one embodiment, the assay is an enzyme-linked immunosorbent assay (ELISA), agglutination test, direct immunofluorescence assay, indirect immunofluorescence assay, or an immunoblot assay.

[0012] In one embodiment of the foregoing aspects, the polypeptide fragment of filamin-A comprises the amino acid sequence NPAEFVVNTSNAGAG (SEQ ID NO: 1) or an antigenic portion thereof.

[0013] In one embodiment, the polypeptide fragment of N-acetylglucosamine-6-sulfatase comprises the amino acid sequence of FEPFFMMIATPAPH (SEQ ID NO: 2) or an antigenic portion thereof.

[0014] In one embodiment of the foregoing aspects, the subject has been or is further tested for one or more of rheumatoid factor, anti-citrullinated protein antibodies (ACPA), and one or more HLA-DR alleles. In one embodiment, the HLA-DR allele is HLA-DRB1*0101 and/or HLA-DRB1*0401. In one embodiment, the subject is at risk for, or is suspected of having, rheumatoid arthritis.

[0015] In one embodiment of the various methods described herein, the method further comprises the step of treating the subject with one or more of a nonsteroidal anti-inflammatory drug (NSAIDs), a steroid, a disease modifying anti-rheumatic drug (DMARD), and a biologic.

[0016] In one aspect described herein is a kit comprising one or more potential antigen and/or potential epitope of filamin-A and/or N-acetylglucosamine-6-sulfatase, and reagents for conducting an assay for detecting the presence of an antibody in a sample that binds to the one or more potential antigen and/or potential epitope of the filamin-A and N-acetylglucosamine-6-sulfatase.

[0017] In one embodiment, the potential antigen and/or potential epitope of filamin-A comprises the amino acid sequence NPAEFVVNTSNAGAG (SEQ ID NO: 1) or an antigenic portion thereof. In one embodiment, the potential antigen and/or potential epitope of N-acetylglucosamine-6-sulfatase comprises the amino acid sequence FEPFFMMIATPAPH (SEQ ID NO: 2) or an antigenic portion thereof.

[0018] In one embodiment of the foregoing aspects, the assay is an enzyme-linked immunosorbent assay (ELISA). In one embodiment, the assay is a western blot.

[0019] Another aspect of the invention relates to a kit or method disclosed above for use in identifying a subject with rheumatoid arthritis.

Definitions

[0020] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0021] As used herein, the terms "diagnose" or "diagnosis" or "diagnosing" refers to determining the nature or the identity of a condition or disease or disorder, detecting and/or classifying the disease and/or disorder in a subject. A diagnosis may be accompanied by a determination as to the severity of the disease. The term also encompasses assessing or evaluating the disease status (progression, regression, stabilization, response to treatment, etc.) in a patient known to have the disease). Diagnosis as it relates to the present invention, relates to the diagnosis of chronic inflammatory arthritis e.g., Rheumatoid arthritis. The diagnosis can be differential diagnosis. As used herein, "differential diagnosis" refers to determination of which two or more diseases with similar symptoms (e.g., RA, osteoarthritis, systemic lupus erythematosus, Lyme arthritis, reactive arthritis, psoriatic arthritis, ankylosing spondylitis) is the one from which a patient is suffering from based on an analysis of clinical data such as for example results obtained from methods described herein.

[0022] As used herein, the term "biological sample" refers to a sample obtained for evaluation *in vitro*. The biological sample can be any sample that is expected to contain antibodies and/or immune cells. The sample can be taken from a part of the body that is specifically affected by the disorder, such as taken specifically from a site of inflammation or pathology in the subject (e.g., synovial fluid, synovial tissue, synovial fluid mononuclear cells (SFMC), spinal fluid, etc.) or can be a more systemic sample (e.g., peripheral blood, peripheral blood mononuclear cells (PBMC), whole blood or whole blood pre-treated with an anticoagulant such as heparin, ethylenediamine tetraacetic acid, plasma or serum). Sample can be pretreated prior to use, such as preparing plasma from blood, diluting viscous liquids, or the like; methods of treatment can also involve separation, filtration, distillation, concentration, inactivation of interfering components, and the addition of reagents.

[0023] As used herein, "immunologically reactive" or "immunoreactive" is defined as the capability of the molecule (e.g., whole protein or polypeptide fragments thereof) of the present invention to induce a specific immune response (e.g. T cell reactivity, B cell response) in appropriate subjects or cells and to bind with specific antibodies. The immunological reactivity can be assayed in a biological sample comprising immunological cells and/or antibodies obtained from a subject by methods described herein.

[0024] As used herein “autoantigen/self-antigen” is any substance normally found within a subject which, in an abnormal situation, is no longer recognized as part of the subject itself by the lymphocytes or antibodies of that subject, and is therefore attacked by the immune system as though it were a foreign substance. An autoantigen can be a naturally occurring molecule such as a protein (e.g., GNS, filamin A) normally produced and used by the subject itself, eliciting an immune response possibly leading to an autoimmune disease (e.g. RA) in the subject.

[0025] The term “antibody,” as used herein, refers to an immunoglobulin molecule which is able to “specifically recognize” and/or “specifically bind” to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically immunoglobulin molecules.

[0026] As used herein, “autoantibody” means an antibody produced by the immune system of a subject that is directed to, and specifically binds to an “autoantigen/self-antigen” or an “antigenic epitope” thereof.

[0027] As used herein, the term "epitope" refers to that portion of any molecule capable of being recognized by, and bound by, a T cell or an antibody (the corresponding antibody binding region may be referred to as a paratope), and/or eliciting an immune response. In general, epitopes consist of chemically active surface groupings of molecules, e.g., amino acids, and have specific three-dimensional structural characteristics as well as specific charge characteristics. As used herein, "immunogenic epitope", as determined by any method known in the art, is defined as a portion of a polypeptide that causes an immune response in a subject. As used herein, "antigenic epitope", as determined by any methods well known in the art, in that a given antibody or T cell receptor specifically recognizes and specifically binds to a given antigen. It can be, and is defined as a portion of a protein (e.g. GNS, filamin A). As used herein, “antigenic portion” refers to the portion of GNS and filamin A that includes the antigenic epitopes.

[0028] “Specifically bind” and/or “specifically recognize” as used herein, refers to the higher affinity of a binding molecule for a target molecule compared to the binding molecule's affinity for non-target molecules. A binding molecule that specifically binds a target molecule does not substantially recognize or bind non-target molecules. e.g., an antibody "specifically binds" and/or “specifically recognize” another molecule, meaning that this interaction is dependent on the presence of the binding specificity of the molecule structure, e.g., an antigenic epitope. For example, an antibody that specifically binds to the antigenic epitope of protein molecules such as GNS, filamin A, and/or polypeptide fragments thereof, instead of indiscriminately binding to cause non-specific binding and/or background binding. As used herein, "non-specific binding" and "background binding" refers to the interaction that does not depend on the presence of specific structure (e.g., a specific antigenic epitopes).

[0029] As used herein, an “immunoassay” refers to any binding assay that uses an antibody capable of binding specifically to a target molecule to detect and quantify the target molecule.

[0030] As used herein, an “appropriate control” refers to one or more biological samples obtained from a subject not afflicted with a disease or disorder that features abnormal level of the molecule and measurement of the molecule therein. An example of an appropriate control can be biological sample from a subject not afflicted from RA. As it relates to the present invention, an appropriate negative control sample would not be positive for T cell reactivity to and/or show presence of autoantibodies to GNS, filamin A and/or polypeptide fragments thereof when tested using the methods described herein.

[0031] The terms “disease”, “disorder”, or “condition” are used interchangeably herein, refer to any alternation in state of the body or of some of the organs, interrupting or disturbing the performance of the functions and/or causing symptoms such as discomfort, dysfunction, distress, or even death to the person afflicted or those in contact with a person. A disease or disorder can also be related to a distemper, ailing, ailment, malady, disorder, sickness, illness, complaint, or affectation.

[0032] As used herein, the terms “treat,” “treatment,” “treating,” or “amelioration” refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of chronic inflammatory arthritis, (e.g., Rheumatoid arthritis), an associated condition and/or a symptom thereof. The term “treating” includes reducing or alleviating at least one adverse effect or symptom of RA. Treatment is generally “effective” if one or more symptoms or clinical markers (e.g. antibodies to one or more of GNS, filamin A or cyclic citrullinated peptide (anti-CCP) and/or rheumatoid factors) are reduced. Alternatively, or in addition, treatment is “effective” if the progression of a disease is reduced or halted. That is, “treatment” includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Accordingly in case of chronic inflammatory arthritis such as RA, “effective treatment” can for example reduce inflammation, swelling and joint pain, bone deformity and/or result in reduction in T or B cell reactivity to GNS, filamin A and/or polypeptides thereof and/or reduction in autoantibodies to GNS, filamin A and/or polypeptides thereof compared to that observed pre-treatment, as determined by methods described herein. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality. For example, treatment is considered effective if the condition is stabilized. The term “treatment” of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[0033] As used herein, a "subject", "patient", "individual" and like terms are used interchangeably and refers to a vertebrate, preferably a mammal, more preferably a primate, still more preferably a human. Mammals include, without limitation, humans, primates, rodents, wild or domesticated animals, including feral animals, farm animals, sport animals, and pets. Primates include, for example, chimpanzees, cynomolgous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include, for example, mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include, for example, cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, and canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. The terms, "individual," "patient" and "subject" are used interchangeably herein. In one embodiment the subject is male. In another embodiment, the subject is female.

[0034] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of conditions or disorders associated with RA. Such models are known in the art and are described in (71). In addition, the compositions and methods described herein can be used to diagnose domesticated animals and/or pets.

[0035] A subject can be one who has been previously diagnosed with or identified as suffering from or under medical supervision for a chronic inflammatory and/or autoimmune disease (e.g., RA). A subject can be one who is diagnosed and currently being treated for, or seeking treatment, monitoring, adjustment or modification of an existing therapeutic treatment, or is at a risk of developing RA, e.g., due to family history, carrying alleles or genotype associated with RA (e.g. HLA-DRB1*0101 and DRB1*0401, HLA-DRB1*0405 and DRB1*0408). The subject can exhibit one or more symptoms of autoimmune disease (e.g., RA) (e.g. swollen joints, joint pains). The subject may have tested positive with other assays for RA associated factor. Such factors include rheumatoid factor, anti-citrullinated protein antibodies (ACPA), one or more of HLA-DR alleles associated with RA, or other autoantigens. The subject may lack one or more symptoms of autoimmune disease. The subject may be identified as testing negative for other RA associated factors (rheumatoid factor, anti-citrullinated protein antibodies (ACPA), one or more of HLA-DR alleles, and other autoantigens).

[0036] As used herein, the terms "protein", "peptide" and "polypeptide" are used interchangeably to designate a series of amino acid residues connected to each other by bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", "peptide" and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g., phosphorylated, glycated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small

polypeptides, but usage of these terms in the art overlaps. The terms "protein", "peptide" and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof.

[0037] The term "statistically significant" or "significantly" refers to statistical significance and generally means a difference of two standard deviations (2SD) or more.

[0038] Definitions of common terms in cell biology and molecular biology can be found in "The Merck Manual of Diagnosis and Therapy", 19th Edition, published by Merck Research Laboratories, 2006 (ISBN 0-911910-19-0); Robert S. Porter et al. (eds.), The Encyclopedia of Molecular Biology, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); Immunology by Werner Luttmann, published by Elsevier, 2006. Definitions of common terms in molecular biology can also be found in Benjamin Lewin, Genes X, published by Jones & Bartlett Publishing, 2009 (ISBN-10: 0763766321); Kendrew et al. (eds.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8) and Current Protocols in Protein Sciences 2009, Wiley Intersciences, Coligan et al., eds.

[0039] Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Sambrook et al., Molecular Cloning: A Laboratory Manual (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1995); Current Protocols in Protein Science (CPPS) (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.), Current Protocols in Cell Biology (CPCB) (Juan S. Bonifacino et. al. ed., John Wiley and Sons, Inc.), and Culture of Animal Cells: A Manual of Basic Technique by R. Ian Freshney, Publisher: Wiley-Liss; 5th edition (2005), Animal Cell Culture Methods (Methods in Cell Biology, Vol. 57, Jennie P. Mather and David Barnes editors, Academic Press, 1st edition, 1998) which are all incorporated by reference herein in their entireties.

[0040] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g., " is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g., " is synonymous with the term "for example."

[0041] As used in this specification and appended claims, the singular forms "a," "an", and "the" include plural references unless the context clearly dictates otherwise. Thus for example, reference to "the method" included one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0042] In this application and the claims, the use of the singular includes the plural unless specifically stated otherwise. In addition, use of "or" means "and/or" unless stated otherwise. Moreover, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms

such as “element” or “component” encompass both elements and components comprising one unit and elements and components that comprise more than one unit unless specifically stated otherwise.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] FIGs. 1A-1D show degree of overlap in HLA-DR-presented peptides and their source proteins identified in the synovial tissue (ST), synovial fluid mononuclear cells (SFMC) or peripheral blood mononuclear cells (PBMC) of RA or Lyme arthritis (LA) patients. The Venn diagrams depict the number of shared peptides or source proteins identified in the ST, SFMC or PBMC of individual RA or LA patients or in the entire RA and/or LA cohort. (FIG. 1A) Shared peptides or (FIG. 1B) shared source proteins identified in patients RA2 or LA5. (FIG. 1C) Shared peptides or (FIG. 1D) shared source proteins identified in the RA, LA, or RA and LA cohorts. All peptide comparisons were based upon consensus-matched, non-redundant identified peptides using in-house generated software. The consensus on source proteins matched between cohorts was assembled by matching the source protein accession numbers using a Microsoft Access query.

[0044] FIGs. 2A-2B show high abundance, high accuracy peptide fragment ions can provide full sequence coverage and clearly distinguish asparagine deamidation from arginine citrullination. (FIG. 2A) CID MS2 spectrum, recorded using the ion trap on the LTQ-Orbitrap XL MS for analysis of the mixture of MHC class II-presented peptides recovered from the synovial tissue of patient RA3. The spectrum was assigned to a modified form of the peptide ²⁷VNDTQFVRFDSDAASPRG⁴⁴ (SEQ ID NO: 5) derived from the source protein B6V6K3 MHC class I antigen (fragment). No sequence-defining fragment ions were observed between the second and eighth residues; nevertheless, the post-translational modification of arginine conversion to citrulline at R8 was assigned by two protein database search programs. (FIG. 2B) HCD MS2 spectrum of the same component, recorded with the Q Exactive plus MS, allowed unambiguous assignment of asparagine deamidation at N2 as the post-translational modification on the peptide sequence. Note: ° loss of H₂O; * loss of NH₃.

[0045] FIGs. 3A-3F show Tandem mass spectra for HLA-DR-presented peptides identified to be immunogenic in patients with RA or LA. (FIG. 3A) ³⁴⁰LGRFERMLAAQGVDPG³⁵⁵ (SEQ ID NO: 6) from endothelial cell growth factor was identified by OMSSA from LA1 ST (22). (FIG. 3B) ⁶⁵⁵IEGNLIFDPNNYLPK⁶⁶⁹ (SEQ ID NO: 7) from apolipoprotein B-100 was consistently identified by Mascot and X!Tandem from LA5 ST (23). (FIG. 3C) ²⁰⁸GTNLFLVAAHELGH²²² (SEQ ID NO: 8) from matrix metalloproteinase-10 (stromelysin 2) was identified by Mascot from LA4 ST (24) (FIG. 3D) ²⁸⁵DKVLIRIMVSRSEVD²⁹⁹ (SEQ ID NO: 9) from annexin A2 was consistently identified by Mascot, OMSSA, and X!Tandem from RA1 ST (25). (FIG. 3E) ²⁴⁴⁶NPAEFVVNTSNAGAG²⁴⁶⁰ (SEQ ID NO: 1) from filamin A was consistently identified by Mascot, OMSSA, and X!Tandem from RA2 ST (the same

peptide was also identified consistently by Mascot, OMSSA, and X!Tandem from RA2 PB) (26) and (FIG. 3F) $^{222}\text{FEPFFMMIATPAPH}^{235}$ (SEQ ID NO: 2) from N-acetylglucosamine-6-sulfatase was consistently identified by Mascot, OMSSA, and X!Tandem from RA2 ST (26) Note: $^\circ$ loss of H₂O; * loss of NH₃.

[0046] FIG. 4 shows clinical characteristics, number of HLA-DR-presented peptides and source proteins identified from the case patient RA2. 1 of 89 HLA-DR presented peptides identified from synovial tissue and 1 of 15 HLA-DR presented peptides identified from her peripheral blood of patient induced the patient's PBMC to secrete IFN γ . These reactive peptides were derived from the proteins GNS and filamin A respectively.

[0047] FIGs. 5A-5B are plots showing T and B cell autoreactivity to Filamin A. (FIG. 5A) PBMC from healthy control (HC) subjects or RA patients were tested for T cell autoreactivity against the filamin A peptide, plus three additional filamin A peptides predicted to be promiscuous HLA-DR binders (promiscuous T cell epitopes). Each assay also included a positive control (PHA) and a negative control (no peptides), T cell reactivity was measured using an IFN γ enzyme linked immunospot (ELISpot) assay. The spot forming units (SFU) per 10^5 were calculated. A positive response was defined as 3 standard deviations above the mean SFU/ 10^6 cells of healthy control subjects (area above the gray shaded region). (FIG. 5B) The levels of IgG anti-filamin A in the sera of HC subjects, RA patients or Lyme arthritis (LA) patients were measured by enzyme-linked immunosorbent assay (ELISA). Plates were coated with recombinant human Filamin A and the wells were incubated with serum samples from each test subject. As controls, wells were also incubated with recombinant IgG anti-filamin A (positive control) or buffer (negative control). Bound IgG anti-filamin antibodies were detected using horseradish peroxidase-conjugated goat anti-human IgG followed by TMB substrate. The shaded gray area corresponds to 3 standard deviations above the mean of HC subjects. Percentage of positive response for each patient group are shown below the graph. P values were calculated using an unpaired t-test. Horizontal line indicates the mean of each dataset. The method used to identify this disease associated autoantigen is described in references (22, 30).

[0048] FIGs. 6A-6B are plots showing T and B cell autoreactivity to N-acetylglucosamine-6-sulfatase. (FIG. 6A) PBMC from healthy control (HC) subjects or RA patients were tested for T cell autoreactivity against the GNS peptide identified from the synovial tissue of patient with RA, plus three additional GNS peptides predicted to be promiscuous HLA-DR binders (promiscuous T cell epitopes). Each assay also included a positive control (PHA) and a negative control (no peptides), T cell reactivity was measured using an IFN γ enzyme linked immunospot (ELISpot) assay. The spot forming units (SFU) per 10^5 were calculated. A positive response was defined as 3 standard deviations above the mean SFU/ 10^6 cells of healthy control subjects (area above the gray shaded region). (FIG. 6B) The levels of IgG anti-GNS in the

sera of HC subjects, RA patients or Lyme arthritis (LA) patients were measured by enzyme-linked immunosorbent assay (ELISA). Plates were coated with recombinant human GNS and the wells were incubated with serum samples from each test subject. As controls, wells were also incubated with recombinant IgG anti-GNS (positive control) or buffer (negative control). Bound IgG anti-GNS antibodies were detected using horseradish peroxidase-conjugated goat anti-human IgG followed by TMB substrate. The shaded gray area corresponds to 3 standard deviations above the mean of HC subjects. Percentage of positive response for each patient group are shown below the graph. P values were calculated using an unpaired t-test. Horizontal line indicates the mean of each dataset. The method used to identify this disease associated autoantigen is described in references (22, 30).

[0049] FIG. 7 shows serum levels of GNS protein determined by sandwich ELISA. Positivity was calculated as >3SD above the mean of normal control (area above the shaded region). HC, healthy control; SLE, systemic lupus; Other A, psoriatic arthritis, ankylosing spondylitis, osteoarthritis; LA, Lyme arthritis; RA, rheumatoid arthritis. 13% of RA patients had high serum GNS protein levels.

[0050] FIG. 8 shows serum levels of filamin A protein determined by sandwich ELISA. Positivity was calculated as >3SD above the mean of normal control (area above the shaded region). HC, healthy control; SLE, systemic lupus; Other A, psoriatic arthritis, ankylosing spondylitis, osteoarthritis; LA, Lyme arthritis; RA, rheumatoid arthritis. 24% of RA patients had high serum filamin A protein levels.

[0051] FIG. 9 shows the amino acid sequence of human GNS (Genbank Accession No. NP_002067.1) (SEQ ID NO: 3).

[0052] FIG. 10 shows the amino acid sequence of human filamin A (See Genbank Accession No. NP_001104026.1) (SEQ ID NO: 4).

DETAILED DESCRIPTION

[0053] Aspects of the invention relate to the finding that N-acetylglucosamine-6-sulfatase (GNS), a lysosomal enzyme, and filamin A, an actin-binding protein, are autoantigenic in subsets of patients with rheumatoid arthritis. GNS and filamin A are targets of T cell and/or B cell responses in RA patients. This was shown by demonstrating that peripheral blood mononuclear cells (PBMC) or synovial fluid mononuclear cells (SFMC) from RA patients are stimulated by autoantigenic peptides of the GNS and filamin A. This was also shown by demonstrating that RA patients have antibodies that specifically bind to the GNS and filamin A. These findings indicate that GNS and filamin A, and immunogenic polypeptides thereof, can be used to identify immunoreactivity in a subject, with that immunoreactivity being indicative of RA disease.

[0054] One aspect of the invention relates to a method of diagnosing rheumatoid arthritis in a subject by determining whether the subject demonstrates immunological reactivity to filamin-A and/or N-acetylglucosamine-6-sulfatase. Immunological reactivity of the subject to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, as compared to an appropriate control, indicates the subject has rheumatoid arthritis.

[0055] Immunoreactivity of a subject to the protein is determined, for example, by obtaining a biological sample from the subject containing antibodies or immune cells (e.g., T cells), and then assaying that biological sample for autoantigen immunoreactivity. Immunoreactivity to a molecule (e.g., a protein) can be determined using the full length molecule or one or more representative fragments or epitopes thereof. The immunological reactivity demonstrated by the subject can be T cell reactivity or B cell reactivity (e.g., has antibodies that specifically bind to the filamin-A and/or N-acetylglucosamine-6-sulfatase). T cell and B cell reactivity of a subject can be determined by various methods known in the art.

[0056] Typically, T cell reactivity is performed with an antigenic fragment of the full length molecule. B cell reactivity can be determined using a full length protein, however in some embodiment, shorter fragments may be used. Any method known in the art can be used to identify the immunological reactivity.

[0057] One aspect of the invention relates to a method for diagnosing rheumatoid arthritis in a subject by identifying the presence of antibodies in the subject that specifically bind to the filamin-A and/or N-acetylglucosamine-6-sulfatase protein or polypeptide fragment thereof. The assay involves contacting a biological sample obtained from the subject with the full length protein or representative fragments thereof (referred to as the test antigen), under conditions that allow an immunocomplex of the antibody and the polypeptide to form, and then assaying for the presence of the immunocomplex. Various methods of detecting the presence of an antibody-antigen complex are available in the art, and are suitable for use in the methods described herein, such as ELISA, agglutination test, direct immunofluorescence assay, indirect immunofluorescence assay, radioimmunoassay and immunoblot assay. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein, (see, e.g., Harlow & Lane, *Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). In one embodiment, the assay involves a detectable label that is used to facilitate detection of the complex through detection of the label. The label present in the test sample is compared to label present in a positive control and/or absent in a negative control. Appropriate controls can be determined by the skilled practitioner. In one embodiment, a negative control is the same type of biological sample obtained, for example, from a healthy subject. In

one embodiment, a negative control is performed with the same sample of the subject, using a protein to which the subject has no immunoreactivity.

[0058] Reactivity that indicates detection of a significant level of the antibody complex above background (e.g., that in a negative control) indicates a positive result. In one embodiment, reactivity is identified by detection of the label. In one embodiment, a determination of reactivity that is ≥ 3 standard deviations (SD) above the mean of a sample of a control (e.g., of a healthy control subject) is considered a positive result. In one embodiment, a result that is at least 2-fold above background (e.g., of the signal of a sample obtained from a healthy control subject) is indicative of positive. In one embodiment, higher levels of detection are used to indicate a positive result (e.g., at least 3-fold, 4-fold, or 5-fold above background).

[0059] One aspect of the invention relates to a method for diagnosing rheumatoid arthritis in a subject by identifying the presence of T cells in the subject that are specifically reactive to GNS and/or filamin A. This is typically accomplished by obtaining a biological sample from the subject that contains T cells, and exposing the cells in the biological sample to one or more polypeptide fragments of filamin-A and/or N-acetylglucosamine-6-sulfatase (also referred to as the test fragment), and assaying for a response to the test fragment such as stimulation or proliferation. In one embodiment, peripheral blood mononuclear cells (PBMC) or synovial fluid mononuclear cells (SFMC) of the subject (e.g., in or obtained from the biological sample) are contacted with the test antigen *in vitro* under conditions conducive to stimulation. The cells are then monitored for a response that indicates stimulation. In one embodiment, T cell proliferation *in vitro* or cytokine production (e.g., IFN- γ) into the supernatant that indicates stimulation is monitored. Identification of a response (e.g., T cell proliferation or cytokine secretion) substantially over that of an appropriate control sample indicates stimulation has occurred. The level of one or more cytokines that are indicative of T cell activation can be monitored in the supernatant of the cells as an indication of T cell activation. One such cytokine is IFN- γ . Other cytokines to monitor include, without limitation IL-17, IL-12, and IL-10. The levels of one or more of such cytokines can be monitored. A significant increase in IFN- γ , IL-17, and/or IL-12, and/or a significant decrease in IL-10 levels indicates activation.

[0060] Detection of the stimulation indicates that the sample comprises immunological cells that are immunologically reactive to the test antigen, which in turn indicates that the subject from whom the sample was obtained is immunologically reactive to the test antigen. Put another way, a positive assay result indicates that the test antigen is an autoantigen in the subject, and that the subject has rheumatoid arthritis. Typical assays for use in this method are T cell proliferation assays, such as 3 H-thymidine incorporation assay, CFSE dilution, or an ELISPOT, and also T cell reactivity assays. Methods of determining T cell reactivity are well known in the art and are described in for example US5750356A.

[0061] Reactivity that indicates a detection of a significant level of stimulation above background (e.g., that in a negative control) is expected to serve as a positive result. In one embodiment, a determination of stimulation that is ≥ 3 standard deviations (SD) above the mean of a sample of a healthy control subject is considered a positive result. In one embodiment, a result that is at least 2-fold above background (e.g., of the signal of a sample obtained from a healthy control subject) is indicative of positive. In one embodiment, higher levels of stimulation is used to indicate a positive result (e.g., at least 3-fold, 4-fold, or 5-fold above background).

[0062] Test antigen for use in the methods herein described include, without limitation, full length GNS, filamin A, and/or polypeptide fragments thereof.

[0063] GNS is a lysosomal enzyme, involved in the catabolism of heparin, heparan sulphate, and keratan sulphate. The GNS of the methods and kits disclosed herein can be full length GNS protein, a polypeptide fragment thereof, a derivative thereof or an antigenic portion thereof. The GNS polypeptide can be mammalian GNS. The GNS can also be an isoform of the full length GNS or a polypeptide fragment thereof. In some embodiments, the GNS of the methods and kits disclosed herein can be derived from human GNS having the amino acid sequence shown in FIG. 9 (SEQ ID NO: 3). (See Genbank Accession No. NP_002067.1, which is incorporated herein in its entirety).

[0064] In some embodiments the GNS of the methods and kits described herein can be a polypeptide fragment including or derived from human full length GNS having the amino acid sequence of SEQ ID NO: 3. The polypeptide fragment refers to fragment of the full length GNS of at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 or more consecutive amino acids of SEQ ID NO:3, that has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of GNS of SEQ ID NO: 3. In some embodiments the polypeptide fragment comprises the amino acid FEPFFMMIATPAPH (SEQ ID NO: 2). The immunological reactivity can be determined by T cell reactivity or B cell response to GNS and can be assayed by the skilled practitioner by the assays described herein. Examples of assays for T cell reactivity include, without limitation, measurement of *in vitro* T cell proliferation, measurement of *in vitro* IFN- γ secretion, induced in PBMC or SFMC of the subject in response to GNS. Example of assays for B cell responses include, without limitation, detection and/or measurement of autoantibodies in a biological sample that specifically recognize and/or specifically bind to GNS.

[0065] The GNS or fragment thereof used can be human GNS. The polypeptide and encoding nucleic acid sequences of GNS and of other isoforms of human origin and those of a number of animals are publically available, e.g., from the NCBI website.

[0066] The GNS or fragment thereof used can be a mammalian homolog of human GNS or a polypeptide fragment thereof. In some embodiments, the GNS polypeptide has an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97% or at least 99% identical to the amino acid sequence of SEQ ID NO: 3 and has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of GNS of SEQ ID NO: 3. In some embodiments, the GNS polypeptide has an amino acid sequence that has at least 85%, at least 90%, at least 95%, at least 97% or at least 99% amino acid sequence homology to amino acid sequence of SEQ ID NO: 3 and has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of GNS of SEQ ID NO: 3. In some embodiments, the GNS is a polypeptide fragment of SEQ ID NO: 3 of at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 consecutive amino acids of SEQ ID NO: 3, that has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of GNS of SEQ ID NO: 3.

[0067] Percent (%) amino acid sequence identity for a given polypeptide sequence relative to a reference sequence is defined as the percentage of identical amino acid residues identified after aligning the two sequences and introducing gaps if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent (%) amino acid sequence homology for a given polypeptide sequence relative to a reference sequence is defined as the percentage of identical or strongly similar amino acid residues identified after aligning the two sequences and introducing gaps if necessary, to achieve the maximum percent homology. Non identities of amino acid sequences include conservative substitutions, deletions or additions that do not affect immunological reactivity of GNS or filamin a. Strongly similar amino acids can include, for example, conservative substitutions known in the art. Percent identity and/or homology can be calculated using alignment methods known in the art, for instance alignment of the sequences can be conducted using publicly available software such as BLAST, Align, ClustalW2. Those skilled in the art can determine the appropriate parameters for alignment, but the default parameters for BLAST are specifically contemplated.

[0068] Filamin A, alpha (filamin A) is an actin-binding protein that crosslinks actin filaments and links actin filaments to membrane glycoproteins. The encoded protein is involved in remodeling the cytoskeleton to effect changes in cell shape and migration. This protein interacts with integrins, transmembrane receptor complexes, and second messengers. The filamin A of the methods and kits disclosed herein can be full length filamin A protein, a polypeptide fragment thereof, a derivative thereof or an antigenic portion thereof. The filamin A or fragment thereof used can be human filamin A.

[0069] The filamin A polypeptide can be mammalian filamin A. The filamin A can also be an isoform of the full length filamin A or a polypeptide fragment thereof. In some embodiments, the filamin A of the

methods and kits disclosed herein can be derived from human filamin A having the amino acid sequence shown in **FIG. 10** (SEQ ID NO: 4). See Genbank Accession No. NP_001104026.1, which is incorporated herein in its entirety).

[0070] In some embodiments the filamin A of the methods and kits described herein can be a polypeptide fragment including or derived from human full length filamin A having the amino acid sequence of SEQ ID NO: 4. The polypeptide fragment refers to fragment of the full length filamin A of at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 consecutive amino acids of SEQ ID NO: 4, that has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of filamin A of SEQ ID NO: 4. In some embodiments the polypeptide fragment comprises the amino acid sequence, NPAEFVVNTSNAGAG (SEQ ID NO: 1). The immunological reactivity can be determined by T cell reactivity or B cell response to filamin A by the skilled practitioner by assays known in the art, some of which are described herein. Examples of assays for T cell reactivity include, without limitation, measurement of *in vitro* T cell proliferation, measurement of *in vitro* IFN- γ secretion, induced in PBMC or SFMC of the subject in response to filamin A (e.g., using an immunogenic polypeptide fragment). Examples of assays for B cell responses include, without limitation, detection and/or measurement of autoantibodies in a biological sample that specifically recognize and/or specifically bind to filamin A. Examples of assays for T cell reactivity include, without limitation, measurement of T cell proliferation and IFN- γ secretion by PBMC or SFMC of the subject by filamin A *in vitro*.

[0071] In some embodiments, human filamin A or a polypeptide fragment thereof is used. The polypeptide and coding nucleic acid sequences of filamin A and of other isoforms of human origin and those of a number of animals are publically available, e.g., from the NCBI website and are well known in the art.

[0072] In some embodiments, the filamin A of the methods and kits described herein is a mammalian homolog of human filamin A or a polypeptide fragment thereof. In some embodiments, the filamin A polypeptide has an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97% or at least 99% identical to the amino acid sequence of SEQ ID NO: 4 and has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of filamin A of SEQ ID NO: 4. In some embodiments, the filamin A polypeptide has an amino acid sequence that has at least 85%, at least 90%, at least 95%, at least 97% or at least 99% amino acid sequence homology to amino acid sequence of SEQ ID NO: 4 and has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of filamin A of SEQ ID NO: 4. In some embodiments, the filamin A is a functional fragment of SEQ ID NO: 4 of at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20 consecutive

amino acids of SEQ ID NO: 4, that has at least about 70%, 80%, 90%, 100% or more than 100% of the immunological reactivity of filamin A of SEQ ID NO: 4.

[0073] The entire full length protein or protein fragments can be used in the assays described herein. Combinations of full length protein and protein fragments can also be used, as can combinations of proteins/fragments that represent the different autoantigens (e.g., testing for one or more of GNS and filamin A reactivity in the same assay, or side by side in the same patient). The appropriate polypeptide or fragment thereof for use in the specific method can be determined by the skilled practitioner. The proteins or fragments can be recombinant, purified, isolated, naturally occurring or synthetically produced. The term “recombinant” when used in reference to a nucleic acid, protein, cell or a vector indicates that the nucleic acid, protein, vector or cell containing them have been modified by introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or a protein, or that the cell is derived from a cell so modified. The term “heterologous” (meaning ‘derived from a different organism’) refers to the fact that often the transferred protein was initially derived from a different cell type or a different species from the recipient. Typically the protein itself is not transferred, but instead the genetic material coding for the protein (often the complementary DNA or cDNA) is added to the recipient cell. Methods of generating and isolating recombinant polypeptides are known to those skilled in the art and can be performed using routine techniques in the field of recombinant genetics and protein expression. For standard recombinant methods, see Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY (1989); Deutscher, Methods in Enzymology 182:83-9(1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, NY (1982).

[0074] Either the full length protein, or protein fragments derived from the full length protein, can be used to identify immunoreactivity in a subject to the proteins disclosed herein. The specific molecules used in the assays described herein (e.g., full length protein(s), representative fragments thereof, or a combination of the full length and fragments) are referred to as the “test antigen” in the assay descriptions. Which fragments of a full length protein to use in the assay can be determined by the skilled practitioner. Typically, a cocktail of fragments derived from the same protein are used for determination of reactivity of a subject to that protein. Depending upon the length, the number of different protein fragments can be determined and optimized by the skilled practitioner. In one embodiment, 2 or more protein fragments are used. In one embodiment, 3 or more, 4, 5, 6, 7, 8, 9, or 10 or more protein fragments are used. In one embodiment, a protein fragment is at least 8 amino acids in length (e.g., ≥ 8 , ≥ 9 , ≥ 10 , ≥ 11 , ≥ 12 , ≥ 13 , ≥ 14 , ≥ 15 amino acids).

[0075] Specific protein fragments can be identified for use in the herein described methods such as those predicted for presentation by HLA-DR. Such protein fragment can be identified by the methods described in the examples and also by a variety of software programs available to the skilled practitioner

(Lin et al. *BMC Immunology* 2008, 9:8). In one embodiment, TEPITOPE 2000 (Sturniolo et al. 1999 *Nature Biotechnology*. 17; 555-561) is used. Other freely accessible online programs offered by the Immune Epitope Database and Analysis Resource (<http://www.iedb.org/>) are also available.

[0076] Examples of specific protein fragments of filamin A include, without limitation NPAEFVVNTSNAGAG (SEQ ID NO: 1).

[0077] Examples of specific protein fragments of GNS include, without limitation FEPFFMMIATPAPH (SEQ ID NO: 2).

[0078] Reactivity to one or both of GNS and filamin A can be assayed in a single subject. In one embodiment, reactivity to both GNS and filamin A are assayed in a subject. In one embodiment, reactivity in a subject to other suspected autoantigens is also performed in combination with the herein described assays. One such autoantigen is cyclic citrullinated peptide (CCP). Several assays for detecting anti-citrullinated protein antibody (ACPAs) are known in the art employing mutated citrullinated Vimentin (MCV-assay), filaggrin-derived peptides (CCP-assay) and viral citrullinated peptides (VCP-assay). Methods for determination of reactivity of a subject for anti-CCP and/or other markers for disease diagnosis are known in the art. Non limiting examples include WO 2005/085858 discloses a method of assessing RA by measuring CCP and serum amyloid A (SAA). WO 2005/064307 and US 2007/0264673 assess RA by measuring CCP and IL-6. WO 2005/029091 and US 2006/094056 provide methods to diagnose, treat, or evaluate inflammatory/autoimmune diseases such as RA by sampling fluids from a human with a suspected diagnosis by detecting CCP. US 2007/0148704 and WO 2007/039280 disclose use of CCP and antibodies as biomarkers in diagnosing RA. WO 2006/008183 discloses various biomarkers for RA.

Symptoms of RA

[0079] A subject suspected of having rheumatoid arthritis can be tested by the methods described herein. The subject may exhibit one or more symptoms of rheumatoid arthritis prior to performance of the methods. Rheumatoid arthritis (RA) is an autoimmune disease that causes the body's immune system to attack joint tissues, which leads to inflammation of joint lining. Inflammation of the joints is the primary manifestation of RA. Early symptoms of RA include affected joints being swollen, tender, warm, painful and stiff especially in the morning. Morning stiffness may last for at least an hour. The pain associated with RA is induced at the site of inflammation. Non limiting examples of other symptoms for RA include presence of rheumatoid nodules in the skin, fatigue, joint deformity and reduction in range of motion of affected joints, dryness of eyes and mouth. Early symptoms associated with RA are also seen in patients with other autoimmune related diseases such as lupus, or in other forms of arthritis, such as osteoarthritis, or in the pain syndrome, fibromyalgia. Accordingly, in some embodiments of the present

invention, the subject exhibits symptoms of RA and/or other autoimmune related disease manifestation known in the art e.g., Tenosynovitis, osteoporosis, carpal tunnel syndrome.

Diagnosis of Rheumatoid arthritis in a subject

[0080] There is no unique test or feature that is pathognomonic for RA. Rather, the diagnosis is made by recognizing a pattern of signs and symptoms. Classification criteria based on symptoms for identifying subjects suffering to early stages of RA and therefore aiding in disease diagnosis are known in the art and are for example set forth in 1987 American College of Rheumatology (ACR) criteria (72) and recently 2010 critieria by ACR and the European League Against Rheumatism (EULAR) (73).

[0081] The diagnostic methods of RA includes X-ray, MRI and/or ultrasound imaging of the affected joints and blood/serological tests. Non limiting examples of blood/serology tests known in the art to be conducted for diagnosis of RA include measurements of ; Erythrocyte Sedimentation Rate (ESR), Rheumatoid factor (RF), Anti-cyclic Citrullinated Peptide (anti-CCP), Antinuclear Antibody (ANA), Uric Acid, complete blood count: measures the numbers of red and white cells in your blood, C-reactive protein (CRP). In RA, 10 HLA-DR alleles, including the HLA-DRB1*0101 and 0401 alleles (6, 7) convey the greatest risk. Accordingly, in some embodiments, the subject has been or is further tested for one or more of rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), and one or more of HLA-DR alleles. Non-limiting examples of HLA-DR alleles that can be tested for include HLA-DRB1*0101, HLA-DRB1*0401.

[0082] In some embodiments, identification of immunoreactivity to one or more of the proteins described herein can be used in diagnosis of RA. In some embodiments, the methods disclosed herein can be used to identify a patient with RA. In some embodiments, the methods disclosed herein can be used for differential diagnosis of RA. Immunoreactivity of a subject to one or more of the proteins can determined *in vitro* using suitable biological samples disclosed herein by a variety of methods available to the skilled practitioner.

[0083] The discoveries presented herein indicate that reactivity to GNS and filamin A can be used as a biomarker for the diagnosis of a condition (e.g., RA and associated symptoms). More specifically, subject immunoreactivity (e.g., autoantigen reactive T cells, and/or anti-autoantigen antibodies) is indicative of the condition. As such, another aspect of the invention relates to a method of diagnosing the condition in a subject (e.g., RA) by determining whether the subject is immunologically reactive with one or more of GNS or filamin A by the herein described methods. The determination may be made in conjunction with the presence of other such symptoms of the condition present in the subject, for example, arthritic symptoms in a subject, or neurologic symptoms in a subject, or cardiac symptoms in a subject. The determination of immunological reactivity of a subject, as compared to an appropriate

control, indicates the subject has, or is likely to develop, the condition. The condition can be further confirmed by the determination of immunological reactivity to additional proteins such as antibodies to citrullinated proteins (ACPA), antinuclear antibody (ANA), anti-neutrophil cytoplasmic antibodies (ANCA) and presence of rheumatoid factors and other diagnostic tests known to those skilled in the art and described above.

[0084] Various assays can be used to identify antibodies present in a serum sample that bind the test antigen (e.g., ELISA, agglutination test, direct immunofluorescence assay, indirect immunofluorescence assay, western blot, an immunoblot assay). For example, the assay could be an immunoblot that carries a recombinant test antigen (full length or peptide fragment(s)).

[0085] The methods described herein can also include determination of rheumatoid factor, ACPA and/or ANA. For example, the test antigens disclosed herein can be included in or used in conjunction with an assay such as the Sure-Vue® RF (Fishersci), RFscan™ Card Test Kit (BD Biosciences), Anti-Rheumatoid Factor IgM ELISA Kit (abcam), AVITEX® -RF (Omega diagnostics) Citrullinated Protein Antibodies IgG ELISA Kit (Omega diagnostics) Anti-CCP EIA (Bio-Rad), BioPlex® 2200 Anti-CCP (Bio-Rad), CCPPoint® (Eurodiagnostica), DIASTAT® anti-nuclear antibody (ANA) test (Eurodiagnostica), DIASTATR PR3-ANCA® (Eurodiagnostica). The protein Filamin A or GNS or fragments thereof may be included in such a kit.

[0086] Test-antigen reactive T cells in PBMC and SFMC can be assessed using a number of assays. For example, reactive T cells in PBMC and SFMC can be assessed using tetramer reagents comprising recombinant HLA-DR molecules and test antigen epitopes.

[0087] The test antigen may comprise naturally occurring or analog or derivative amino acids, as long as the immunoreactive or immunostimulatory nature of the peptide is retained to sufficient degree to allow T cell activation and/or antibody binding. Thus, some amino acids may be added to or subtracted from the native protein or polypeptide fragments as known in the art. Additionally, some amino acids of the native human protein or polypeptide fragments may be substituted with amino acids that occur in other species, or be substituted as known in the art. Amino acid substitution exchange groups and empirical similarities between amino acid residues, can be found in standard texts such as Schulz et al., Principles of Protein Structure, 14-16 (Springer-Verlag, New York, 1979). There is a limit to how much substitution can be tolerated before the original tertiary structure is lost. Typically, tertiary structure conservation would be lost when the amino acid sequence varies by more than 50%. See, e.g., Chothia & Lesk, *Relation between the divergence of sequence & structure in proteins*, 5 EMBO J. 823 (1986). Guidance concerning which amino acid changes are likely to be phenotypically silent is found in Bowie et al., 247 Science 1306 (1990). Amino acids that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis. Cunningham et al.,

244 Science 1081 (1989). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as antibody binding and/or T cell stimulation. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallography, nuclear magnetic resonance, or photoaffinity labeling. Smith et al., 224 J. Mol. Biol. 899 (1992); de Vos et al., 255 Science 306 (1992).

[0088] The genes and encoded proteins of the autoantigens described herein have been sequenced and are available at numerous sources. Further, the genes are conserved in human, chimpanzee, rat, and zebrafish. Hence, these peptides can include those derived from non-human sources or appropriate sequence information. In an aspect of the invention, the test peptide is predicted to be presented by HLA-DR molecules associated with chronic inflammatory arthritis (e.g., RA).

[0089] As noted above, generally, amino acid substitutions should be made conservatively; i.e., a substitute amino acid should replace an amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Variants within the scope of this invention may also, or alternatively, contain other modifications, including the deletion or addition of amino acids, that have minimal influence on the stimulatory properties, antibody binding, tertiary structure of the peptide. Thus, for example, conservative substitutions in a protein fragment can be made with the proviso that functional activity is retained to a meaningful degree such that the particular assay (e.g., T cell reactivity or immunoassay) works as intended to provide evidence in diagnosing subjects (e.g., with RA).

[0090] Moreover, peptides often contain amino acids other than the twenty “naturally occurring” amino acids. Further, many amino acids, including the terminal amino acids, may be modified by natural processes, such as processing and other post-translational modifications, or by chemical modification techniques well known in the art. Known modifications include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0091] Such modifications are well known to those of skill in the art and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as Creighton, PROTEINS - STRUCTURE & MOLECULAR PROPERTIES (2nd ed., W.H. Freeman & Co., New York, 1993). Many detailed reviews are available on this subject, such as by Wold, POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, 1-12 (Johnson, ed., Academic Press, New York, 1983); Seifter et al. 182 Meth. Enzymol. 626 (1990); Rattan et al., 663 Ann. N.Y. Acad. Sci. 48 (1992). Accordingly, the peptides of the present invention also encompass derivatives or analogs in which a substituted amino acid residue is not one encoded by the genetic code.

[0092] Further, "derivatives" of a test antigen contain additional chemical moieties not normally a part of the protein. Covalent modifications of the autoantigens are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. For example, derivatization with bifunctional agents, well-known in the art, is useful for cross-linking the antibody or fragment to a water-insoluble support matrix or to other macromolecular carriers. Derivatives also include radioactively labeled peptides that are labeled, for example, with radioactive iodine (125I, 131I), carbon (14C), sulfur (35S), tritium (3H) or the like; conjugates of peptides with biotin or avidin, with enzymes, such as horseradish peroxidase, alkaline phosphatase, β -D-galactosidase, glucose oxidase, glucoamylase, carboxylic acid anhydride, acetylcholine esterase, lysozyme, malate dehydrogenase or glucose 6-phosphate dehydrogenase; and also conjugates of monoclonal antibodies with bioluminescent agents (such as luciferase), chemoluminescent agents (such as acridine esters), or fluorescent agents (such as phycobiliproteins).

[0093] Structural analogs of the autoantigens identified herein are provided by known method steps based on the teaching and guidance presented herein. Knowledge of the three-dimensional structures of proteins is crucial in understanding how they function. The three-dimensional structures of hundreds of proteins are currently available in protein structure databases (in contrast to the thousands of known protein sequences in sequence databases). Analysis of these structures shows that they fall into recognizable classes of motifs. It is thus possible to model a three-dimensional structure of a protein based on the protein's homology to a related protein of known structure. Many examples are known where two proteins that have relatively low sequence homology, can have very similar three dimensional structures or motifs.

[0094] It is possible to determine the three dimensional structures of proteins of up to about 15 kDa by nuclear magnetic resonance (NMR). The technique only requires a concentrated solution of pure protein.

No crystals or isomorphous derivatives are needed. The structures of a number of proteins have been determined by this method. The details of NMR structure determination are well-known in the art. See, e.g., Wuthrich, NMR of Proteins & Nucleic Acids (Wiley, N.Y., 1986); Wuthrich, 243 Science 45 (1989); Clore et al., 24 Crit. Rev. Bioch. Molec. Biol. 479 (1989); Cooke et al., 8 Bioassays 52 (1988).

[0095] Thus, according to the present invention, use of NMR spectroscopic data can be combined with computer modeling to arrive at structural analogs of at least portions of the autoantigen (peptides or epitopes) based on a structural understanding of the topography. Using this information, one of ordinary skill in the art can achieve structural analogs of the autoantigens such as by rationally-based amino acid substitutions allowing the production of peptides in which the binding affinity or avidity is modulated in accordance with the requirements of the expected diagnostic use of the molecule, for example, the achievement of greater binding specificity or affinity.

[0096] The herein described methods may further be used to indicate a therapy for the subject who tests positive. The identification of GNS and/or filamin A for a biomarker in RA, as provided herein, is an important addition to the clinician's arsenal in combating chronic inflammatory arthritis, and assists the clinician in choosing the course of therapy. For example, when RA is diagnosed, nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g., ibuprofen, naproxen sodium) or steroids (e.g., corticosteroid medication such as prednisone); or disease modifying anti-rheumatic drugs (DMARDs), such as hydroxychloroquine, sulfasalazine or methotrexate, may be prescribed. Additionally or alternatively, anti-TNF therapy (e.g., HUMIRA® (adalimumab), ENBREL® (etanercept), may be beneficial. Other non-limiting examples include Orencia® (abatacept), Kineret® (anakinra), certolizumab® (Cimzia), Simponi® (golimumab), Remicade® (infliximab), Actemra® (tocilizumab) and Xelijanz® (tofacitinib). It is also contemplated that GNS and/or filamin A-specific therapy, such as targeting GNS and/or filamin A e.g., GNS and/or filamin A -binding antibodies, may also be beneficial. In one embodiment, the subject is provided one or more therapies following an indication that they are immunoreactive to an autoantigen. As such, another aspect of the invention relate to a method of treating a subject diagnosed with a RA. The method comprises diagnosing the subject for the disorder by one or more of the herein described methods, and then treating the subject for said condition. Diagnosis may further include assessing the subject for other symptoms such as a neurological condition or heart condition typically seen with secondary RA symptoms. In some embodiments of the present invention, the methods to determine immunoreactivity to autoantigens disclosed herein can be conducted prior to, during and/or after a therapeutic treatment and therefore aid in assessment of effectiveness of the treatment and/or monitor disease progression.

[0097] Another aspect of the invention relates to a kit for identifying a subject with a condition such as chronic inflammatory arthritis. The kit comprises one or more of GNS, filamin A, or a portion or

fragment thereof, such as a set of synthesized peptides, fragments, or epitopes thereof, and reagents necessary for conducting an assay capable of detecting the presence of immunoreactivity of a subject to that test antigen (e.g., an antibody in a sample obtained from said subject that binds to GNS and/or filamin A). The kit can be designed for any of the various assays described herein. In one embodiment, the assay in the kit is an enzyme-linked immunosorbent assay (ELISA) or immunoblot, the components for which are well-known in the art. The peptides may be synthesized or obtained from natural or recombinant sources, each of which is well-known in the art. The kit may further include other autoantigens known in the art, such as those described herein. (e.g., citrullinated protein/peptides such as citrullinated filaggrin, fibrinogen, fibronectin, α -enolase, collagen type II, histones, vimentin or fragments/epitopes thereof). The kit may alternately further comprise buffers, enzymes, and/or containers for performing the reactions or analyses. The various reagents within the kit may be provided separately or together as is convenient in a container such as a vial, test tube, flask, bottle or even syringe. The components may be suitably aliquotted for performance of the methods. The kit may further contain one or more positive and/or negative controls. The antigens or other components of the kit may be labeled with a detectable marker.

[0098] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0099] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[00100] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used to describe the present invention, in connection with percentages means $\pm 1\%$.

[00101] In one respect, the present invention relates to the herein described compositions, methods, and respective component(s) thereof, as essential to the invention, yet open to the inclusion of unspecified elements, essential or not (“comprising”). In some embodiments, other elements to be included in the description of the composition, method or respective component thereof are limited to those that do not materially affect the basic and novel characteristic(s) of the invention (“consisting essentially of”). This applies equally to steps within a described method as well as compositions and components therein. In other embodiments, the inventions, compositions, methods, and respective components thereof, described

herein are intended to be exclusive of any element not deemed an essential element to the component, composition or method (“consisting of”).

[00102] All patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00103] Embodiments of various aspects described herein can be defined in any of the following numbered paragraphs:

1. A method of diagnosing rheumatoid arthritis in a subject comprising determining whether the subject is immunologically reactive with one or more of filamin-A and N-acetylglucosamine-6-sulfatase, wherein immunological reactivity of the subject to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, as compared to an appropriate control, indicates the subject has rheumatoid arthritis.
2. The method of paragraph 1, wherein determining is by evaluating a biological sample obtained from the subject for immunological reactivity with the one or more of filamin-A and N-acetylglucosamine-6-sulfatase.
3. The method of any one of paragraphs 1-2, wherein determining immunological reactivity is by detecting the presence of T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, comprising the steps:
 - a) stimulating peripheral blood mononuclear cells (PBMC) of the subject or the synovial fluid mononuclear cells (SFMC) of the subject *in vitro* with one or more of filamin-A and N-acetylglucosamine-6-sulfatase, whole protein or polypeptide fragments;
 - b) measuring T cell proliferation *in vitro* or secretion of IFN- γ into cell culture supernatants; and
 - c) identifying the subject as having T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when T cell proliferation or secretion of IFN- γ is measured as significantly increased over that of an appropriate control.

4. The method of paragraph 3, wherein step b) is measuring T cell proliferation *in vitro*, and the subject is identified as having T cell reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when T cells proliferation is measured as significantly increased over that of an appropriate control.

5. The method of paragraph 3, wherein step b) is measuring T cell secretion of IFN- γ into cell culture supernatants, and the subject is identified as having T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when secretion of IFN- γ is measured as significantly increased over that of an appropriate control.

6. The method of any one of paragraphs 1-5, wherein determining immunological reactivity comprises determining if the subject has a B-cell response to one or more of filamin-A and N-acetylglucosamine-6-sulfatase resulting in the production of autoantibodies that specifically recognize the one or more filamin-A and N-acetylglucosamine-6-sulfatase, by contacting the sample with filamin-A protein or a polypeptide fragment thereof and/or N-acetylglucosamine-6-sulfatase protein or a polypeptide fragment thereof, under conditions that allow an immunocomplex of the antibody and the filamin-A or N-acetylglucosamine-6-sulfatase form, and detecting the presence or absence of an immunocomplex, wherein the presence of an immunocomplex indicates the subject presents a B-cell response to filamin-A and/or N-acetylglucosamine-6-sulfatase and wherein the absence of an immunocomplex indicates the subject fails to present a B-cell response to filamin-A and/or N-acetylglucosamine-6-sulfatase.

7. The method of paragraph 6, wherein the assay is an enzyme-linked immunosorbent assay (ELISA), agglutination test, direct immunofluorescence assay, indirect immunofluorescence assay, or an immunoblot assay.

8. The method of any one of paragraphs 3-7, wherein the polypeptide fragment of filamin-A comprises the amino acid sequence NPAEFVVNTSNAGAG (SEQ ID NO: 1) or an antigenic portion thereof.

9. The method of any one of paragraphs 3-8, wherein the polypeptide fragment of N-acetylglucosamine-6-sulfatase comprises the amino acid sequence of FEPFFMMIATPAPH (SEQ ID NO: 2) or an antigenic portion thereof.

10. The method of any one of paragraphs 1-9 wherein the subject has been or is further tested for one or more of rheumatoid factor, anti-citrullinated protein antibodies (ACPA), and one or more HLA-DR alleles.
11. The method of paragraph 10, wherein the HLA-DR allele is HLA-DRB1*0101 and/or HLA-DRB1*0401.
12. The method of any one of paragraphs 1-11, wherein the subject is at risk for, or is suspected of having, rheumatoid arthritis.
13. The method of any one of paragraphs 1-12, further comprising the step of treating the subject with one or more of a nonsteroidal anti-inflammatory drug (NSAIDs), a steroid, a disease modifying anti-rheumatic drug (DMARD), and a biologic.
14. A kit comprising, one or more potential antigen and/or potential epitope of filamin-A and/or N-acetylglucosamine-6-sulfatase, and reagents for conducting an assay for detecting the presence of an antibody in a sample that binds to the one or more potential antigen and/or potential epitope of the filamin-A and N-acetylglucosamine-6-sulfatase.
15. The kit of paragraph 14, wherein the potential antigen and/or potential epitope of filamin-A comprises the amino acid sequence NPAEFVVNTSNAGAG (SEQ ID NO: 1) or an antigenic portion thereof.
16. The kit of paragraph 14, wherein the potential antigen and/or potential epitope of N-acetylglucosamine-6-sulfatase comprises the amino acid sequence FEPFFMMIATPAPH (SEQ ID NO: 2) or an antigenic portion thereof.
17. The kit of any one of paragraphs 14-16, wherein the assay is an enzyme-linked immunosorbent assay (ELISA).
18. The kit of any one of paragraphs 14-16, wherein the assay is a western blot.
19. The kit or method of any one of paragraphs 1-18 for use in identifying a subject with rheumatoid arthritis.

[00104] The invention is further illustrated by the following examples, which should not be construed as further limiting.

EXAMPLES

[00105] The following examples illustrate some embodiments and aspects of the invention. It will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be performed without altering the spirit or scope of the invention, and such modifications and variations are encompassed within the scope of the invention as defined in the claims which follow. The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

Example 1

Background

[00106] Identification of autoantigens has been a great challenge in autoimmune diseases such as rheumatoid arthritis (RA). To address this issue, a new method that combines discovery-based proteomics to identify HLA-DR-presented peptides in patients' synovial tissues, joint fluid or peripheral blood (PB) and translational research to determine the immunogenicity of the identified peptides and their source proteins is described herein. With this approach, endothelial cell growth factor was recently identified as a target of T and B cell responses in Lyme arthritis (LA) (22) and annexin A2 as such a target in both LA and RA (26).

Objectives

[00107] To identify novel disease-associated autoantigens which induce T and B cell responses in patients with RA.

Methods

[00108] HLA-DR-presented self-peptides were isolated and identified from RA patients' tissue or fluids by tandem mass spectrometry, synthesized and tested for T cell autoreactivity with the matching patient's peripheral blood mononuclear cells (PBMC). Immunoreactive peptides or their source proteins were then tested for T cell autoreactivity by IFN- γ ELISpot assay and for autoantibody responses by ELISA using cells and sera from our large cohort of RA patients and control subjects. All RA patients met the 2010 ACR/EULAR criteria for RA.

Results

[00109] In the RA patient, 1 of 89 HLA-DR-presented peptides identified from her synovial tissue and 1 of 15 HLA-DR-presented peptides identified from her PB induced her PBMC to secrete IFN- γ . These reactive peptides were derived from the proteins N-acetylglucosamine-6-sulfatase (GNS), a lysosomal enzyme, and from filamin A (filamin A), an actin-binding protein, respectively. It was found that 8 of 25 RA patients (32%) had T cell reactivity with GNS that was >3 SD above the mean value of 10 healthy controls (HC) ($p=0.004$), and 13 of the 25 patients (52%) had T cell reactivity to filamin A ($p=0.002$). In addition, 35% of 92 RA patients had elevated autoantibody responses to GNS that were >3 SD above the mean value of 50 HC ($p<0.0001$), and 32% of the patients had high autoantibody responses to filamin A ($p<0.0001$).

[00110] Of the 92 patients, 52 (56%) had a response to one of these autoantigens, and 10 (11%), including the case patient, had reactivity with both autoantigens. Compared with patients with RA, none of 94 patients with other rheumatic diseases, including systemic lupus (SLE), psoriatic arthritis, spondyloarthritis, Lyme arthritis, or osteoarthritis had B cell reactivity with GNS (in each instance, $p<0.0001$), and only 3 patients with SLE ($p=0.003$) and 1 with psoriatic arthritis ($p<0.0001$) had responses to filamin A that were slightly above the cut-off value.

Conclusions

[00111] This approach is an effective way to identify novel autoantigens associated with autoimmune forms of arthritis. With this method, two previously unrecognized autoantigens were identified, each of which is a target of T and B cell responses in about 1/3 of patients with RA, but rarely in those with other types of arthritis. These findings suggest that testing for these newly recognized autoantibodies may have diagnostic utility in RA.

Example 2

[00112] With the advent of highly sensitive, nanoflow LC-MS/MS-based peptide sequencing (31), it has become possible to identify peptides presented by HLA-DR molecules in individual patients' tissues or cells. In 1992, Hunt et al. were the first to use LC-MS/MS to identify class I major histocompatibility complex (MHC) molecules in tumor tissue culture systems (32). They have continued to lead this rapidly growing field, and the identification of MHC-presented peptides has been widely undertaken primarily using cell lines (33-41). Moreover, advances in mass spectrometry techniques and bioinformatics have improved the efficiency and accuracy for identification of MHC-presented molecules (42, 43).

[00113] As a next step, a few laboratories have undertaken the direct analysis of class II HLADR-presented peptides in clinical samples of mixed cell lines (44-49). In previous study, 57-104 non-redundant, HLA-DR-presented self-peptides per patient from the ST of four patients, two with RA and two with LA were identified (30). However, large amounts of synovial tissue can only be obtained when patients undergo surgical procedures, such as synovectomy or joint replacement, and this limits the analysis to patients at the severe end of the spectrum, usually seen late in the illness.

[00114] In present study, the number of patients studied were increased to thirteen, five with RA and eight with LA. Moreover, it was demonstrated that HLA-DR-presented peptides could be identified in individual patients - not only from ST, but also from synovial fluid mononuclear cells (SFMC) and peripheral blood mononuclear cells (PBMC), which allow the analysis not only of a greater number of patients, but also of patients seen earlier in the illness. In addition, the increasing sensitivity of new mass spectrometers and improvements in computer programs for spectrum-to-peptide matches resulted in the identification of greater numbers of HLA-DR-presented peptides from each sample. In the current study, 18 new ST, PBMC and SFMC samples were analyzed and previous LC-MS/MS data were re-analyzed to compare with new data from SFMC and PBMC samples.

[00115] Altogether, from 22 samples that originated from 13 patients, we identified 1,593 nonredundant HLA-DR-presented, self-peptides derived from 870 source proteins. This experience shows that LC-MS/MS now has the sensitivity and specificity to identify large numbers of HLADR- presented self-peptides in tissues and fluids with mixed cell populations, obtained from individual patients. After the peptides were synthesized and tested with the matching patient's PBMC, it was determined that among these peptides were novel immunogenic T cell epitopes derived from the source proteins endothelial cell growth factor (ECGF) (22) apolipoprotein B-100 (apoB-100) (23), matrix metalloproteinase-10 (MMP-10) (24) in LA; from annexin A2 in both RA and LA (25) and from *N*-acetylglucosamine-6-sulfatase and filamin A in RA (26). Furthermore, it was shown that these epitopes or their source proteins were targets of T and B cell responses in many patients (22-26).

Results

[00116] Patients — All five patients with RA were women, ages 37 to 70 (Table 1), a distribution which reflects the gender bias associated with RA. Three of the five patients had classic, seropositive RA. They had the HLA-DRB1*0101 or 0401 alleles, which code for the RA shared epitope; these three patients had ACPA and two had rheumatoid factor (RF). The remaining two RA patients had chronic seronegative RA. They did not have shared epitope alleles, and their tests were negative for ACPA and RF. In four

patients, ST from a hip or knee was obtained when arthroscopic synovectomy or joint replacement surgery was performed three to 66 years after disease onset. For one patient (RA2), samples were available from all three sites (ST, SFMC and PBMC), and, for patient RA4, SFMC and PBMC samples (but not ST) were collected at the time of diagnosis, prior to therapy with disease modifying anti-rheumatic drugs (DMARDs).

[00117] The eight patients with antibiotic-refractory LA included four teenage boys, three adult men and one woman (Table 1). Four of the eight patients had the HLA-DR*0101, 0401 or 1501 alleles, which are known to be increased in frequency in patients with refractory LA (58). All eight patients were treated with \geq two months of oral antibiotics, seven also received \geq one month of IV antibiotics, and seven subsequently received methotrexate. Due to incomplete resolution of synovitis, the eight patients underwent arthroscopic synovectomies of an affected knee six months to four years after the onset of arthritis, and ST samples were obtained during the surgeries. Additionally, SFMC samples were available from four patients and PBMC from two patients.

[00118] **Table 1: Clinical characteristics, number of MS/MS spectra, peptide matches, decoy hits, non-redundant peptides, and source proteins identified from each patient's HLA-DR molecules in synovial tissue, synovial fluid or peripheral blood.**

Patient	Rheumatoid Arthritis						Lyme Arthritis						
	R41	R42	R43	S44	R45	L41	L42	L43	L44	L45	L46	L47	L48
Gender	F	F	F	F	F	M	M	M	M	F	M	M	M
Age (years)	70	57	45	63	68	72	48	18	58	51	18	56	55
Arthritis Duration (years)	66	5	7	1	3	1.5	1.5	0.5	1.5	4	0.35	0.85	0.75
Anti-citrylinated protein antibodies	neg	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg
Rheumatoid Factor	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
HLA-DR ⁺ ES genotype	0402/1104	0305/0401	0301/0302	0301/0302	1003/0301	0301/1101	0301/1101	0301/1101	0303/0301	0303/0301	0403/1101	0302/1101	0302/1101
Synovial tissue, No. of													
MS/MS spectra	1647 ¹	1717 ²	5487 ⁴⁰	10220 ²		10275 ³	2237 ⁵	1684 ³	9837 ³	11940 ²	14141 ²	5892 ³	9195 ³
spectrum-to-peptide matches	1021	1223	337	2538		1852	1682	986	4516	2521	2453	821	4445
consensus matched peptides	248	388	107	471		143	282	185	481	215	389	88	312
Decoy hits	5	9	0	1		0	0	0	0	0	0	0	0
consensus non-decoy non-redundant peptides	95	92	61	372		68	97	57	167	135	135	36	84
peptides tested for immune responses identified by only one program	18	8					12	2	10	18	12		
nonimmunogenic peptides	1	2					NPD	1	0	1	1	NPD	NPD
Source proteins	80	78	45	126		51	79	41	126	108	109	38	70
Synovial fluid mononuclear cells, No. of													
MS/MS spectra	10979 ²	5326 ³		55189 ²			16248 ²			13030 ²		1606 ³	5948 ⁴
spectrum-to-peptide matches	3581	252		1155			3594			1388		120	358
consensus matched peptides	30	51		68			2			357		8	113
Decoy hits	5	0		0			5			0		0	0
consensus non-decoy non-redundant peptides	15	52		32			2			61		5	57
peptides tested for immune responses identified by only one program	0	0		0			0			18			
nonimmunogenic peptides	0	0		0			0			0		NPD	NPD
Source proteins	14	23		32			2			89		5	48
Peripheral blood mononuclear cells, No. of													
MS/MS spectra	20931 ²	8227 ⁴		12060 ³						25146 ²	3518 ⁴		
spectrum-to-peptide matches	1897	372		1228						1321	181		
consensus matched peptides	22	60		7						25	40		
Decoy hits	0	0		0						0	0		
consensus non-decoy non-redundant peptides	14	53		7						31	38		
peptides tested for immune responses identified by only one program	1	1		0						0	NPD		
nonimmunogenic peptides	11	24		7						0	11		

¹ MS/MS data acquired with a Sciex QSTAR mass spectrometer; ² Thermo LTQ-Orbitrap XL mass spectrometer; ³ Agilent 6530 Q-TOF mass spectrometer; and ⁴ Thermo Q Exactive plus mass spectrometer.

⁵ Results from a second time immune purification from the tissue cell lysate.

NPD: not available; NND: not yet done.

[00119] Number of Peptides and Source Proteins Identified from HLA-DR-Presented Self Peptides —

Overall, 1,647 to 40,390 MS/MS spectra were generated per sample from the *in vivo* HLA-DR-presented peptides isolated from the 22 samples of ST, SFMC or PBMC from the 13 patients (Table 1). Using three different MS/MS spectra search engines (Mascot, OMSSA, and X!Tandem), 161 to 29,993 spectrum-to-peptide matches were obtained per sample. The peptides identified by consensus match (i.e., \geq two search programs) are reported in Table 1. The spectra for these peptides were manually inspected and given priority for inclusion in immunological assays; this number ranged from 2 to 2,363 peptides per sample. As noted in Table 1, expert manual inspection of peptide MS/MS spectra assigned with high scores by only a single program led to the addition of 90 peptides to the inclusion list for immunological assays.

[00120] At this stringency level, 59 to 801 non-decoy, non-redundant HLA-DR-presented self-peptides were identified in the 12 individual ST samples; these peptides were derived from 42 to 493 different source proteins (Table 1). Using this same methodology for the six SFMC and four PBMC samples, 2 to 71 non-redundant peptides derived from 2 to 49 source proteins were identified per patient from SFMC,

and 7 to 34 non-redundant peptides derived from 7 to 24 source proteins were identified per patient from PBMC. The lengths of the MHC class II HLA-DR-presented peptides ranged from seven to 27 amino acids; the typical length of the peptides was 13-17 amino acids. Altogether, from all three sites, 1,593 non-redundant HLA-DR-presented, self-peptides were identified; these were derived from 870 source proteins.

[00121] In the translational component of the research, all non-redundant peptides were synthesized and tested for reactivity with the matching patient's PBMC. To date, this step has been completed for three patients with RA and five with LA (Table 1). One immunogenic HLA-DR-presented peptide was identified in ST from patient RA1, and one immunogenic peptide was identified in ST from patient RA2 (25, 26). In this patient, an additional peptide was also found in both ST and PBMC in three different analyses. Similarly, among the five LA patients, at least one unique immunogenic HLA-DR-presented peptide was identified in each of three patients (LA1, LA4, and LA5) (22-24). Four of the six immunogenic peptides were identified by consensus match, and two were identified by manual inspection of sequences identified by only one program.

[00122] Comparison of Results from Different LC-MS/MS Instruments in the Identification of HLA-DR-Presented Self Peptides — During the 7-year period of this study, newer high sensitivity LC-MS/MS instruments became available in the laboratory. Therefore, over the course of the study, HLA-DR-presented peptides samples were analyzed by four types of LC-MS/MS instruments (Table 2). The average numbers of unique-consensus peptides identified from LA or RA patients' ST was 239 using the QSTAR, 313 using the LTQ-Orbitrap XL, 571 using the 6550 Q-TOF, and 2,226 using the Q Exactive plus MS. The average numbers from patients' SFMC were 51 using the LTQ-Orbitrap XL, 6 using the 6550 Q-TOF, and 70 using the Q Exactive plus MS. From patients' PBMC, the average numbers were 15 using the LTQ-Orbitrap XL, and 50 using the Q Exactive plus MS. Thus, as the sensitivity and data handling capacity of the LC-MS/MS instruments improved during the study, the number of identified HLA-DR-presented peptides steadily increased.

[00123] Table 2: Comparison of the spectra generated and identified by the four different mass spectrometers.

	Synovial tissue	Synovial fluid	Peripheral blood
QSTAR	N=4*		
# MS/MS spectra†	1815		
# spectrum-to-peptide matches	1033		
# consensus matched peptides	239		
# Decoy hits	0		
# non-decoy non-redundant peptides	95		
# Source Proteins	70		
LTQ-Orbitrap XL	N=7	N=4	N=3
# MS/MS spectra	9756	16106	19379
# spectrum-to-peptide matches	2246	1670	1498
# consensus matched peptides	313	51	15
# Decoy hits	0	0	0
# non-decoy non-redundant peptides	121	31	11
# Source Proteins	93	24	9
6550 iFunnel Q-TOF	N=3	N=1	
# MS/MS spectra	19633	1000	
# spectrum-to-peptide matches	13106	120	
# consensus matched peptides	571	6	
# Decoy hits	3	0	
# non-decoy non-redundant peptides	203	5	
# Source Proteins	141	5	
Q Exactive plus	N=1	N=3	N=2
# MS/MS spectra	10936	6125	4922
# spectrum-to-peptide matches	9827	255	267
# consensus matched peptides	2226	70	50
# Decoy hits	3	0	0
# non-decoy non-redundant peptides	765	38	30
# Source Proteins	479	28	18

*Number of different samples analyzed. Only data for the first pass analysis are included.

†All numbers given indicate the average number of spectra identified based upon the number of samples analyzed.

[00124] Comparison of HLA-DR-presented Peptides and Source Protein Repertoires in ST, SFMC, and PBMC — The identified HLA-DR-presented peptides from ST of RA1, RA2, LA1, and LA2 using IPI database were showed in reference (30). Since the SFMC sample from LA2, and SFMC and PBMC samples from RA2 were analyzed in this study, the original data from the four previous samples, which were reanalyzed using UniProt database, were included herein for comparison of the HLA-DR-presented peptides repertoires found in the three sample sites now being analyzed. In one patient each with RA or LA (RA2 and LA5), samples were available from all three compartments (ST, SFMC and PBMC), and this allowed for the degree of overlap in peptide presentation among these three sites to be determined for each of these patients (FIG. 1A). For patient RA2, 53% of the HLA-DR-presented peptides identified in SFMC and 53% of those found in PBMC were shared with those in ST, but only 6% were identified at all three sites. Analysis of the source proteins from which the peptides were derived gave similar frequencies

(FIG. 1B). In comparison, patient LA5 had a smaller percentage of peptides shared between SFMC and ST (21%) than between PBMC and ST (36%), and the degree of overlap of source proteins between SFMC and ST (16%) was similar between PBMC and ST (22%). When the degree of overlap was compared between pairs of sample types available from the five RA patients or eight LA patients, a similar picture was found. In general, more than half of the peptides identified in SFMC were also found in ST (FIG. 1C). In RA, about half of the peptides identified in PBMC were also found in ST, whereas in LA, in which many more peptides were identified in ST, only about one-quarter of the PBMC peptides were also found in ST. There was a trend toward a lower percentage of source proteins than peptides being the same in SFMC or PBMC as in ST (FIG. 1D). Thus, in both diseases, HLA-DR-presented peptides in SFMC or PBMC provided some reflection of the peptides presented in ST, but also revealed additional peptides.

[00125] Source Proteins for HLA-DR-presented Peptides — The complete lists of consensus peptides were identified by at least two search engines from each site for each patient and the non-redundant HLA-DR-presented peptides and their source proteins were identified as described in methods. The most common source proteins of peptides from ST that were found in at least four patients, regardless of disease, are listed in alphabetical order in Table 3. These peptides (in order of frequency) were derived from cathepsin S, collagen α -1(I) chain, syntenin-1, serine carboxypeptidase CPVL (probable), albumin, pro-low-density lipoprotein receptor-related protein 1, apolipoprotein A2, complement C3, cytochrome c oxidase subunit 5B (mitochondrial), filamin-A, monocyte differentiation antigen CD14, and stromelysin-1. Since fewer SFMC and PBMC samples were analyzed, the list from these sites shows the source proteins identified in at least two patients rather than four patients. In SFMC, the list included keratin (type II cytoskeletal 1) and nuclease-sensitive element-binding protein 1. Keratin was commonly identified in both SFMC and PBMC. Because of the keratin type and the fact that the samples were not subjected to proteolysis during processing, it is unlikely that these peptides were sample-handling artifacts.

[00126] Table 3. The most abundant source proteins for the identified HLA-DR-presented peptides from three sites in RA and LA patients.

Synovial tissue*

Protein Name	Accession	RA#	LA#	Protein Name	Accession	RA#	LA#
Albumin	P02782	2	5	Filamin-A	P21333	2	4
Alpha-1B-glycoprotein	P04217	1	3	Fructose-bisphosphate aldolase A	P04075	2	2
Alpha-2-macroglobulin	P01023	2	3	Hemopexin	P02790	1	3
Alpha-endopeptidase	P06733	1	3	HLA class I histocompatibility antigen, A-2 alpha chain	P01892	2	2
Angiotensinogen	P01019	1	3	Inter-alpha-trypsin inhibitor heavy chain H4	Q14824	1	3
Annexin A2	P07355	1	3	Legumain	Q99536	1	3
Apolipoprotein A-II	P02852	3	3	Lysozyme-associated membrane glycoprotein 1	P11279	2	4
C4b-binding protein alpha chain	P04003	1	3	Macrophage mannose receptor 1	Q5VSK2	1	3
Cathepsin	P27797	1	3	Monocyte differentiation antigen CD14	P08571	2	4
Cathepsin S	P25774	3	5	Olfactomedin-like protein 2B	Q688L6	2	4
Caveolin-1	Q03135	1	3	Perilipin-3	Q68864	2	4
Chondroadherin	Q15335	1	3	Phosphatidylcholine-sterol acyltransferase	P04180	2	2
Coagulation factor XIII A chain	P00388	2	2	Phosphoglycerate kinase 1	P08556	3	1
Coffin-1	P23528	2	3	Plexin domain-containing protein 2	Q8UX71	3	2
Collagen alpha-1(I) chain	P02452	3	5	Profilin-1	P07737	2	3
Collagen alpha-1(III) chain	P12007	2	2	Protein-density lipoprotein receptor-related protein 1	Q07854	3	4
Collagen alpha-2(I) chain	P08123	2	3	Proteoglycan 4	Q92854	1	3
Collagen alpha-2(V) chain	P05897	2	3	Rho GDP-dissociation inhibitor 2	P52566	1	4
Complement C1q subcomponent subunit B	P02746	6	4	60S ribosomal protein L30	P62988	3	1
Complement C1s subcomponent	P09871	1	4	60S ribosomal protein L8	Q02878	2	2
Complement C3	P01024	3	3	Serine carboxypeptidase CPVL (probable)	Q8H325	3	4
Complement C5	P01031	1	4	SPARC-like protein 1	Q14515	2	4
C-reactive protein	P02741	2	3	Stablin-1	Q3NY15	2	3
Cytochrome c oxidase subunit 5B, mitochondrial	P10606	2	4	Stromelysin-1	P08254	2	4
Cytochrome c oxidase subunit 5A, mitochondrial	P10176	2	2	Syntenin-1	Q08560	2	6
Decorin	P07585	1	4	Talin-1	Q8Y490	2	5
Epididymal secretory protein E1	P01918	1	3	Vascular cell adhesion protein 1	P19020	2	4
ER-Golgi intermediate compartment protein 1	Q999X5	1	3	Vitronectin	P04024	2	3
Fibrinogen beta chain	P02875	3	2				

Synovial fluid†

Protein Name	Accession	RA#	LA#	Protein Name	Accession	RA#	LA#
Albumin	P02788	1	1	Keratin, type I cytoskeletal 9	P35537	1	1
Alpha-2-macroglobulin	P01023	1	1	Keratin, type II cytoskeletal 1	P04264	1	2
Annexin A6	P08133	1	1	Lysosomal alpha-mannosidase	Q00754	1	1
C4b-binding protein alpha chain	P04083	1	1	MHC class II antigen	P01889	1	1
Cathepsin S	P25774	1	1	Nuclease-sensitive element-binding protein 1	P04229	1	1
Coffin-1	P23528	1	1	Plasminogen	P67802	1	2
Complement C3	P01024	1	1	Plexin domain-containing protein 2	P00747	1	1
Fibrinogen alpha chain	P02671	1	1	Stromelysin-1	Q8LKK71	1	1
Ficolin-1	Q08602	0	2	T-cell surface glycoprotein CD4	P01730	1	1
Glutathione S-transferase P	P09211	1	1	Toll-like receptor 2	Q60603	1	1
Hemoglobin subunit alpha	P08985	1	1				
Histone H1	Q02539	0	2				

Peripheral Blood‡

Protein Name	Accession	RA#	LA#	Protein Name	Accession	RA#	LA#
Alpha-1-antitrypsin	P01009	0	2	Keratin, type I cytoskeletal 9	P35537	1	2
Inter-alpha-trypsin inhibitor heavy chain H4	Q14824	1	3	Keratin, type II cytoskeletal 1	P04264	1	1
Keratin, type I cytoskeletal 10	P13645	1	1	Nuclease-sensitive element-binding protein 1	P67802	1	1

The numbers in "RA#" and "LA#" columns indicate the number of patients from whom each source protein was identified.

*Source proteins identified in ST in at least four of the twelve patients.

†Source proteins identified in SF in at least two of six patients tested.

‡Source proteins identified in PB in at least two of four patients tested.

[00127] In most RA patients, the identified HLA-DR-presented peptides were derived from source proteins that are thought to serve as potential autoantigens in RA. These included cartilage glycoprotein 39, various types of collagen, enolase, fibrinogen, fibronectin, immunoglobulin, and vimentin (**Table 4**). Interestingly, HLA-DR-presented peptides from these source proteins were also commonly identified in patients with LA.

[00128] **Table 4: Source proteins found both in RA and LA that are known or proposed to serve as autoantigens in RA***

	Rheumatoid Arthritis Patient	Lyme Arthritis Patient
Cartilage glycoprotein 39	RA2	LA1/LA8
Collagen -		
type I, α -1	RA1/RA3	LA3/LA5/LA6/LA7/LA8
type I, α -2	RA2 [†] /RA3	LA3/LA5/LA8
type IV, α -1		LA8
type V, α -1	RA5	LA3/LA8
type V, α -2	RA2/RA3	LA5/LA7/LA8
type VI, α -1		LA8
type VI, α -3	RA3	LA3/LA5/LA7/LA8
type XI, α -1	RA2/RA3	LA5/LA8
type XII, α -1	RA3/RA5	LA1/LA5/LA8
type XIV, α -1	RA2	LA3/LA4/LA5/LA6/LA7/LA8
type XV, α -1	RA3	LA7/LA8
Enolase	RA4 [‡] /RA5	LA2/LA5 [†] /LA6/LA7/LA8
Fibrinogen -		
alpha	RA2 [†] /RA3/RA5	LA3/LA4/LA5/LA7 [†] /LA8 [†]
beta	RA2/RA3/RA5	LA1/LA4/LA6/LA7 [‡] /LA8
gamma	RA2 [†] /RA3/RA4 [‡]	LA2/LA4/LA5/LA7 [†] /LA8
Fibronectin	RA1/RA2/RA3/RA5	LA1/LA2/LA3/LA4/LA5/LA6/LA7 [†] /LA8
Immunoglobulin	RA2/RA3	LA1/LA3/LA4/LA8
Vimentin	RA1/RA2/RA3/RA5	LA1/LA3/LA7/LA8 [†]

*Unless marked otherwise, peptides from these source proteins were identified only in synovial tissue.

[†]Peptide(s) from this source protein identified in synovial tissue and synovial fluid

[‡]Peptide(s) from this source protein identified only in synovial fluid

[§]Peptide(s) from this source protein identified in synovial tissue, synovial fluid, and peripheral blood

[00129] Search for citrullinated HLA-DR-presented peptides — Protein citrullination is considered to be an important post-translational modification in RA. The strong association between RA shared-epitope alleles and ACPA-positive RA has been hypothesized to result from the binding and presentation of citrullinated peptides by HLA-DR molecules (11). Therefore, the MS/MS spectra were searched carefully for evidence of this post-translational modification, which results in the gain of 0.984 D (NH₂ vs. OH) when arginine is converted to citrulline. The search algorithms assigned several observed MS/MS spectra that had incomplete fragment ion series to citrullinated peptides. However, in each case, manual inspection and targeted tandem mass spectrometry yielded full sequence information that unambiguously defined that the peptide modification actually corresponded to hydrolysis of asparagine to aspartic acid or glutamine to glutamic acid.

[00130] For example, arginine conversion to citrulline at R8 of the peptide VNDTQFVRFDSDAASPRG (SEQ ID NO: 5), that corresponds to residues 27 to 44 in the source protein B6V6K3 MHC class I antigen (fragment), which was originally detected in the ST of patient RA3, was assigned as the citrullinated peptide by two protein database search programs during analysis of LC-MS/MS data acquired on the LTQ-Orbitrap XL MS. However, since conversion of arginine to citrulline and hydrolysis of asparagine to aspartic acid result in the same change in elemental composition and thus to the same peptide mass shift, the molecular weight change and partial sequence coverage should not be considered definitive. In this case, the signal for the MS/MS spectrum shown in **FIG. 2A** was recorded using the low resolution ion trap of the LTQ-Orbitrap XL MS; the series of sequence-determining fragment ions was incomplete and left room for ambiguity. The fragment ion at m/z 589.9 matches both the y11 ions of arginine to citrulline modification and hydrolysis of asparagine to aspartic acid modification within 0.5 Da error tolerances. Therefore, despite the agreement of the two database searches, it was really not possible to distinguish between these two post-translational modifications on the basis of these data. Later, when we had the opportunity to apply targeted tandem MS on the Q Exactive plus MS, the full series of high accuracy, high abundance diagnostic fragment ions allowed unambiguous assignment of the modification as asparagine deamidation at N2 (**FIG. 2B**). Similar results were obtained for each of the other tentative assignments of citrullination that had been based on low resolution MS/MS spectra containing incomplete ion series.

[00131] Identification of Immunogenic peptides and source proteins - In five of the eight patients for whom sample testing have been completed, one to two immunogenic peptides (T cell epitopes) were identified from the peptides identified from each patient. When these peptides or their source proteins were tested for reactivity with PBMC or serum samples from many patients, it was found that 10-50% of RA or LA patients had T and/or B cell reactivity with each immunogenic peptide or its source protein. The immunological data and key MS/MS data for each individual autoantigen have been shown in references (22-26), the contents of which are incorporated herein in their entireties. Here, in an effort to integrate and make available the full mass spectrometry dataset, presented are complete list of peptides identified from each patient that correspond to assignments indicated on the MS/MS spectra. The peptides found to be immunogenic were derived from the source proteins endothelial cell growth factor (ECGF) (22), apolipoprotein B-100 (apoB-100) (23), matrix metalloproteinase-10 (MMP-10) (24) in LA; from annexin A2 in both RA and LA (25) and from N- acetylglucosamine-6-sulfatase and filamin A in RA (26). The MS/MS spectra and peptide sequences determined for these autoantigens are shown in **FIG. 3**.

[00132] The immunogenic HLA-DR-presented peptide derived from filamin A was identified in both ST and PBMC, whereas the peptides from ECGF, apoB-100, annexin A2, and N- acetylglucosamine-6-sulfatase were identified only in ST. Although annexin A2 had been previously shown to serve as an

autoantigen in several rheumatic diseases, including about 10% of patients with RA (59), it had not been previously identified as an autoantigen in LA (25). Likewise, neither of the other three autoantigens in LA (ECGF, MMP-10, and apoB-100) nor the two in RA (N-acetylglucosamine 6-sulfatase and filamin A) had been previously identified as autoantigens in those diseases. Moreover, for proteins in this group that had been reported to be immunogenic prior to this investigation, the precise epitopes had not previously been determined.

[00133] In 14 patients with antibiotic-refractory LA, ST was available for histopathologic correlations with autoantibody responses. Autoantibody reactivity with endothelial cell growth factor correlated strongly with obliterative microvascular lesions (36) anti-apoB-100 autoantibodies correlated with greater numbers and activation of endothelial cells and more synovial fibroblast proliferation (23) anti-MMP-10 autoantibodies correlated with measures of cell proliferation, including greater synovial lining layer thickness and greater numbers of synovial fibroblasts (24) and anti-annexin A2 autoantibodies correlated with greater synovial fibroblast proliferation (25). Thus, the identification of T cell epitopes led to the identification of B cell responses that correlated with pathophysiological consequences.

DISCUSSION

[00134] In this study, large numbers of HLA-DR-presented peptides are identified directly from clinical samples of ST, SFMC or PBMC of patients with RA or LA. The challenge inherent in this approach exceeds that presented by the already difficult task of identifying HLA-DR-presented peptides in tissue culture systems, in which large numbers of a single cell type can be grown and harvested for analysis. Nevertheless, it was the impressive success that had been realized from tissue culture studies which led to undertaking the direct identification from clinical samples that are reported herein. Tandem mass spectrometry was first used to identify MHC molecules in well-defined tissue culture systems (32); for such analyses, at least 5×10^8 cells expressing 2×10^5 MHC molecules per cell was regarded as the minimum sample requirement (43). Current reports from the Hunt group and other groups continue to improve and exploit this strategy very effectively to identify very large numbers of MHC- or HLA-bound peptides. These studies have identified both MHC Class I and Class II antigenic peptides in various tumor types, and have probed the mechanisms underlying the generation of HLA- presented peptides (37-39). However, the repertoire of HLA-DR-presented peptides in tissue culture systems contains many peptides derived from source proteins in the tissue culture medium (40, 41). It is thus quite different from direct analysis of clinical samples, as we have found (30). Previously, *in vivo* presented peptides have been identified in patients' spleen (44), colon (45), kidney (46), pooled bronchiolavage fluid (47), thyroid (48), or thymus (49). These studies, on average, have identified only ~20-40 peptides per sample, and in some instances, samples from different patients needed to be pooled to identify peptides. An important

exception was the identification of thousands of peptides, including some cancer-related peptides, presented by soluble HLA complexes that were immunoprecipitated from the plasma of patients and healthy controls (33). In previous study of two patients each with RA or LA, in which samples were analyzed using the QSTAR mass spectrometer, >80 non-redundant peptides were identified in each ST sample (30). In comparison, when the same type of strategy was applied to the sample obtained from our most recent patient, use of the Q Exactive plus MS led to the identification of 801 non-redundant peptides, a substantial increase. Even with recent increases in sensitivity and improvements in data analysis tools, identification of HLA-DR-presented peptides by LC-MS/MS still offers technical challenges. First, not all HLA-DR complexes are quantitatively recovered from a given sample. Additionally, some of the isolated peptides have low ionization efficiency and/or are not selected for MS/MS analysis, and not all spectra can be assigned by the search algorithms, due to low signal intensity, incomplete component resolution, and/or the presence of unusual PTMs. However, as shown here, the performance of MS/MS instruments and search algorithms continues to move forward (60).

[00135] In the current study, the greater sensitivity now available was taken advantage of, by analyzing not only ST, but also SFMC and PBMC as additional sources of *in vivo* HLA-DR-presented peptides that can be determined in individual patients. Although a substantial number of the peptides identified were the same in ST as in SFMC or, to a lesser extent, in PBMC, a number of unique peptides were only identified in SFMC or PBMC. As expected, the number of peptides identified in ST was greater than in SFMC or PBMC. Yet, 67% of the peptides identified in SFMC were also found in ST, and 55% of the peptides identified in PBMC were found in ST. Therefore, the analyses of SFMC and PBMC each provided a window into the ST repertoire, but also yielded antigenic peptides not found in ST. The data herein indicate that a more complete picture in an individual patient can be obtained by analyzing tissue or fluids from more than one site. The addition of SFMC and/or PBMC to the analytical scheme has three advantages. First, a more complete picture of an individual patient can be obtained by analyzing samples from multiple sites. Second, since ST can usually only be obtained from patients who undergo surgical procedures, frequently late in the disease, use of SFMC and/or PBMC allows evaluation of patients early in the disease when immune responses may be more robust. Third, given that ST is usually not available, one may obtain at least some information about events in ST, the target of the immune response in chronic inflammatory forms of arthritis. Thus, the testing of SFMC or PBMC opens up the possibility of testing a broader spectrum of patients at earlier stages of their diseases, and increasing the yield of information about immune reactivity in individual patients.

[00136] In RA patients, HLA-DR-presented peptides were identified from many source proteins previously reported to be autoantigens in RA, including vimentin, enolase, fibrinogen and collagen. Interestingly, peptides from many of these same source proteins were frequently found in the joints of LA

patients, where they are not known to be immunogenic. In RA, many proteins that contain citrulline have been identified, the non-standard amino acid that results from the enzymatic post-translational modification of arginine, such as vimentin, enolase, fibrinogen, collagen, fibronectin, calreticulin, etc. (11, 61-67). Antibodies to these or other citrullinated proteins, known as ACPA, are strongly associated with RA, particularly in patients with HLA-DR alleles that encode for the RA shared epitope (11-13), a highly homologous amino acid sequence at positions 70-74 (Q K/R RAA) in the B1 chain of the HLA-DR molecule. These residues contribute to the P4 anchoring pocket, giving it an overall positive charge. A disease-linked peptide containing citrulline (neutral R-group) has been shown to have greater affinity for the HLA shared epitope in the P4 pocket, as compared to the unmodified analog that contains arginine (positively charged R-group); this citrullinated peptide has been reported to induce T cell activation in DR4-IE transgenic mice (11). Therefore, the MS/MS spectra herein were searched carefully for evidence of peptides that could contain arginine-to-citrulline conversions. Our search algorithms assigned several peptides as forms containing citrulline residues. However, closer manual inspection of the initially obtained spectra and targeted tandem mass spectra recorded on the Q Exactive plus MS, the most advanced instrument employed in this study, determined that, for each of these peptides, the one-Dalton gain was actually due to deamidation at asparagine. The search algorithms did not distinguish correctly between these two different post-translational modifications because they result in the same peptide mass, and the initial MS/MS spectra contained incomplete series of fragment ions that lacked diagnostic peaks between the residues in question. Asparagine deamidation, in addition to being the product of biological processes, can also result from exposure of the sample to high temperature and/or low pH (68), the very conditions used to elute peptides from the MHC molecules, and thus this modification could, in some cases, be an artifact generated during sample processing. A library of citrullinated proteins has been compiled via LC-MS/MS analysis of RA patients' synovial tissue/fluid (69, 70). Manual search of our LC-MS/MS data according to this inventory did not result in the detection of any citrullinated HLA-DR-presented peptide.

[00137] In conclusion, the data disclosed herein show that LC-MS/MS is now sensitive enough to identify large numbers of HLA-DR-presented peptides directly from clinical samples of ST, SFMC and PBMC in individual patients with chronic inflammatory arthritis. In present study, discovery-based LC-MS/MS identification of HLA-DR-presented peptides from an individual patient's tissues or fluids, provided a bridge for the identification of novel autoimmune responses. Of the three autoantigens identified in RA patients, only one had been described previously, and all of the four autoantigens identified in LA patients were novel. A principal advantage of autoantigen identification starting with an immunogenic T cell epitope is that it often focuses the search on more broadly applicable T and B cell responses in many patients that result in sustained high-titer autoantibodies, and these responses correlate

with pathophysiological consequences. Furthermore, the identification of *in vivo* HLA-DR-presented peptides from PBMC, which can be readily obtained from any patient, offers a precision, personalized approach for the identification of potentially pathogenic immune responses in a patient with any immune-mediated disease.

Materials and Methods

[00138] Study Patients — The study was approved by the Human Investigations Committee at Massachusetts General Hospital, and all participants gave written informed consent. The five RA patients met the 2010 criteria of the American College of Rheumatology/ European League against Rheumatism (ACR/EULAR) definition for the diagnosis of rheumatoid arthritis (50), and the eight LA patients met the criteria of the Centers for Disease Control and Prevention for the diagnosis of Lyme disease (51). All eight LA patients had antibiotic-refractory LA, defined as persistent arthritis for \geq three months after the start of \geq two months of appropriate oral antibiotic therapy and \geq one month of IV antibiotic therapy (52). In LA patients, synovial tissue was obtained at the time of therapeutic, arthroscopic synovectomies, and, in RA patients, the tissue was collected at the time of synovectomy or joint replacement surgery. Sufficient quantities of SFMC were available for analysis in six patients and PBMC were available in four patients.

[00139] ST, SFMC, and PBMC Preparation — All the reagents were purchased from Sigma- Aldrich (St. Louis, MO) except where noted. ST samples (8-10 g) were prepared using the protocol described previously in reference (30), the contents of which are incorporated hereby in its entirety. Heparinized SFMC and PBMC (\sim 3 to 8×10^7 cells) were obtained by Ficoll-Hypaque (MP Biomedicals) separation and stored in liquid nitrogen prior to analysis. On the day of purification, SFMC and PBMC samples were quickly thawed by placing in a 37 °C water bath for two minutes. Cells were washed twice in phosphate buffered saline (PBS) (Life Technologies) by centrifuging at $800 \times g$ for 10 min. The pellet was re-suspended with 10 mL lysis buffer: 150 mM sodium chloride, 20 mM tris(hydroxymethyl)aminomethane•HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid disodium solution, 0.04% sodium azide, 1 mM 4- (2-aminoethyl)benzenesulfonyl fluoride•HCl, 10 μ g/mL leupeptin, 10 μ g/mL pepstatin A, 5 μ g/mL aprotinin, and 1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate. Immunoaffinity purifications of HLA-DR complexes from SFMC and PBMC were performed using the protocol previously described for synovial tissue samples in reference (30).

[00140] LC-MS/MS — High performance LC-MS/MS was performed on purified HLA-DR-presented peptides after desalting using C₁₈ ZipTip (Millipore, Billerica, MA). The methods used for each mass spectrometer are described below.

[00141] LTQ-Orbitrap XL mass spectrometer — A nanoAcquity UPLC system (Waters Corp., Milford, MA) was coupled to an LTQ-Orbitrap XL MS (ThermoFisher Scientific, San Jose, CA) through a TriVersa.

[00142] NanoMate ion source (Advion, Ithaca, NY) — Peptide enrichment was performed with a trapping column (180 μ m \times 20 mm, 5 μ m 100 \AA Symmetry C₁₈, Waters Corp) at a flow rate of 15 μ L/min for 1 min, and separation was achieved with a capillary column (150 μ m \times 10 cm, 1.7 μ m 130 \AA BEH C₁₈, Waters Corp). Buffer A contained 1% acetonitrile (ACN) and 0.1% formic acid in water, and buffer B contained 1% water and 0.1% formic acid in ACN. A linear gradient of buffer B from 2% to 40% over 52 min was used at a flow rate of 0.5 μ L/min.

[00143] The capillary voltage was set to 1.7 kV using the NanoMate, and the capillary temperature was set to 120 $^{\circ}$ C. The mass spectra were recorded over the range m/z 300-2000 at a resolution of 60,000 (the width of the peak at half its maximum height at m/z 300) at a scan rate of approximately 1.2 s/spectrum. Tandem MS was performed for the five most abundant, multiply- charged species in the mass spectra that had a signal intensity threshold of 8000 NL. The normalized collision energy for CID was set to 35%, and helium was used as the collision gas. MS/MS spectra were recorded with the LTQ XL linear ion trap. All spectra were recorded in profile mode. Two to five LC-MS/MS runs were performed for each sample, and all the RAW files for each sample were combined into one Mascot generic file using Proteome Discoverer 1.3 software (ThermoFisher Scientific).

[00144] Q Exactive plus mass spectrometer — A nanoAcquity UPLC system was coupled to a Q Exactive plus MS (ThermoFisher Scientific, San Jose, CA) through a TriVersa NanoMate ion source. The same peptide enrichment and separation columns, HPLC solvents and gradients, and NanoMate capillary voltage and temperature used on the LTQ-Orbitrap XL MS were applied on the Q Exactive plus MS. Survey MS scans were recorded over the range m/z 400-2000 with a resolution of 70,000 (at m/z 300). The 15 most abundant, multiply-charged ions were selected for higher-energy collisional induced dissociation (HCD) MS/MS with a resolution of 17,500, an isolation width of 1.5 m/z, and a normalized collision energy of 27%. All spectra were recorded in profile mode. One LC-MS/MS run was performed for each sample, and the RAW file for each sample was converted to a Mascot generic file using Proteome Discoverer 1.3 software (ThermoFisher Scientific).

[00145] 6550 iFunnel QTOF LC/MS — Two 1260 HPLC systems were coupled to a 6550 iFunnel QTOF MS through a chip cube interface (Agilent Technologies, Santa Clara, CA). A Polaris-HR-3C18 chip was used for peptide enrichment and separation. The flow rate on the enrichment column was set at 2 μ L/min with sample flush volume set as 4 μ L. With the same solvents used on the Waters UPLC systems, a gradient of buffer B (from 2% to 5% over 0.1 min, 5% to 20% over 74.9 min, 20% to 40% over 10 min,

40% to 90% over 10 min, held at 90% buffer B for 5 min, returned to 2% buffer B and held for 10 min) was used at a flow rate of 0.3 μ L/min for peptide separation. The capillary voltage was set to 1.7 to 1.95 kV, and the gas temperature was set to 225 °C. The survey mass spectra were recorded over the range m/z 295-1700 with acquisition rate of 8 spectra/second. Tandem mass spectra were recorded over the range m/z 50-1700 with an acquisition rate of 3 spectra/second. Precursor ion isolation was performed with a narrow isolation window of 1.3 m/z. Tandem MS was performed for the 20 most abundant, multiply-charged species in the mass spectra that had a signal intensity threshold of 5000 counts or a relative threshold of 0.001%. The collision energy for CID was set using the formula $[3.1 \times (m/z)/100 + 1]$ for doubly charged precursor ions and $[3.6 \times (m/z)/100 - 4.8]$ for triply and higher charged precursor ions. Active exclusion was enabled after recording one tandem spectrum and released after 0.15 min. All spectra were recorded in the centroid mode. One to two LC-MS/MS runs were performed for each sample, and all the data files for each sample were combined into one Mascot generic file using MassHunter Qualitative Analysis B.05.00 (Agilent Technologies, Santa Clara, CA).

[00146] Protein Database Searching — In previous study, the International Protein Index (IPI) database (72,155 protein sequences) was used to identify HLA-DR-presented peptides from ST samples from two patients each with RA or LA (identified here as patients RA1, RA2, LA1 and LA2 (30). Because the IPI database is now closed and no longer maintained, the spectra obtained for the samples from these four patients were reanalyzed using the UniProt protein database (95,500 entries, August 2010) and the findings were compared to our previous study reference (30). Using the UniProt database, ~96-98% of the peptides identified were the same as those previously assigned using the IPI database. Since an SFMC sample from LA2, and SFMC and PBMC samples from RA2 were available for analysis in this study, the re-analyzed LC-MS/MS data from ST of RA1, RA2, LA1, and LA2 were included here for comparison of the HLA-DR-presented peptides repertoires in ST, SFMC, and PBMC. In the current study, HLA-DR-presented self-peptides were identified by searching the Mascot generic file from each sample against the UniProt human database concatenated with a decoy database. The decoy databases were generated by randomizing each protein sequence in the database using the Perl script decoy.pl (Matrix Science). Mascot 2.4.0 (Matrix Science), OMSSA Browser 2.1.1 (NCBI), and X!Tandem tandem-win-12-10-01-1 (The Global Proteome Machine Organization www.thegpm.org) were used for database searches. For inclusion of a peptide sequence in our database, a consensus match was required (assignment of the sequence by >two search engines) or a match by only one program that was judged acceptable upon review by two experts. Cysteinylation of cysteine, deamidation of glutamine and asparagine, pyroglutamic acid from amino-terminal glutamine and glutamic acid, and oxidation of methionine were specified as the variable modifications in all searches. S-cysteinylated peptides have been identified in MHC class I- and class II-presented peptides (53-56) and in our previous study (30). In addition, the

conversion of arginine to citrulline was included as a variable post-translational modification; peaks exhibiting the difference in molecular weight corresponding to arginine vs. citrulline ($\Delta = 0.98402$ Da) were searched for each arginine residue in the database.

[00147] For data acquired using the LTQ-Orbitrap XL MS, precursor ion error tolerance was set as 0.01 Da and fragment ion error tolerance was set as 0.5 Da; for data acquired using Q Exactive plus MS, precursor ion error tolerance was set as 10 ppm and fragment ion error tolerance was set as 20 mmu; for data acquired using 6550 iFunnel QTOF MS, precursor ion error tolerance was set as 20 ppm and fragment ion error tolerance was set as 50 mmu. “No enzyme” was specified for all the protein database searches. Mascot score cutoff was set at ≥ 20 ; OMSSA e-value cutoff was set to ≤ 0.01 ; and X!Tandem e-value cutoff was set at ≤ 10 . In addition, consensus identifications of peptides by at least two search programs were correlated using a Microsoft Access query. Over the course of the study, UniProt human protein database was continuously updated. To validate the results obtained with the UniProt database presented herein, the spectra obtained from a recent PBMC sample from an RA patient were reanalyzed using a Swiss-Prot protein database (20,100 entries, January 2016) and 93% of the peptides identified with the updated database were the same as those assigned using the UniProt database used here.

[00148] Removal of redundant peptide sequences – The endogenous HLA-DR-presented peptides derived from the same source protein often contain overlapping sequences with different peptide lengths. For example, $^{116}\text{DHLKYVMLPVA}^{126}$ (SEQ ID NO: 10), $^{116}\text{DHLKYVMLPVAD}^{127}$ (SEQ ID NO: 11), and $^{116}\text{DHLKYVMLPVADQ}^{128}$ (SEQ ID NO: 12) from HP protein (accession Q6PEJ8) were all identified from ST of LA8, and $^{116}\text{DHLKYVMLPVA}^{126}$ (SEQ ID NO: 13) and $^{116}\text{DHLKYVMLPVAD}^{127}$ (SEQ ID NO: 14) were considered redundant peptides of $^{116}\text{DHLKYVMLPVADQ}^{128}$ (SEQ ID NO: 15). To address this issue, the lists of identified consensus peptides were submitted to software generated in-house that collates and sets aside redundant peptides that have amino acid sequences that overlap in a core sequence. Peptide sequences with at least six continuous overlapping amino acid residues were considered redundant, and the peptide with the longest amino acid sequence was output from the software program. This software was also used to compare the consensus peptides among different samples. Peptide sequences with at least six continuous overlapping amino acid residues were considered consensus.

[00149] Identification of source proteins – All of the consensus peptides identified by at least two protein database search engines, and peptides assigned by a single program whose identifications passed close manual inspection, were submitted to another software program generated in-house, that screens the peptide sequences against the UniProt human protein database (not including decoy sequences). All the accession numbers of the source proteins containing the screened peptide sequence are displayed as a text string concatenating the accession numbers by semicolons in Microsoft Excel. This search showed that a

number of identified peptides could be derived from more than one source protein, the consensus source proteins among different samples were correlated by matching the text strings containing protein accession numbers using a Microsoft Access query. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (57) via the PRIDE partner repository with the dataset identifier PXD003051.

[00150] Determinations of immunogenicity of peptides and source proteins — For the translational component of the protocol, all non-redundant peptides identified by LC-MS/MS were synthesized and evaluated against the matching patient's PBMC using ELISpot assays. Immunogenic peptides were then tested for T cell reactivity by ELISpot assay, and the source proteins of the peptides were assayed for B cell responses by ELISA in many patients and control subjects. To date, the determinations of peptide immunogenicity have been completed for three RA and in five LA patients (22-26). Further such analyses are ongoing.

[00151] Statistics – All p values are two-tailed. A p value of ≤ 0.05 was considered statistically significant.

Example 3 - ELISPOT assays can be performed with GNS and/or filamin A peptides suspected of being autoantigens in a subject.

[00152] Enzyme-linked immunosorbent spot (ELISPOT) assays are performed using ELISpot^{plus} for human IFN- γ kits (Mabtech Inc., # 3420-2AW-Plus). Briefly, PBMC collected using Ficoll-Hypaque density centrifugation and stored in liquid nitrogen are thawed quickly and plated in round bottom, 96-well plates (Costar, # 3799) at 2×10^5 per well in 200 μ l of complete media (RPMI-1640, 2 mM glutamine, 100 units/ml penicillin 100 μ g/ml streptomycin, 10 mM HEPES (all from Invitrogen) and 10% human AB serum (Cellgrow). Full length protein or shorter peptide fragments are added at a concentration of 1 μ M in duplicate wells. Positive and negative controls consist of 1% PHA (Invitrogen, # 10576-015) and no antigen, respectively. After 5 days at 37°C and 5% CO₂, cells are transferred to ELISPOT plates (Mabtech), previously coated with IFN- γ capture antibody, and incubated overnight. All subsequent steps are performed as detailed in the manufacturer's protocol. Images of wells are captured using ImmunoSpot series 3B analyzer and spots counted using ImmunoSpot 5.0 academic software (Cellular Technology Limited).

Example 4 - ELISA assays can be performed with GNS and/or filamin A peptides suspected of being autoantigens in a subject.

[00153] Serum anti-peptide antibody ELISA: EasyWash ELISA plates (CoStar) are coated with 100 µl of 0.5 µg/ml carrier free, recombinant human GNS and/or filamin A full length protein or peptide fragments dissolved in PBS and incubated overnight at 4°C. All subsequent steps are performed at room temperature with plates on a platform shaker set at 200 rpm. The next day, plates are washed three times with PBST (phosphate buffered saline and 0.05% Tween-20) then incubated with 200 µl of blocking buffer (5% nonfat dry milk in PBST) for 1 hr. Afterwards, wells are washed three times with PBST and 100 µl of each patient's serum sample diluted 1/100 with blocking buffer is added to individual wells and incubated for 1 hr. As a control, serum from eight healthy subjects is added to each plate to be used for inter-plate standardization. After three more washes with PBST, 100 µl goat anti-human IgG conjugated to horseradish peroxidase (KPL # 074-1006) diluted 1:7500 in blocking buffer is added to each well and incubated for 1 hr. Plates are then washed three times with PBST, followed by three times with PBS and incubated with 100 µl of a 1:1 mixture of the substrate 3,3',5,5'-tetramethylbenzidine and 0.01% hydrogen peroxide (TMB substrate reagent kit, # 555214) (BD Biosciences). The reaction is stopped after 3 min with 100 µl of 2N sulfuric acid (LabChem Inc., # LC25790-2). Absorbance values (OD₄₅₀) for each well were determined using a microplate reader (Bio-Rad, model 550).

[00154] Synovial fluid autoantigen sandwich ELISA: EasyWash ELISA plates are coated with 50 µl of the capture antibody, goat anti-human autoantigen (human GNS and/or filamin A full length protein or peptide fragments) diluted in PBS (5 µg/ml) and incubated overnight at 4°C. All subsequent steps are performed at room temperature. The next day, plates are washed three times with PBS and incubated with blocking buffer for 30 min. Afterwards, plates are washed three times with PBS and 100 µl of each patients' synovial fluid sample diluted 1:10 with blocking buffer are added to individual wells and incubated for 2 hr. In order to quantify results, recombinant human autoantigen serially diluted with blocking buffer is also added to each plate to generate a standard curve. After washing the plates three times, wells are filled with 150 µl of blocking buffer, gently vortexed and washed again three times with PBS to ensure removal of all unbound proteins. Plates are then incubated with 50 µl of the mouse anti-human autoantigen antibody diluted in blocking buffer (5 ng/ml) for 2 hr. Plates are again washed with PBS and 50 µl of the detection antibody, goat anti-mouse IgG conjugated to horse radish peroxidase (Santa Cruz, #SC-2005) diluted in blocking buffer (1:1000) is added to plates and incubated for 1 hr. After plates are washed three times with PBS, 100 µl of TMB was added for ~6 min and then the reaction is stopped with 100 µl of 2N sulfuric acid. Plates are read as described above.

Example 5 - Immunoblotting can be performed with GNS and/or filamin A peptides suspected of being autoantigens in a subject.

[00155] Autoantigen (e.g, human GNS and/or filamin A full length protein or peptide fragments, such as human recombinant autoantigen) 12 µg is electrophoresed through a 10% mini-PROTEAN TGX gels (Bio-Rad) then transferred to nitrocellulose membranes. All subsequent steps are performed at room temperature with rocking. Membranes are cut into strips, individually placed into eight channel reservoir liners (Costar, #4878) and incubated for 1 hr in 1.5 ml blocking buffer (5% nonfat dry milk, 0.1% Tween-20 in 20 mM Tris, 500 mM sodium chloride; pH 7.5). Afterwards, strips are washed three times for 1 min intervals with rinse buffer (0.1% Tween-20 in 20 mM Tris, 500 mM sodium chloride; pH 7.5) and each individual strip is incubated for 1 hr with patient's serum diluted 1:100 in blocking buffer. Strips are again washed three times with rinse buffer and incubated for 1 hr with goat anti-human IgG antibody conjugated to alkaline phosphatase (KPL, #4751-1006) diluted 1:2000 in blocking buffer. Strips are washed three times with rinse buffer and another three times with 20 mM Tris, 500 mM sodium chloride; pH 7.5. Bands are visualized by incubation with NBT/BCIP substrate solution (Roche Diagnostics GmbH, # 11681451001) for 3-5 min after which the strips are washed with copious amounts of water to stop the reaction. Bands are considered positive if darker than the pre-determined positive control sample included in each assay.

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CLAIMS

1. A method of diagnosing rheumatoid arthritis in a subject comprising determining whether the subject is immunologically reactive with one or more of filamin-A and N-acetylglucosamine-6-sulfatase, wherein immunological reactivity of the subject to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, as compared to an appropriate control, indicates the subject has rheumatoid arthritis.
2. The method of claim 1, wherein determining is by evaluating a biological sample obtained from the subject for immunological reactivity with the one or more of filamin-A and N-acetylglucosamine-6-sulfatase.
3. The method of any one of claims 1-2, wherein determining immunological reactivity is by detecting the presence of T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase, comprising the steps:
 - a) stimulating peripheral blood mononuclear cells (PBMC) of the subject or the synovial fluid mononuclear cells (SFMC) of the subject *in vitro* with one or more of filamin-A and N-acetylglucosamine-6-sulfatase, whole protein or polypeptide fragments;
 - b) measuring T cell proliferation *in vitro* or secretion of IFN- γ into cell culture supernatants; and
 - c) identifying the subject as having T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when T cell proliferation or secretion of IFN- γ is measured as significantly increased over that of an appropriate control.
4. The method of claim 3, wherein step b) is measuring T cell proliferation *in vitro*, and the subject is identified as having T cells reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when T cell proliferation is measured as significantly increased over that of an appropriate control.
5. The method of claim 3, wherein step b) is measuring T cell secretion of IFN- γ into cell culture supernatants, and the subject is identified as having T cell reactive to one or more of filamin-A and N-acetylglucosamine-6-sulfatase when secretion of IFN- γ is measured as significantly increased over that of an appropriate control.
6. The method of any one of claims 1-5, wherein determining immunological reactivity comprises determining if the subject has a B-cell response to one or more of filamin-A and N-acetylglucosamine-6-sulfatase resulting in the production of autoantibodies that specifically recognize the one or more filamin-

A and N-acetylglucosamine-6-sulfatase, by contacting the sample with filamin-A protein or a polypeptide fragment thereof, and/or N-acetylglucosamine-6-sulfatase protein or a polypeptide fragment thereof, under conditions that allow an immunocomplex of the antibody and the filamin-A or N-acetylglucosamine-6-sulfatase form, and detecting the presence or absence of an immunocomplex, wherein the presence of an immunocomplex indicates the subject presents a B-cell response to filamin-A and/or N-acetylglucosamine-6-sulfatase and wherein the absence of an immunocomplex indicates the subject fails to present a B-cell response to filamin-A and/or N-acetylglucosamine-6-sulfatase.

7. The method of claim 6, wherein the assay is an enzyme-linked immunosorbent assay (ELISA), agglutination test, direct immunofluorescence assay, indirect immunofluorescence assay, or an immunoblot assay.

8. The method of any one of claims 3-7, wherein the polypeptide fragment of filamin-A comprises the amino acid sequence NPAEFVVNTSNAGAG (SEQ ID NO: 1) or an antigenic portion thereof.

9. The method of any one of claims 3-8, wherein the polypeptide fragment of N-acetylglucosamine-6-sulfatase comprises the amino acid sequence of FEPFFMMIATPAPH (SEQ ID NO: 2) or an antigenic portion thereof.

10. The method of any one of claims 1-9 wherein the subject has been or is further tested for one or more of rheumatoid factor, anti-citrullinated protein antibodies (ACPA), and one or more HLA-DR alleles.

11. The method of claim 10, wherein the HLA-DR allele is HLA-DRB1*0101 and/or HLA-DRB1*0401.

12. The method of any one of claims 1-11, wherein the subject is at risk for, or is suspected of having, rheumatoid arthritis.

13. The method of any one of claims 1-12, further comprising the step of treating the subject with one or more of a nonsteroidal anti-inflammatory drug (NSAIDs), a steroid, a disease modifying anti-rheumatic drug (DMARD), and a biologic.

14. A kit comprising, one or more potential antigen and/or potential epitope of filamin-A and/or N-acetylglucosamine-6-sulfatase, and reagents for conducting an assay for detecting the presence of an antibody in a sample that binds to the one or more potential antigen and/or potential epitope of the filamin-A and N-acetylglucosamine-6-sulfatase.
15. The kit of claim 14, wherein the potential antigen and/or potential epitope of filamin-A comprises the amino acid sequence NPAEFVVNTSNAGAG (SEQ ID NO: 1) or an antigenic portion thereof.
16. The kit of claim 14, wherein the potential antigen and/or potential epitope of N-acetylglucosamine-6-sulfatase comprises the amino acid sequence FEPFFMMIATPAPH (SEQ ID NO: 2) or an antigenic portion thereof.
17. The kit of any one of claims 14-16, wherein the assay is an enzyme-linked immunosorbent assay (ELISA).
18. The kit of any one of claims 14-16, wherein the assay is a western blot.
19. The kit or method of any one of claims 1-18 for use in identifying a subject with rheumatoid arthritis.

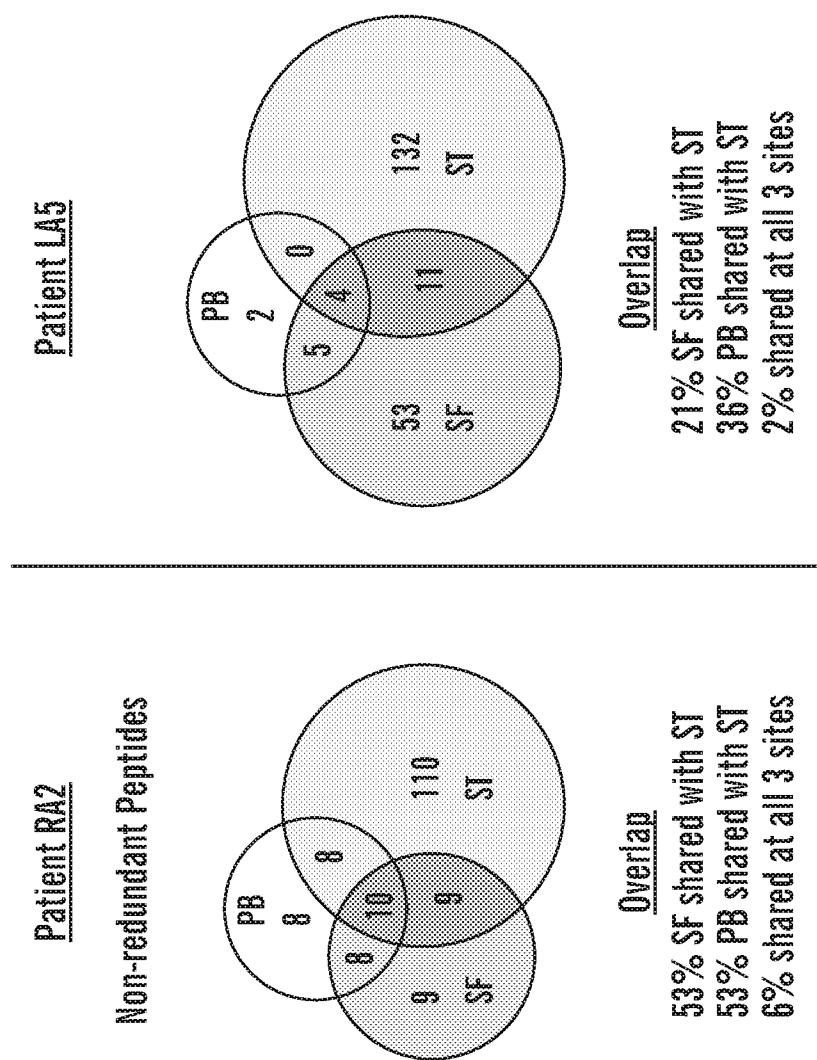


FIG. 1A

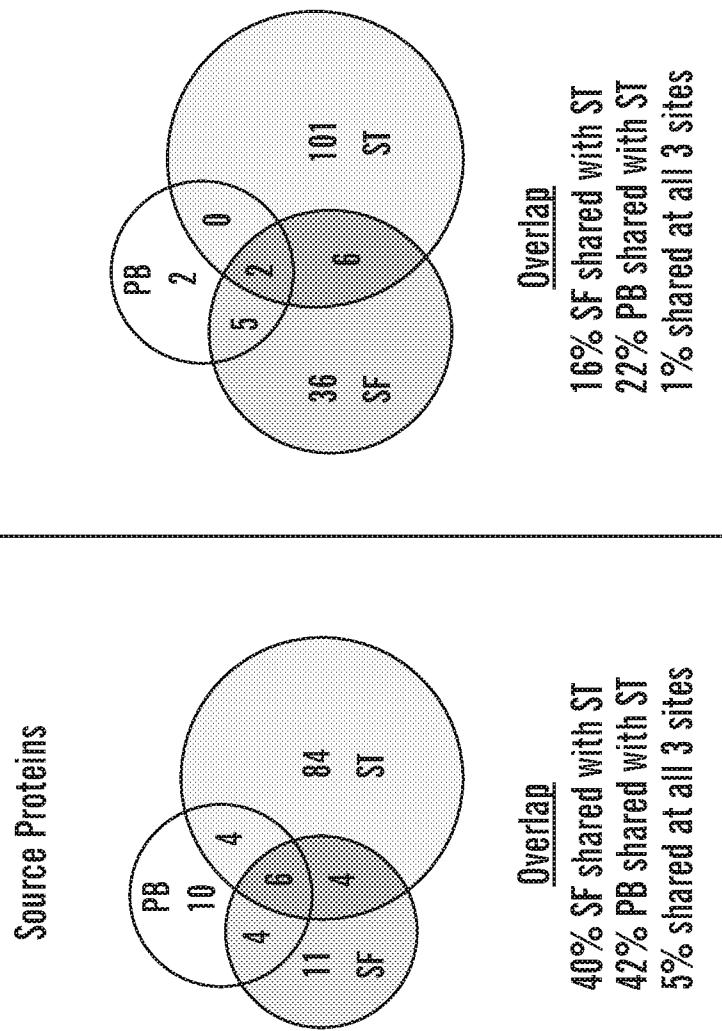


FIG. 1B

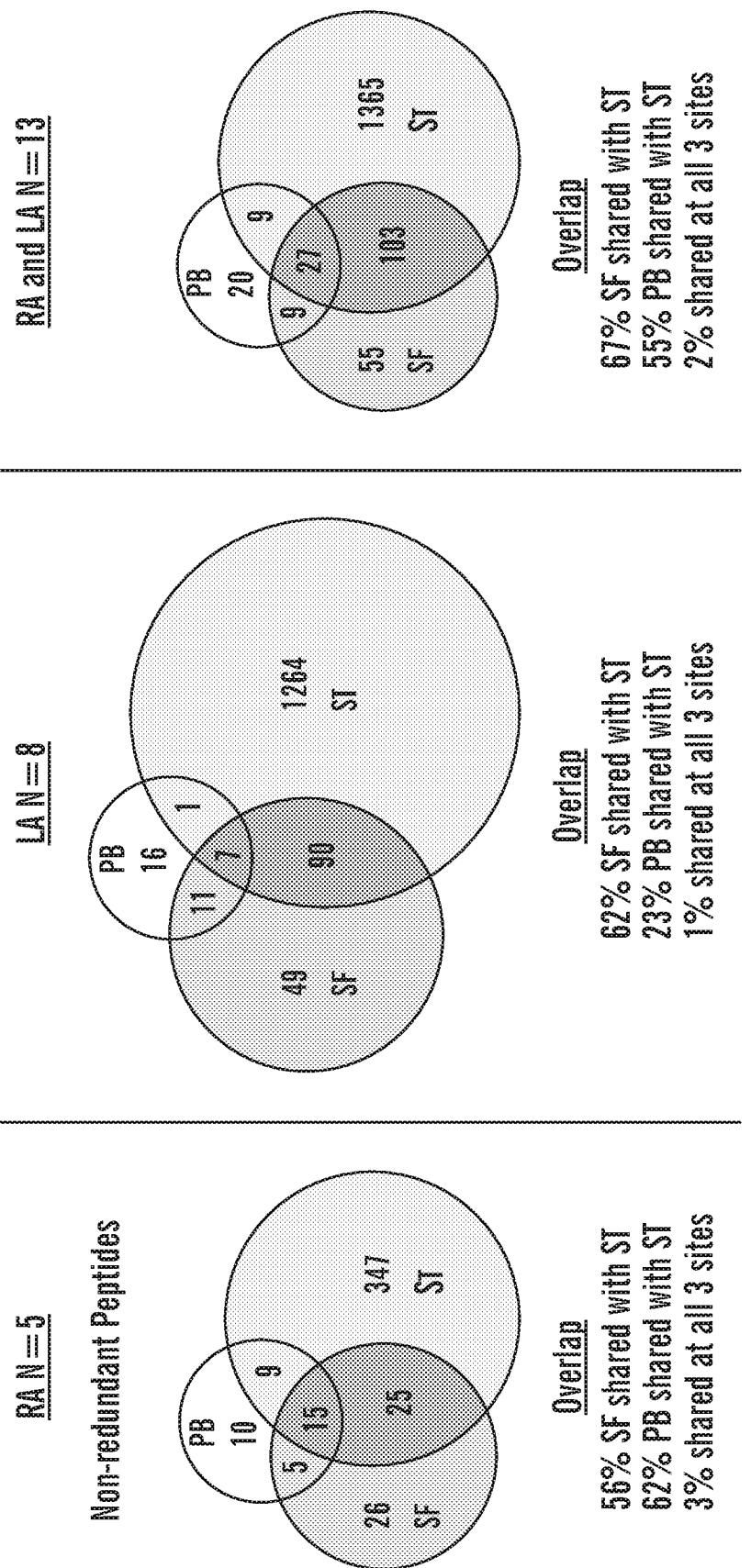


FIG. 1C

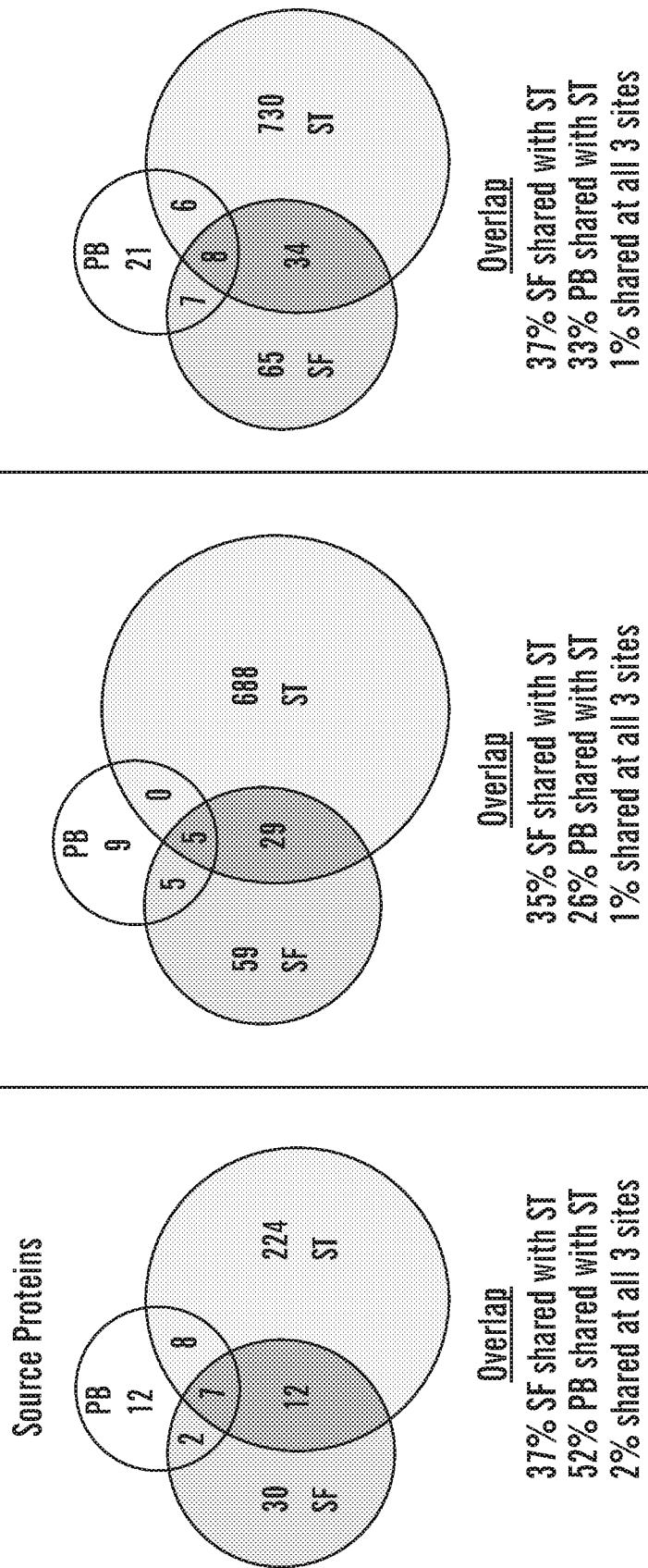


FIG. 1D

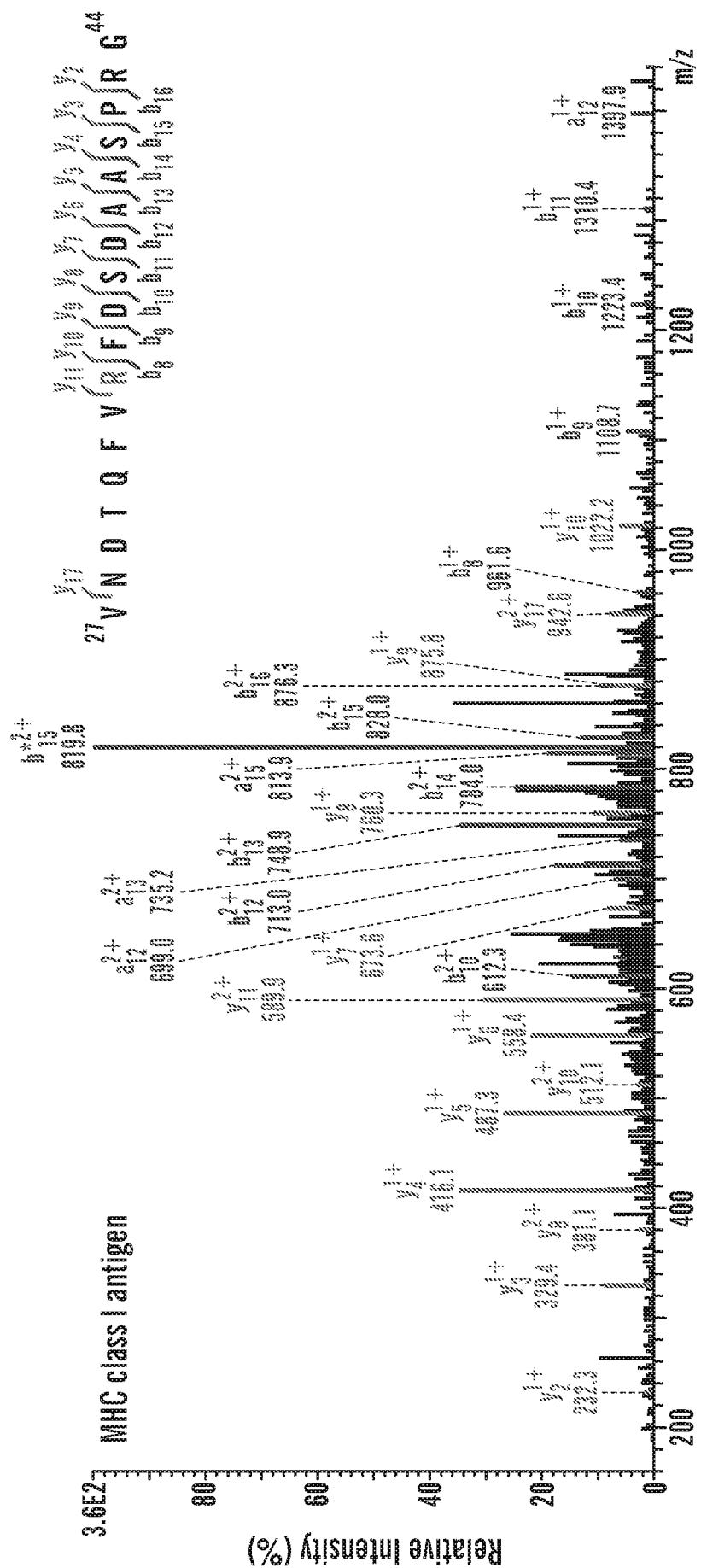


FIG. 2A

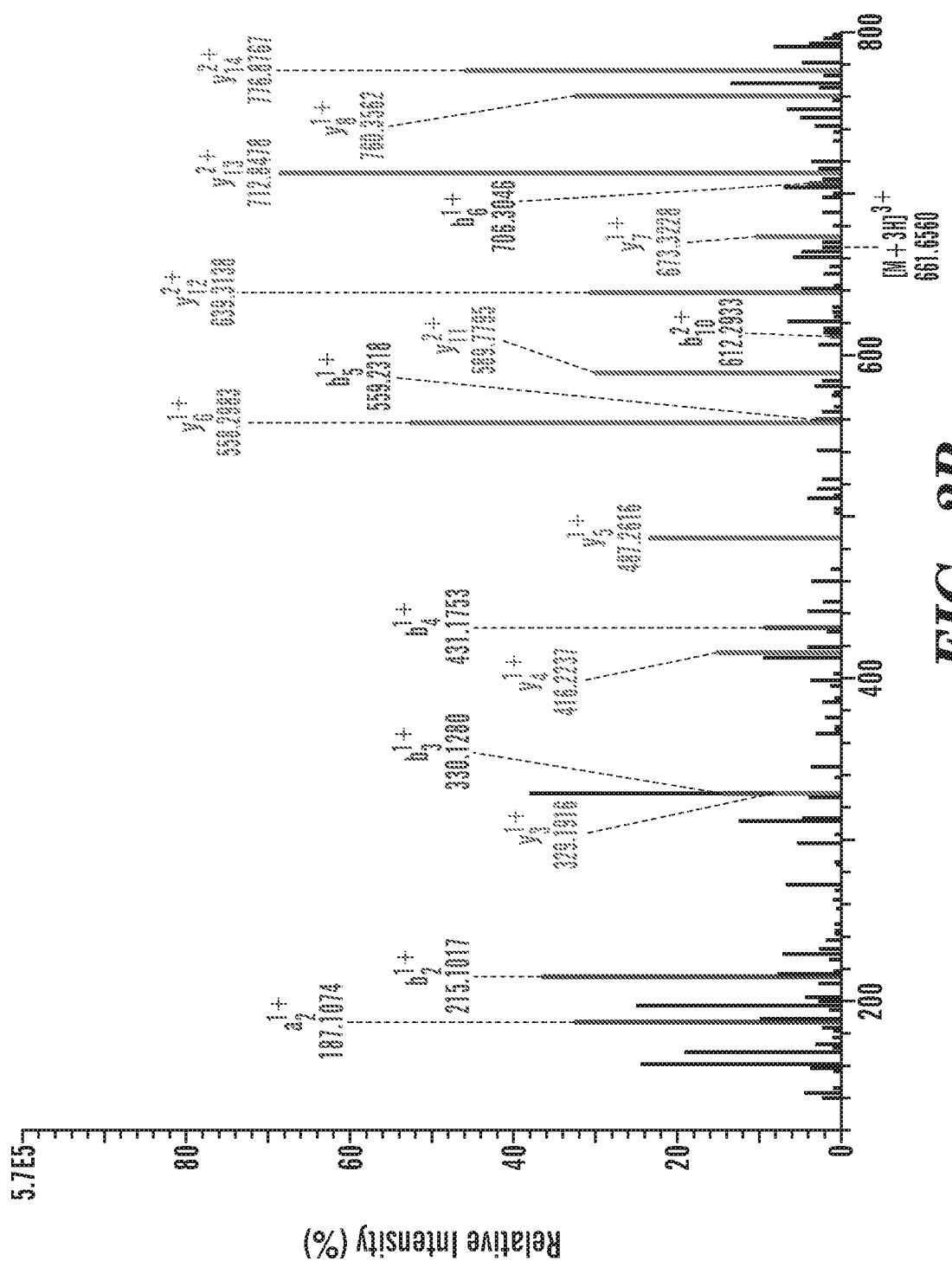


FIG. 2B

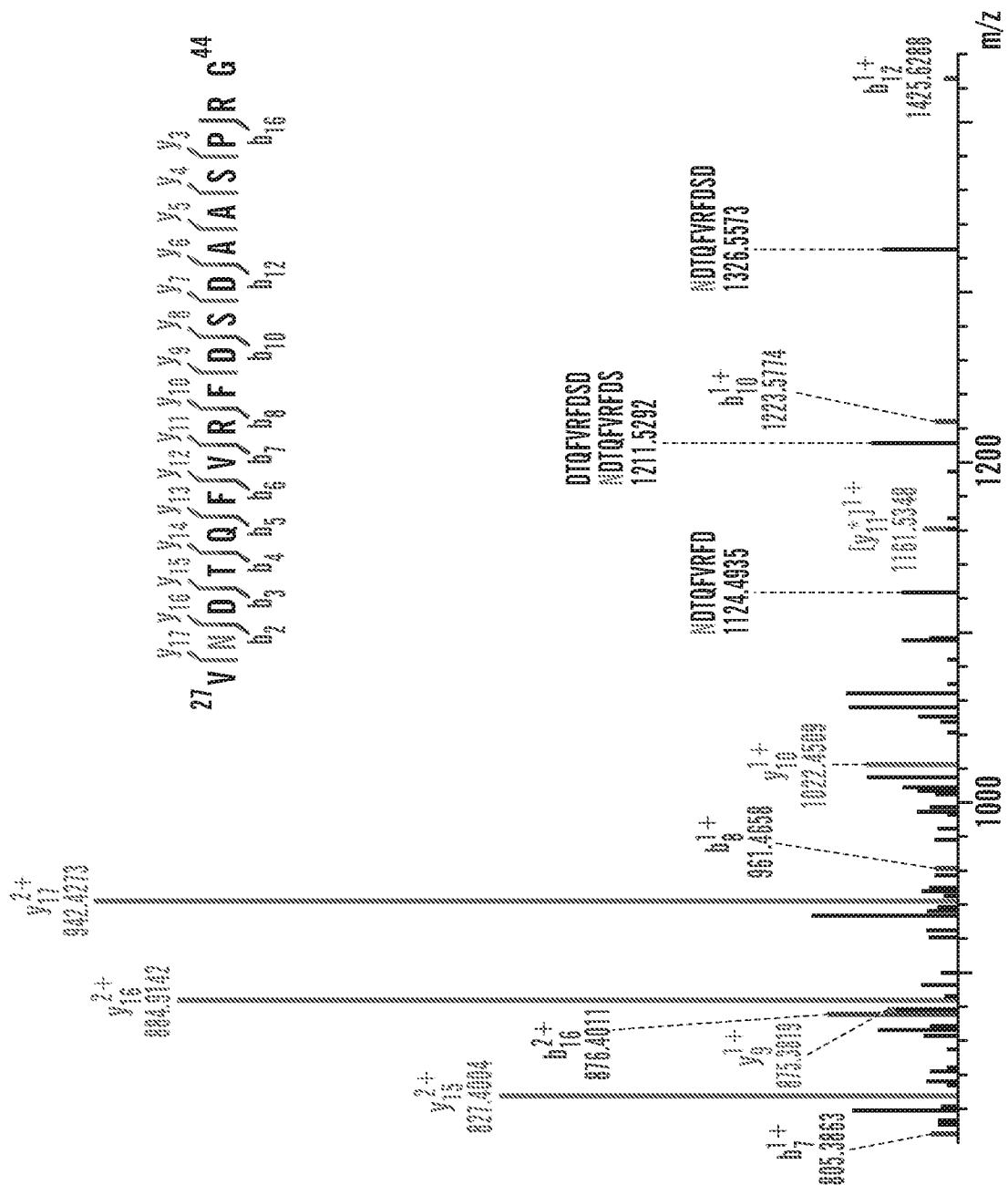


FIG. 2B (cont.)

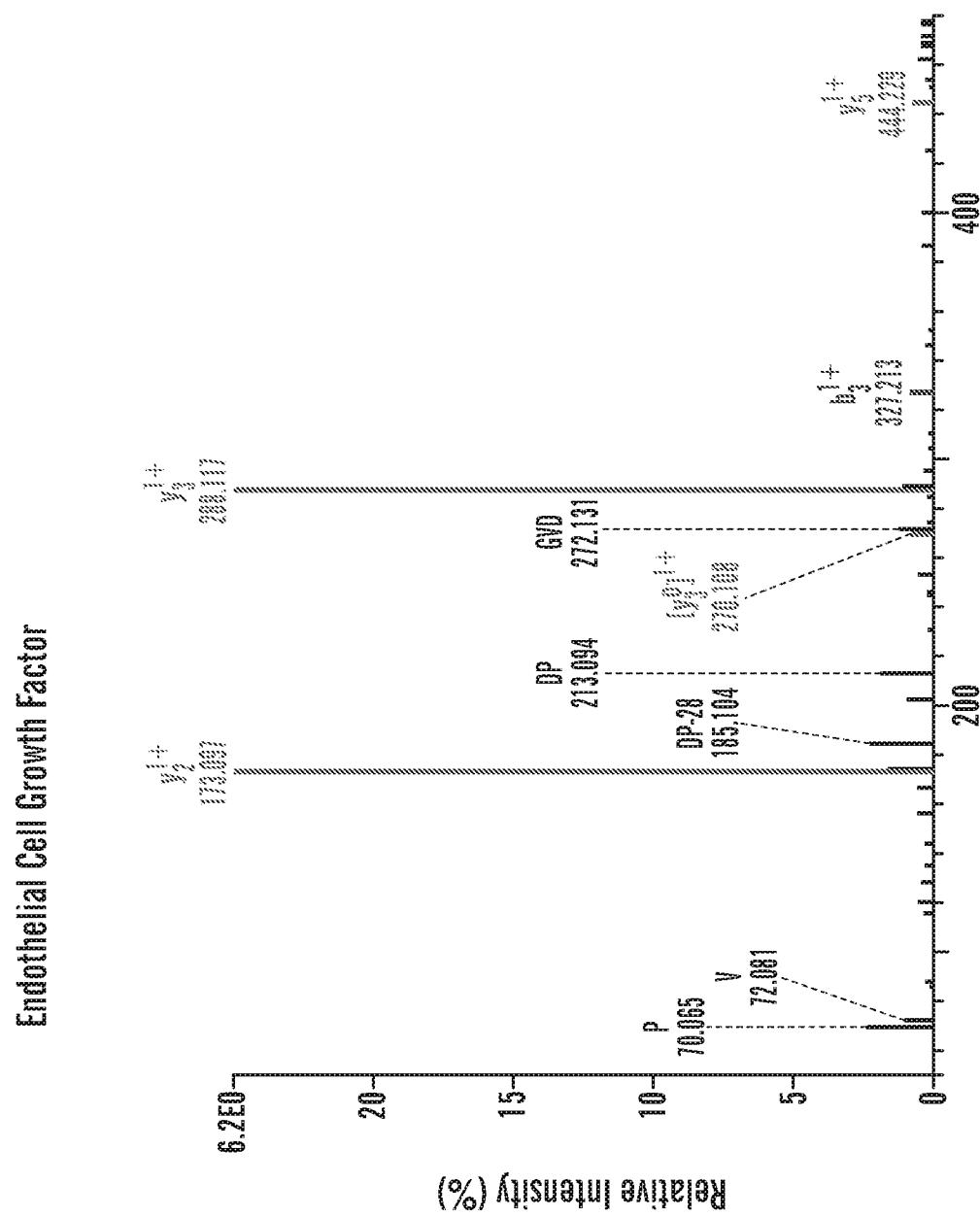


FIG. 3A

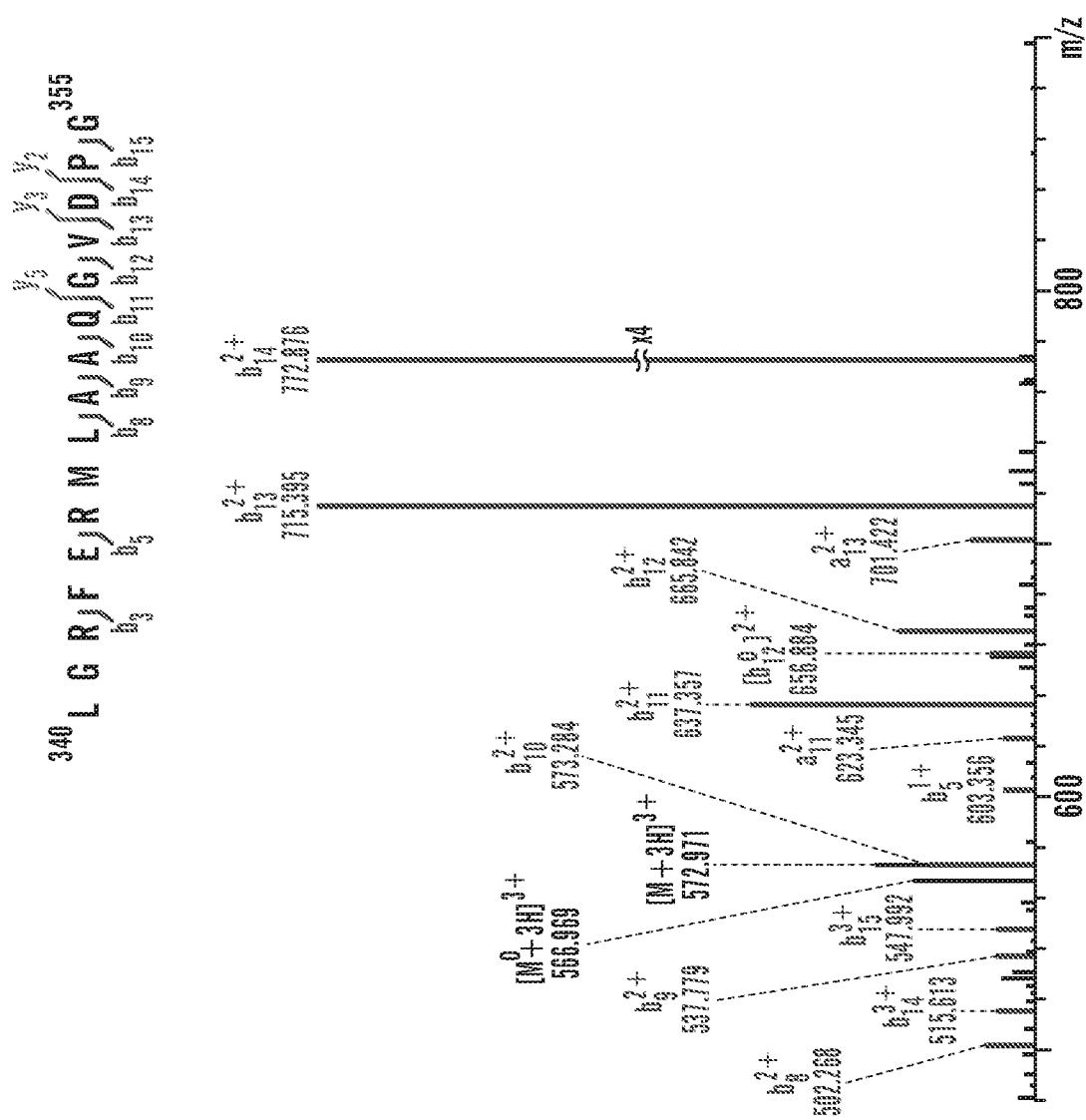


FIG. 3A (*cont.*)

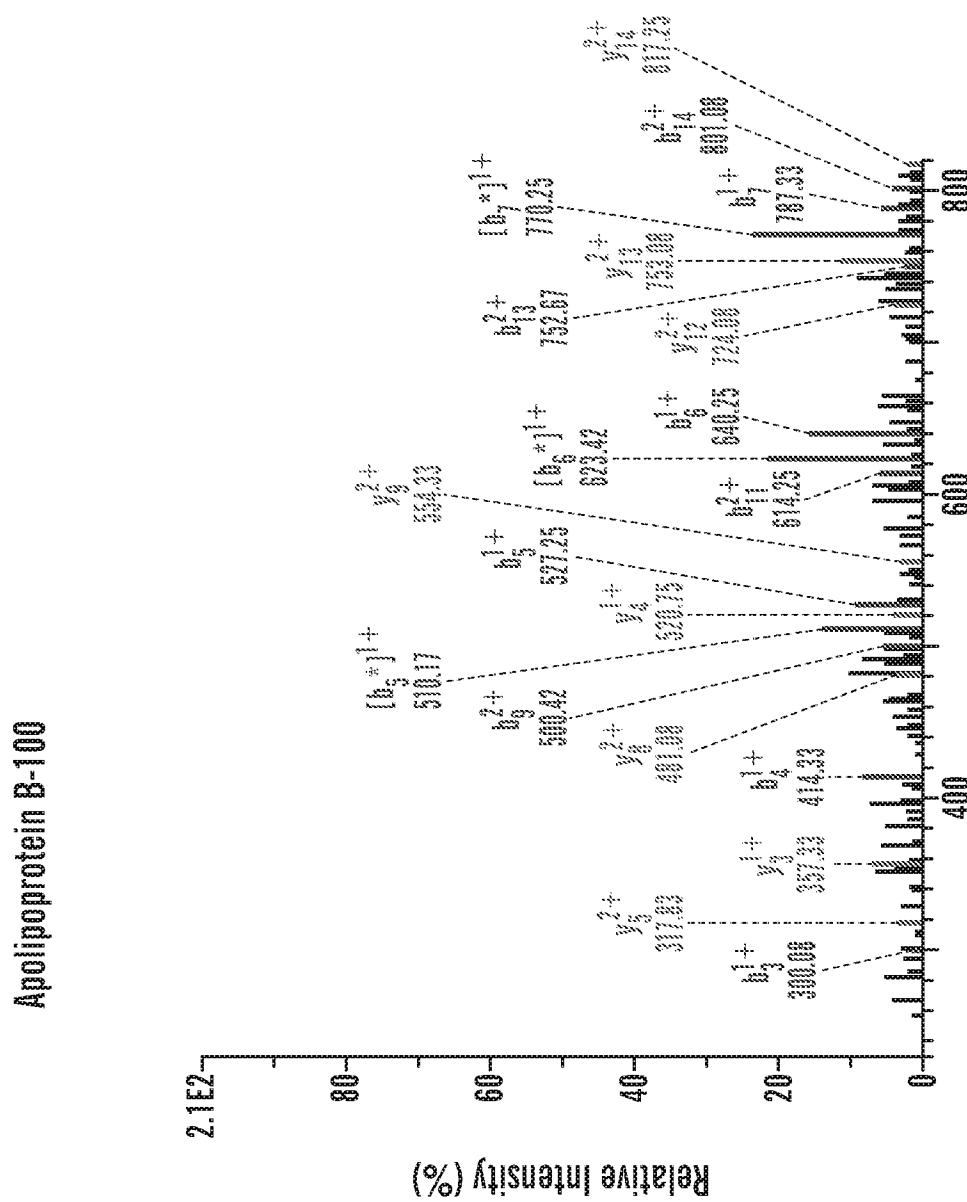


FIG. 3B

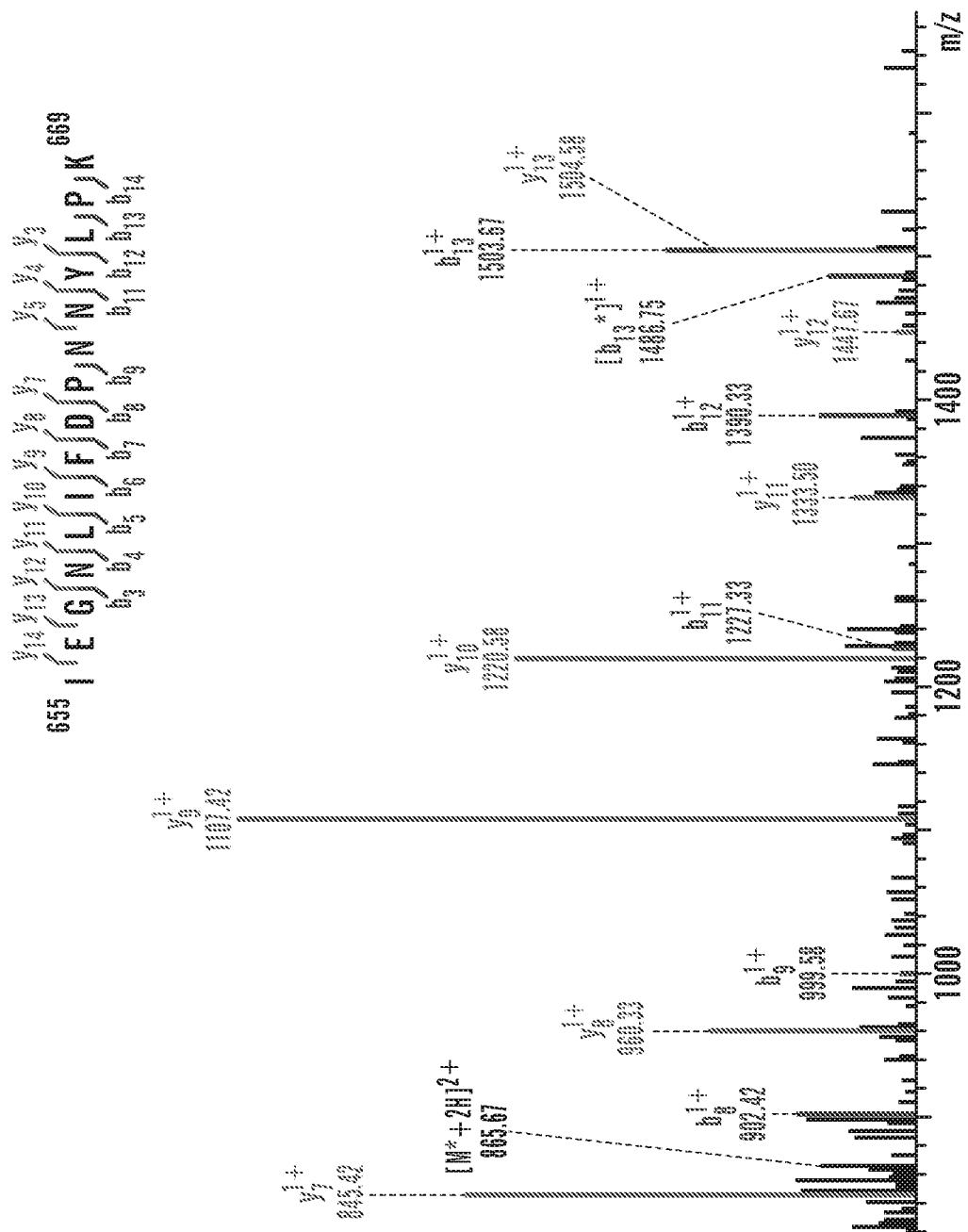


FIG. 3B (cont.)

Matrix Metalloproteinase-10

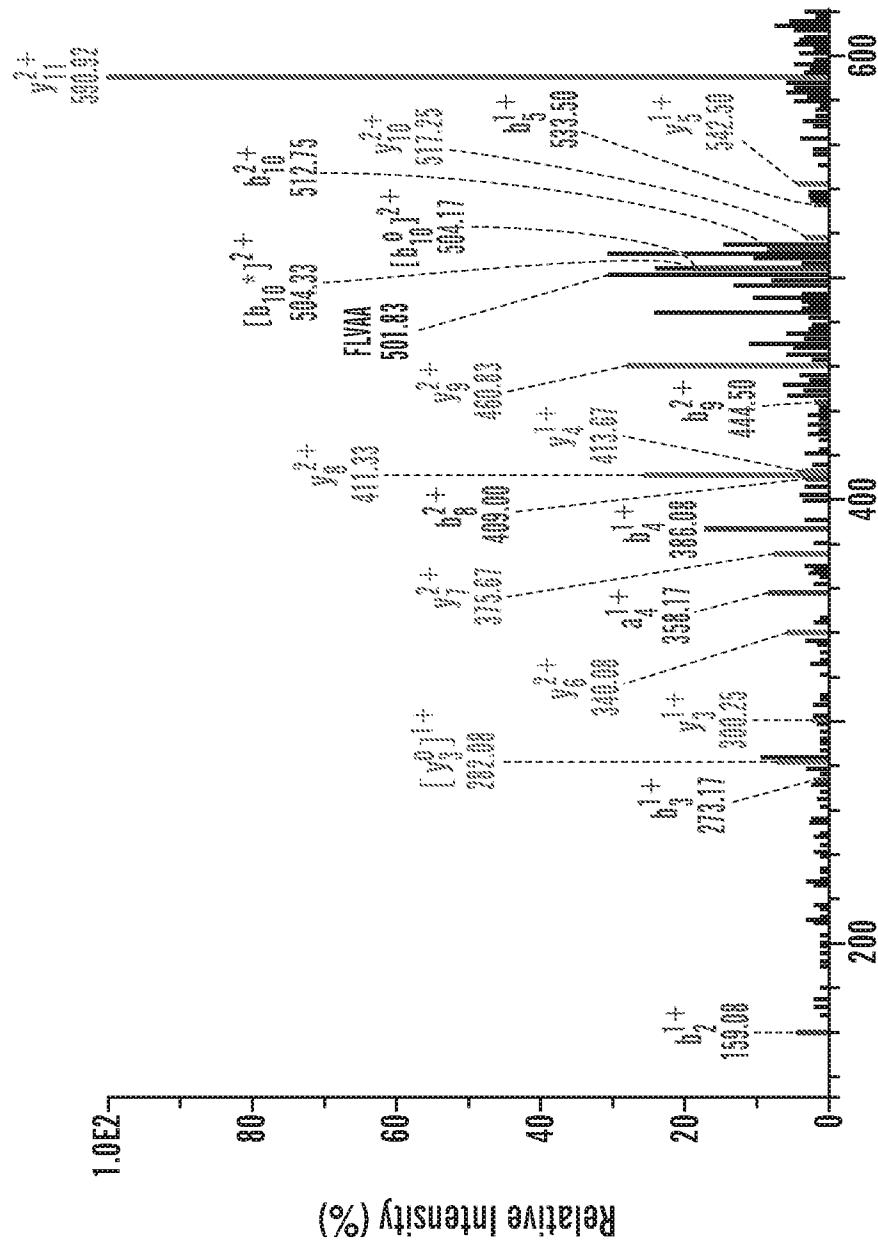


FIG. 3C

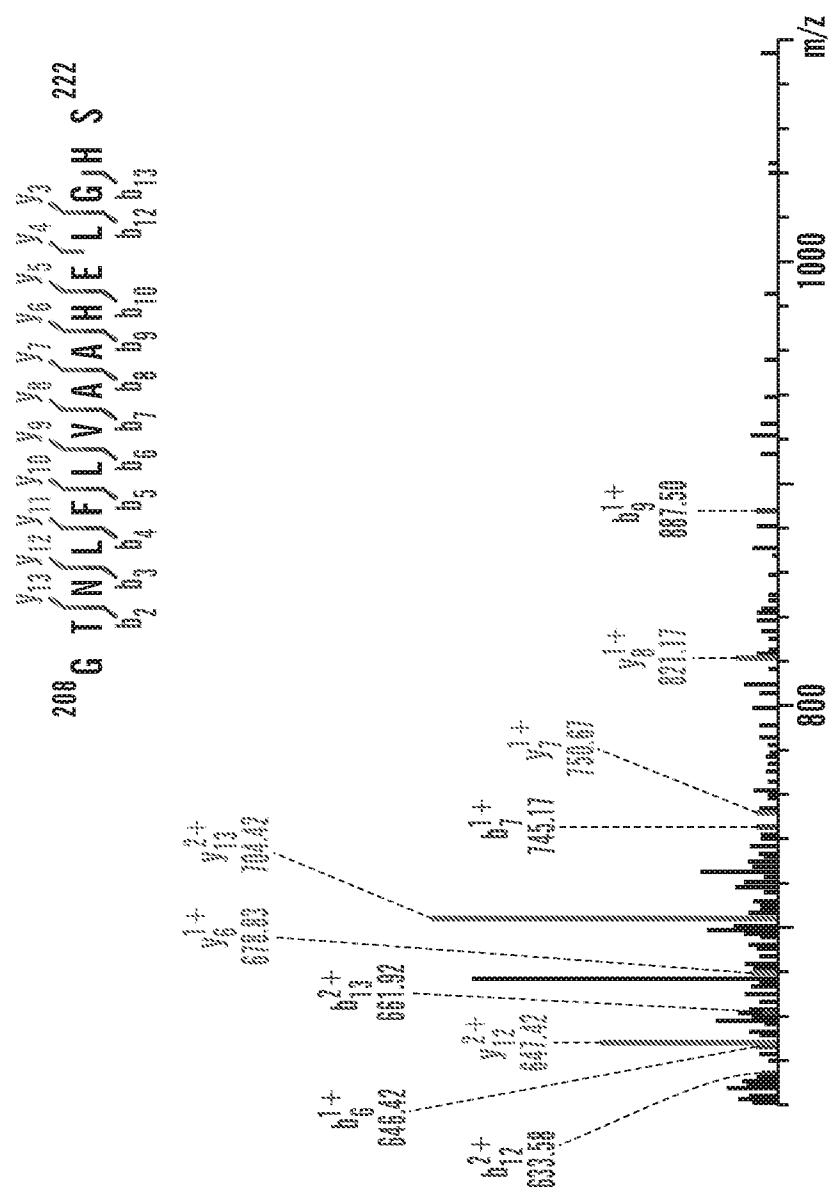


FIG. 3C (cont.)

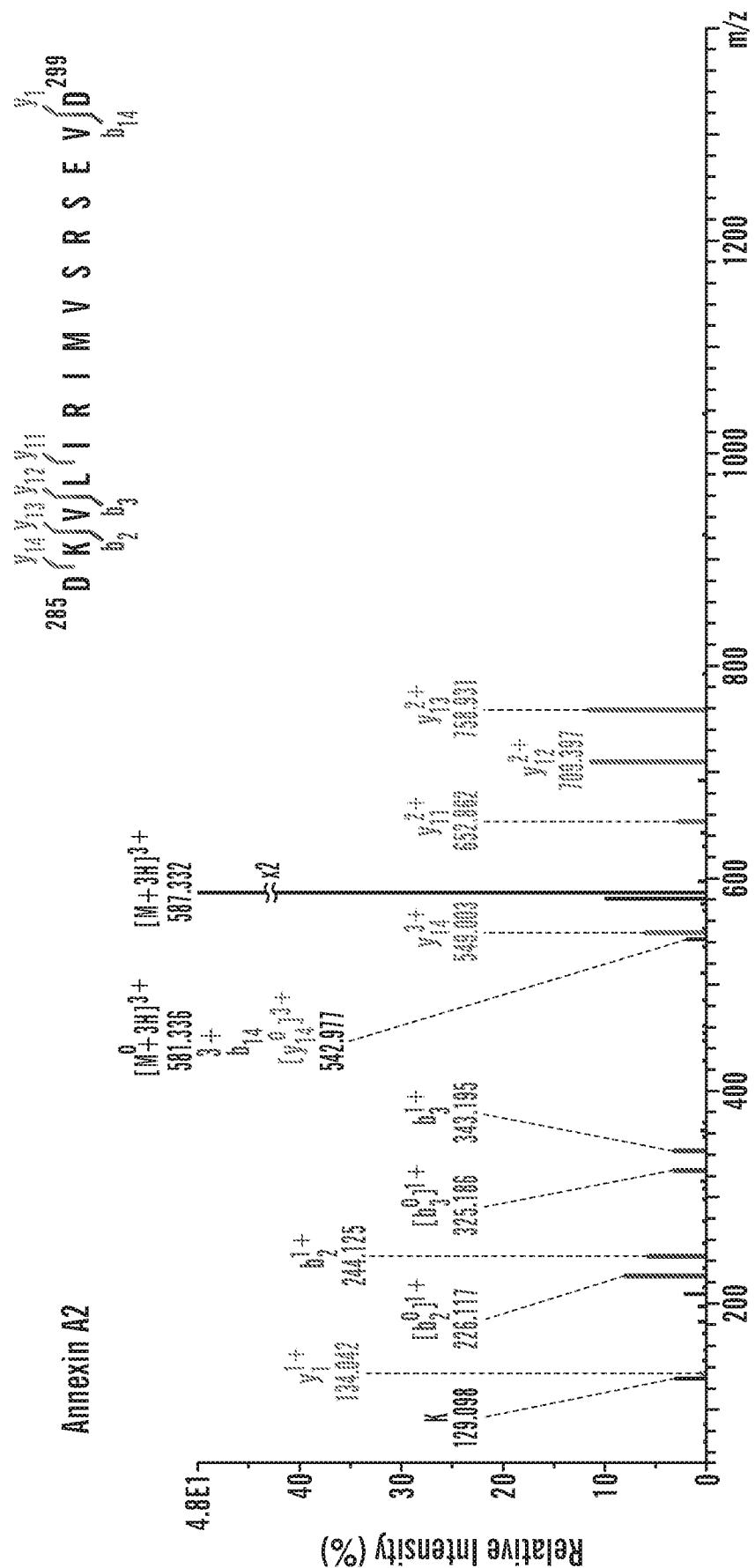


FIG. 3D

Vitamin A

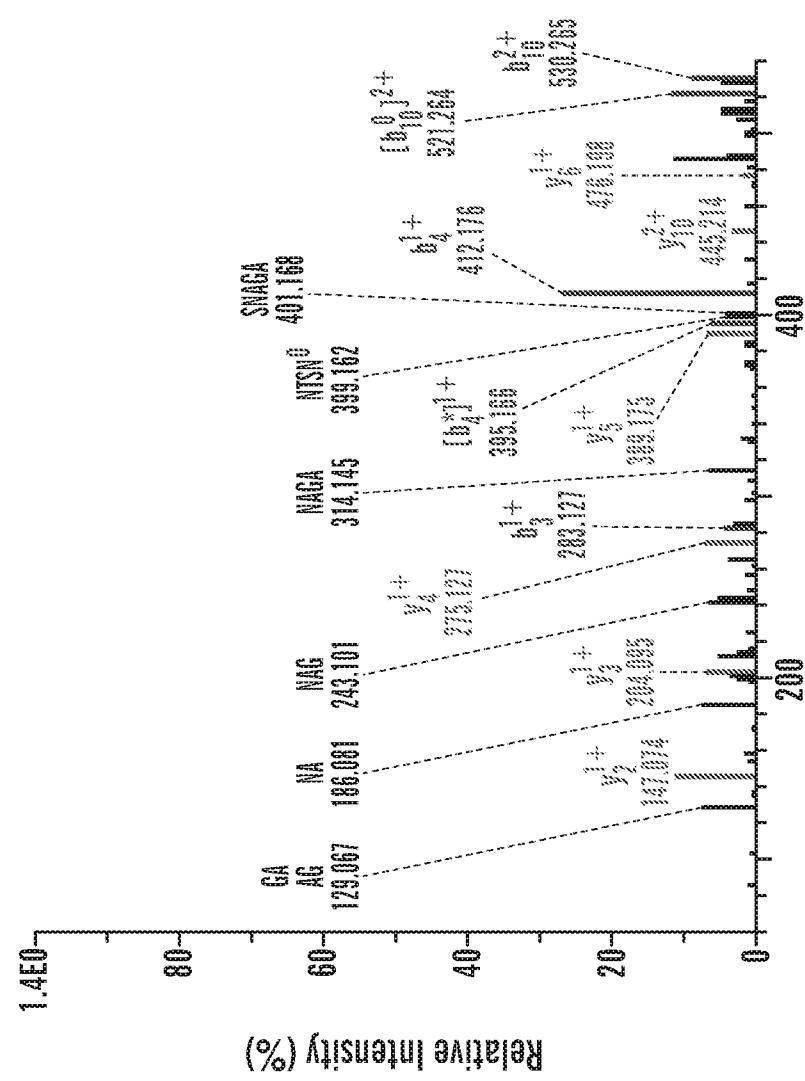


FIG. 3E

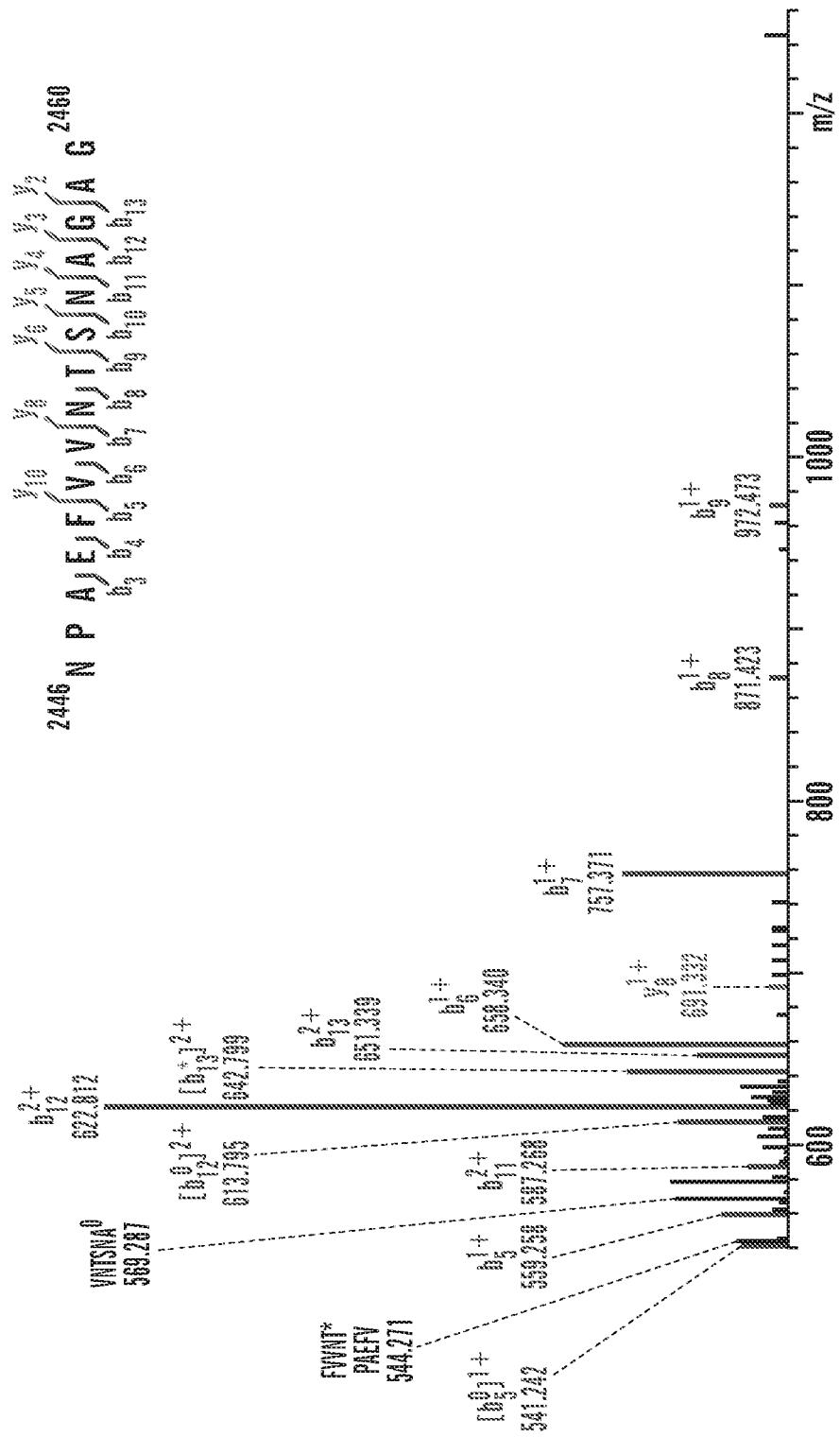


FIG. 3E (cont.)

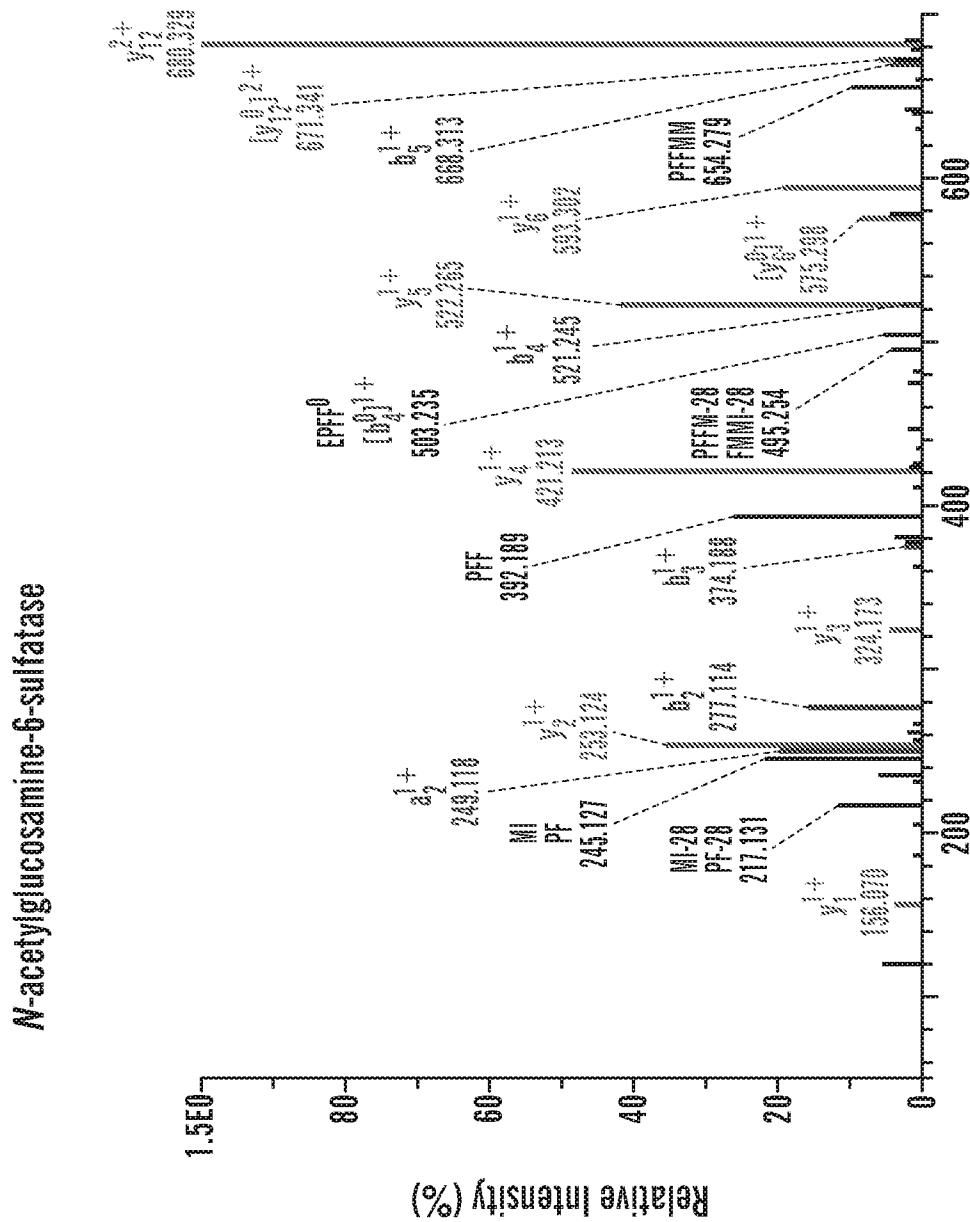


FIG. 3E

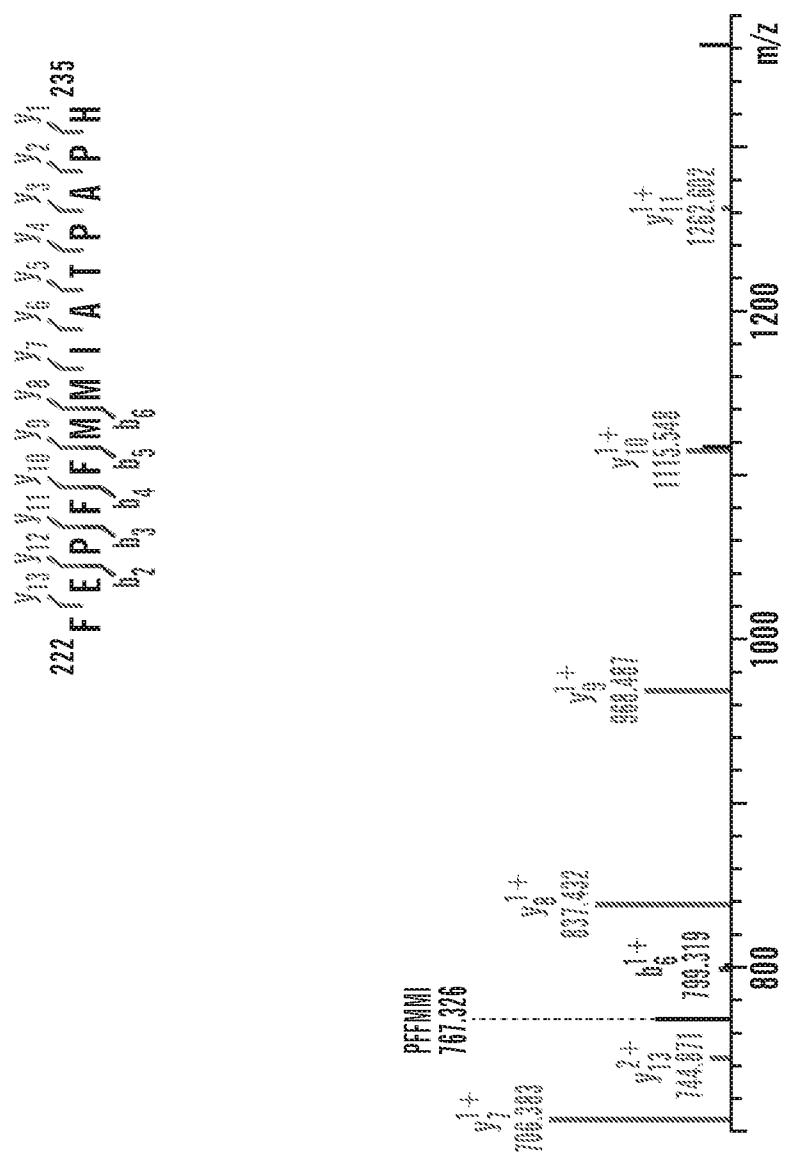


FIG. 3F (cont.)

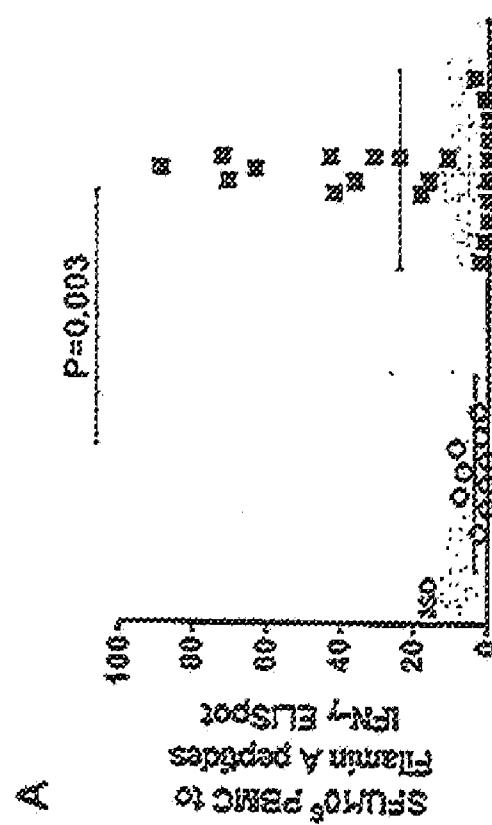
FIG. 4

Case Patient	RA2
Gender	F
Age (years)	37
Arthritis Duration (years)	3
Rheumatoid Factor	pos
ACPA*	pos
HLA-DR genotype	0101/0401
Synovial tissue	# HLA-DR peptides # source Proteins # reactive peptides
	89 86 1 (GNS)
Synovial fluid	# HLA-DR peptides # source proteins # reactive peptides
	19 13 0
Peripheral blood	# HLA-DR peptides # source proteins # reactive peptides
	15 11 1 (FLNA)

*anti-citrullinated protein antibodies; # Number of; GNS, N-acetylglucosamine-6-sulfatase; FLNA, filamin A.

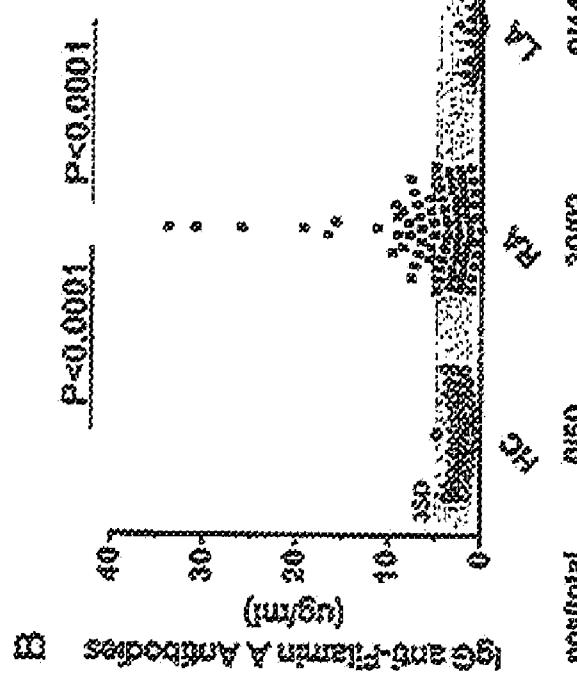
FIGs. 5A-5B

T Cell Response



No. positive
Percent
0/10
0%
1/102
10%
1/102
10%

B Cell Response



No. positive
Percent
0/50
0%
3/50
30%
3/50
30%

FIGs. 6A-6B

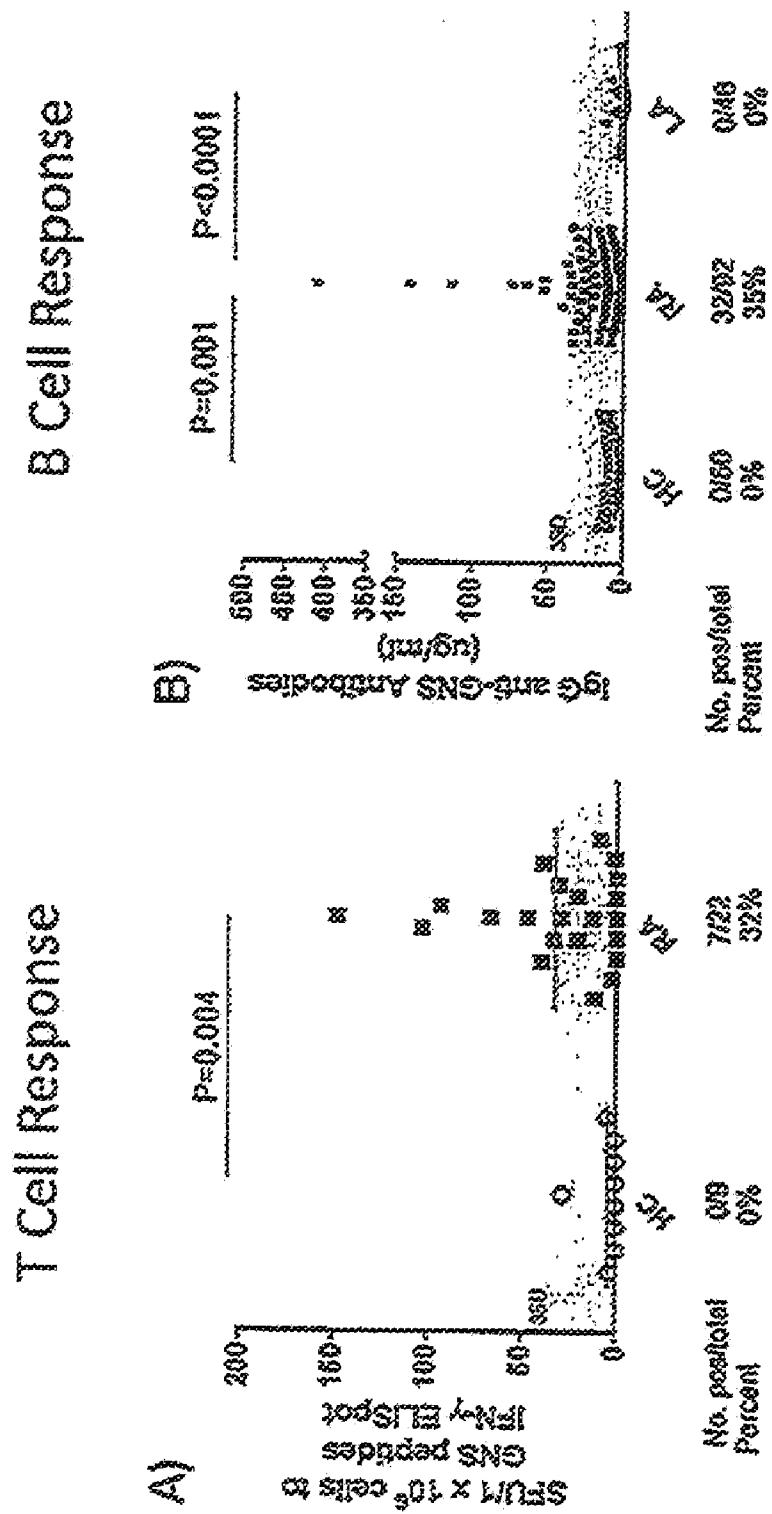
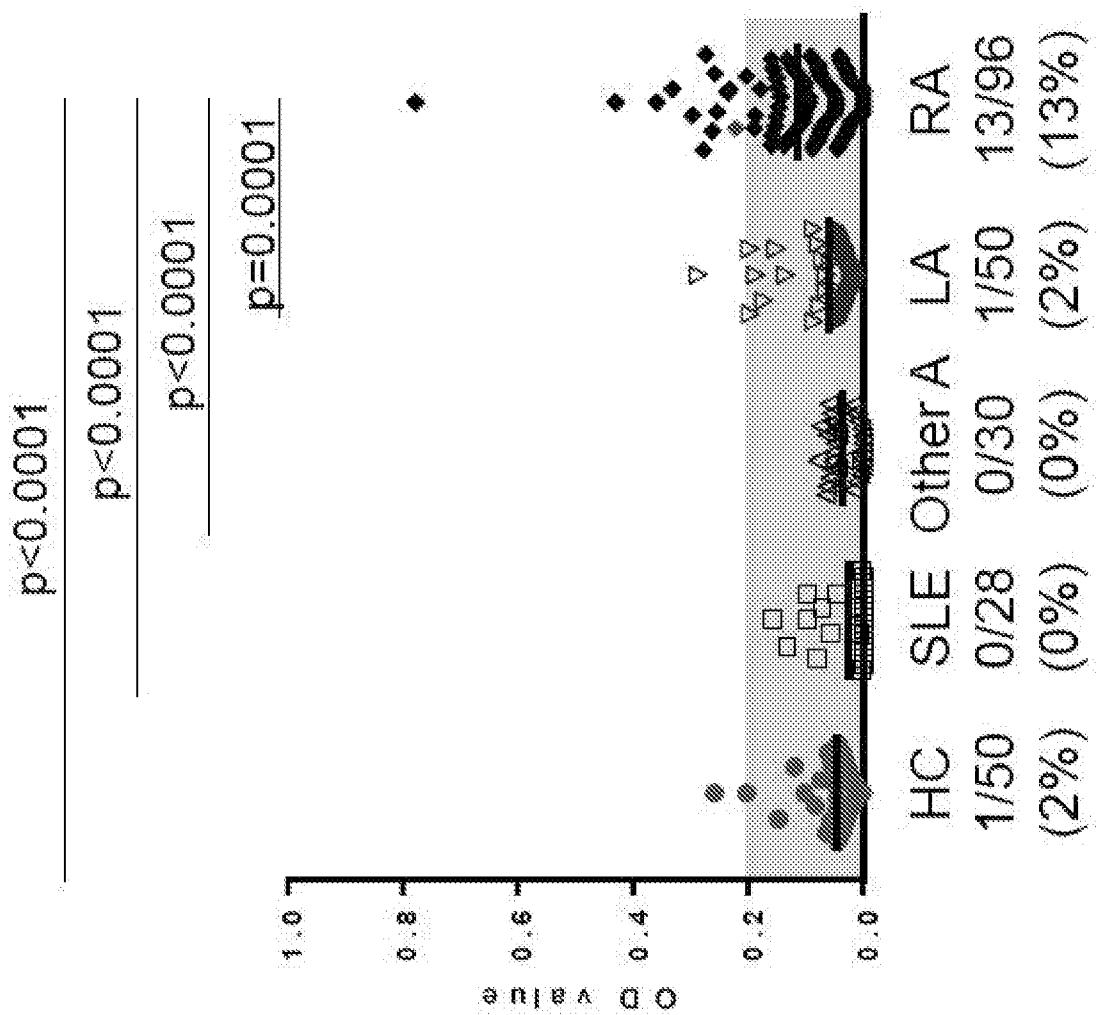


FIG. 7

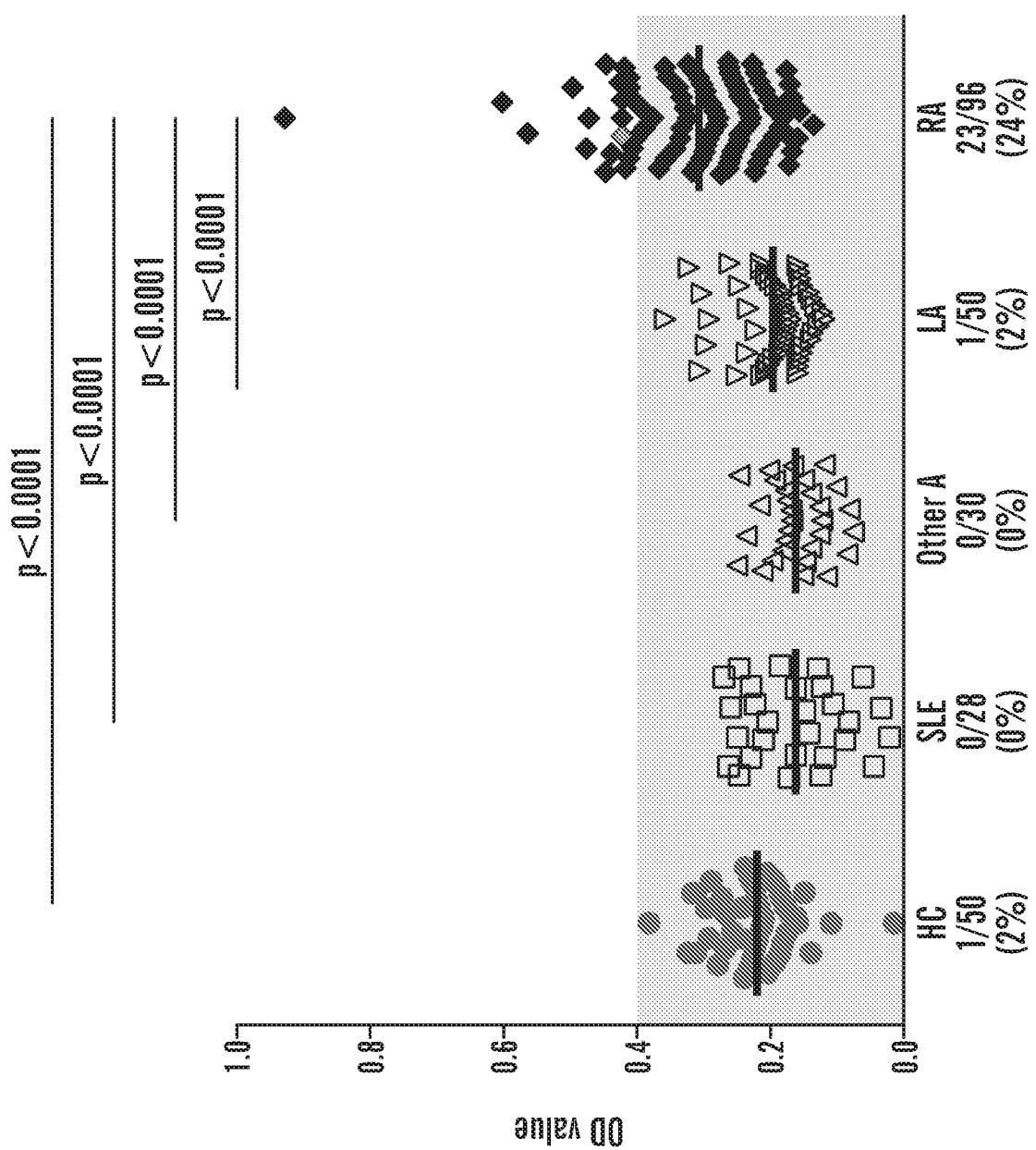


FIG. 8

FIG. 9

1 MRLPLAPGR LRGSPRHLR SCSPALLLV LGGCLGVFGV AAGTRRPNW LLTDDQDEV
61 LGGMTPPLKKT KALIGEMGMT FSSAYVPSAL CCPRSRASILT GKYPHNHHWV NNTLEGNCSS
121 KSWQKIQEPN TFPAILRSMC GYQTFFAGKY LNEYGAPDAG GLEHVPLGWS YWYALEKNSK
181 YYNYTLSING KARKHGENYS VDYLTDVLAN VSLDFLDYKS NFEPEFFMMIA TPAPHSPWTA
241 APQYQKAFQN VFAPRNKNFN IHGTONKHWLI RQAKTPMTNS SIQFLDNAFR KRWQTLLSVD
301 DLVEKLVKRL EFTGELNNTY IFYTSDDNGYH TGQFSLPIDK RQLYEFDIKV PLLVRGPGIK
361 PNQTSKMLVA NIDLGPTILD IAGYDLNKTQ MDGMSLLPIL RGASNLTWRS DVIVEYQGEG
421 RNVTDPTCPS LSPGVSQCCFP DCVCEDAYNN TYACVRTMSA LWNLQYCEFD DQEVFVEVYN
481 LTADPDQITN IAKTIDPELL GKMNYRLMML QSCSGPTCRT PGVFDPGYRF DPRLMFSNRG
541 SVRTRRFSKH LL

FIG. 10

1 MSSSHSragQ SAAGAAPGGG VDTRDAEMPA TEKDLAEDAP WKKIQQNTFT RWCNEHLKCV
 61 SKRIANLQTD LSDGLRLIAL LEVLSQKKMH RKHNQRPFTFR QMQLENVNSVA LEFLDRESIK
 121 LVSIDSKAIV DGNLKLILGL IWTLILHYSI SMPMWDEEED EEAKKQTPKQ RLLGWIQNL
 181 PQLPITNFSR DWQSGRALGA LVDSCAPGLC PDWDSWDAK PVTNAREAMQ QADDWLGIPQ
 241 VITPEEIVDP NVDEHSVMTY LSQFPKAKLK PGAPLRPKLN PKKARAYGPG IEPTGNMMVK
 301 RAEEFTVETRS AGQGEVLVYV EDPAGHQEEA KVTANNDKNR TFSVWVVPFV TGTHKVTVLF
 361 AGQHIAKSPF EVYVDKSQGD ASKVTAQGPG LEPSGNIAINK TTYFEIFTAG AGTGEVEVWI
 421 QDPMGQKGTV EPQLEARGDS TYRCSYQPTM EGVHTVHVTFF AGVPIPRSPY TVTVGQACNP
 481 SACRAVGRRGL QPKGVRVKET ADFKWTKGAGSGELKVTVK GPKGEERVKQ KDLGDDGVYGF
 541 EYPMVPGTY IVTITWGGQN IGRSPFEVKV GTECGNQKVR AWGPGLEGGV VGKSADFVVE
 601 AIGDDVGTLG FSVEGPSQAK IECDDKGDGSC DVRYWPQEA GEYAVHVLCN SEDIRLSPFM
 661 ADIRDAPQDF HPDRVVKARGP GLEKTVAVN KPAEFTVDAK HGGKAPLRVQ VQDNNEGCPVE
 721 ALVKDNGNGT YSCSYVPRKP VKHTAMVSWG GVSIPNSPFR VNVGAGSHPN KVVKVYGPVVA
 781 KTGLKAHEPT YFTVDCAEAG QGDVSIGIKC APGVVGPSEA DIDFDIIRND NDTFTVKYTP
 841 RGAGSYTIMV LFADQATPTS PIRVKVEPSH DASKVKAEGP GLSRTGVELG KPTHFTVNAK
 901 AAGKGKLDVQ FSGLTKGDAV RDVDIIDHHHD NTYTVKYTPV QQGPVGVNVT YGGDPIPCKP
 961 FSVAVSPSLD LSKIKVSGLG EKVDVGKDQE FTVKSKGAGG QGKVASKIVG PSGAAVPCKV
 1021 EPGLGADNSV VRFLPREEGP YEVEVTVYDGV PVPGSPFPLE AVAPTKPSKV KAFGPGLQGG
 1081 SAGSPARFTI DTKGAGTTGGL GLTVEGPCEA QLECLDNGDG TCSVSYVPT PGDYNINILF
 1141 ADTHIPGSPF KAHVVPFCDA SKVKCSPGGL ERATAGEVGGQ FQVDCSSAGS AELTIEICSE
 1201 AGLPAEVYIQ DHGDGTHTIT YIPLCPGAYT VTIKYGGQPV PNFP SKLQVE PAVDTSGVQC
 1261 YGPGIEGQGV FREATTEFSV DARALTQTTGG PHVKARVANP SGNLTETYVQ DRGDGMYKVE
 1321 YTPYEEGLHS VDVTYDGSPV PSSPFQVPVT EGCDPSRVRV HGPGIQSGTT NKPKNKFTVET
 1381 RGAGTGGLGL AVEGPSEAKM SCMDNKGSC SVEYIPIYEAG TYSLNVTYGG HQVPGSPFKV

FIG. 10 cont'd

1441 PVHDVTDASK VKCSGPGLSP GMVRANLPQS FQVDTSKAGV APLQVKVQGP KGLVEPVDV
1501 DNADGTQTVN YVPSREGPYS ISVLYGDEEV PRSPFKVKVL PTHDASKVKA SGPGNNTGV
1561 PASLPVEFTI DAKDAGEGLL AVQITDPEGK PKKTHIQDNH DGTYTWAYVP DVTGRYTI
1621 KYGGDEIPFS PYRVRRAWPTG DASKCTVTVS IGGHGLGAGI GPTIQIGEET VITVDTKAAG
1681 KGKVTCVCT PDGSEVDVVDV VENEDGTFDI FYTAPQPGKY VICVRFGEH VPNSPFQVTA
1741 LAGDQPSVQP PLRSQQQLAPQ YTYAQGGQQT WAPERPLVGV NGLDVTSLRP FDLVIPFTIK
1801 KGEITGEVRM PSGKVAQPTI TDNKDGTVTV RYAPSEAGLH EMDIRYDNMH IPGSPLQFVV
1861 DYVNCGHVTY YGPGLTHGVV NKPATFTVNT KDAGEGGLSL AIEGPSKAEI SCTDNQDGTC
1921 SVSYLPVLPG DYSILVKYNE QHVPGSPFTA RVTGDDSMRM SHLKVGSAAD IPINISETDL
1981 SLLTATVPP SGREEPCLLK RLRRNGHVGIS FVPKETGEHL VHVKKNGQHV ASSPIPVVIS
2041 QSEIGDASRV RVSGQQLHEG HTFEPAEFII DTRDAGYGGGL SLSIEGPSKV DINTEDLEDG
2101 TCRVTVCPTE PGNYIINIKF ADQHVPGSPF SVKVTGEGRV KESITRRRRRA PSVANVGSHC
2161 DLSLKIPIS IQDMTAQVTS PSGKTHEAEI VEGENHTYCI RFVPAEMGTH TVSVKYKGQH
2221 VPGSPFQFTV GPLGEGGGAHK VRAGGPGLER AEAGVPAEFS IWTREAGAGG LAIAVEGPSK
2281 AEISFEDRKD GSCGVAYVQ EPGDYEVSVK FNEEHIPDSP FVVPVASPSG DARRLTVSSL
2341 QESGLKVNQP ASFAVSINGA KGAIDAKVHS PSGALEECCYV TEIDQDKYAV RFIPRENGVY
2401 LIDVKFNGTH IPGSPFKIRV GEPGHGGDPG LVSAYGAGLE GGVGTGNPAEF VVNTSNAGAG
2461 ALSVTIDGPS KVKKMDCQECP EGYRVTYTPM APGSYLSIK YGGPYHIGGS PFKAKVTGPR
2521 LVSNHSIHET SSVFVDSLTK ATCAPQHGAP GPGPADASKV VAKGLGLSKA YVGQKSSFTV
2581 DCSKAGNNML LVGVHGPRTP CEEILVKHVG SRLYSVSYLL KDKGEYTLVV KWGDEHIPGS
2641 PYRVVVVP