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- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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- (54) **Title:** HUMAN MONOCLONAL ANTIBODIES THAT ENHANCE PAD4 FOR USE IN AUTOIMMUNE DISEASES

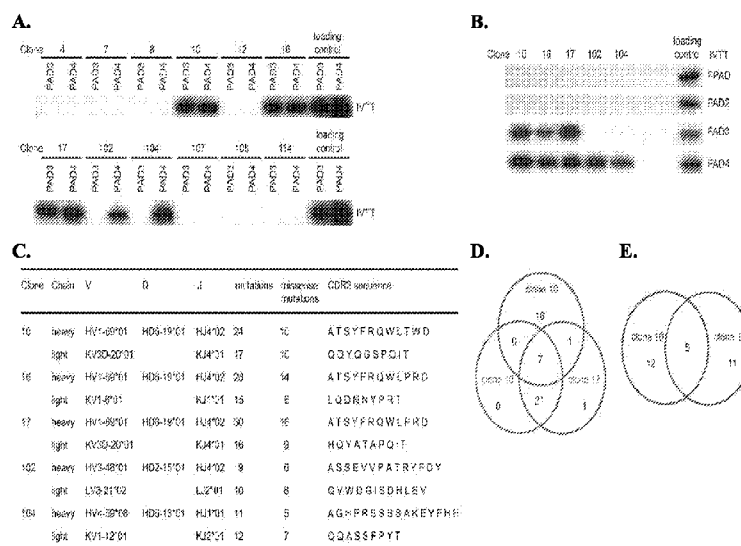


FIG. 1A-E

- (57) **Abstract:** Disclosed herein are antibodies and compositions comprising monoclonal antibodies that can be used to enhance PAD4 activity in a subject.

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HUMAN MONOCLONAL ANTIBODIES THAT ENHANCE PAD4 FOR USE IN AUTOIMMUNE DISEASES

RELATED APPLICATION INFORMATION

[0001] This application claims priority to U.S. Application No. 63/464,249, filed on May 05, 2023, the contents of which are herein incorporated by reference.

SEQUENCE LISTING STATEMENT

[0002] The contents of the electronic sequence listing titled JHU-43096-601-ST26.xml (Size: 14,536 bytes; and Date of Creation: April 24, 2024) is herein incorporated by reference in its entirety.

FIELD

[0003] The present disclosure relates to monoclonal antibodies that enhance PAD4 activity in a subject, compositions comprising these antibodies, and methods of using these antibodies to develop treatments for disease, such as, for example, the treatment in autoimmune diseases.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0004] None.

BACKGROUND

[0005] The examination of monoclonal antibodies, derived from cases in which patients with autoimmune diseases, has yielded crucial insights into the origins of the humoral response against self-antigens¹⁻⁵ and mechanisms of autoantibody pathogenicity⁶⁻⁸. While autoantibodies in rheumatic diseases were first described in rheumatoid arthritis (RA)⁹, the molecular characterization of RA-specific antibodies lagged significantly behind other diseases. The discovery that citrullinated proteins are major targets of the humoral response in RA has renewed interest in further characterizing the autoantibody repertoire in this disease^{3,10-14}.

[0006] Citrullinated proteins are generated post-translationally by the peptidylarginine deiminases (PADs), a family of five calcium-dependent enzymes (termed PAD1-4 and 6) that convert arginine residues to citrulline residues¹⁵. At least two major groups of autoantibodies have been linked to the pathogenic role of citrullination in RA: 1) anti-citrullinated protein antibodies (ACPAs), which likely drive effector functions by forming immune complexes¹⁶ and

2) anti-PAD4 antibodies that assist in the production of citrullinated antigens by enhancing the activity of PAD4¹⁷. Antibody-mediated PAD4 activation is driven by a subgroup of anti-PAD4 antibodies initially recognized by their cross-reactivity with PAD3¹⁷. These PAD3/PAD4 cross-reactive antibodies (anti-PAD3/4XR) enhance PAD4 activity by increasing the sensitivity of the enzyme to calcium. Together, ACPAs and antibodies to PAD4 may create a feed-forward loop that promotes effector functions via immune complex formation and autoantigen production.

SUMMARY

[0007] The present disclosure relates to an isolated or purified monoclonal antibodies comprising:

[0008] a heavy variable chain comprising SEQ ID NO:1 and a light variable chain comprising SEQ ID NO:2;

[0009] a heavy variable chain comprising SEQ ID NO:3 and a light variable chain comprising SEQ ID NO:4;

[0010] a heavy variable chain comprising SEQ ID NO:5 and a light variable chain comprising SEQ ID NO:6;

[0011] a heavy variable chain comprising SEQ ID NO:7 and a light variable chain comprising SEQ ID NO:8; or

[0012] a heavy variable chain comprising SEQ ID NO:9 and a light variable chain comprising SEQ ID NO:10.

[0013] In some aspects, the above described monoclonal antibody is a human monoclonal antibody or a derivative or variant thereof. In another aspect the monoclonal antibody is a humanized antibody or a derivative or variant thereof. In still another aspect, the monoclonal antibody is a chimeric antibody or a derivative or variant thereof. In still yet another aspect, the monoclonal antibody is a bi-specific antibody or a derivative or variant thereof. In some aspects the monoclonal antibody is an IgG antibody.

[0014] In another aspect, the present disclosure relates to a composition comprising at least one of the above monoclonal antibodies.

[0015] In yet another aspect, the composition of the present disclosure further comprises an excipient or at least one pharmaceutically acceptable carrier.

[0016] In still a further aspect, the present disclosure relates to a kit comprising one or more of the above described compositions.

[0017] In still yet a further aspect, the present disclosure relates to a method of increasing PAD4 in a subject, the method comprising administering an effective amount of one of the above antibodies or compositions to a subject (such as a human, mouse, rat, rabbit, etc.).

[0018] In still yet a further aspect, the present disclosure relates to a method of screening for an agent that inhibits PAD4. In this aspect, the method comprises:

[0019] a. administering to an animal at least one of the above-described monoclonal antibodies or compositions to an animal (e.g., a mouse, rat, rabbit, goat, dog, cat, etc.) to increase the level of PAD4 in the animal;

[0020] b. administering at least one agent to the animal having an increased level of PAD4; and

[0021] c. determining if the level of PAD4 in the animal has decreased, increased, or remained the unchanged after administration of the agent to the animal.

[0022] In some aspects, the agent to administered to the animal is a small molecule, a biologic, an antibody-drug conjugate, a peptide, a microRNA, a short interfering RNA, or any combinations thereof.

Other aspects and embodiments of the disclosure will be apparent in light of the following detailed description and accompanying figures.

DESCRIPTION OF THE FIGURES

[0023] **FIG. 1** shows the characterization of anti-PAD4 monoclonal antibodies derived from RA memory B cells. (FIG. 1A) Monoclonal antibodies with reactivity to PAD3/4 or PAD4 were screened by IP using [³⁵S]methionine-labeled PAD3 and PAD4 as antigens. Representative data from 12 of 32 monoclonal antibodies are shown. (FIG. 1B) IP of radiolabelled *P. gingivalis* PAD (PPAD), PAD2, PAD3 and PAD4 using anti-PAD3/4XR and anti-PAD4-only monoclonal antibodies (3.4 nM antibody per IP assay). Radiolabeled products directly resolved by SDS-PAGE are shown to demonstrate loading. (FIG. 1C) Ig gene usage, mutation number and CDR3 amino acid sequences of monoclonal antibodies to PAD3/4 (clones 10, 16 and 17) and antibodies with reactivity only to PAD4 (clones 102 and 104). (FIG. 1D and FIG. 1E) Venn diagrams show the number of SHM shared by clones 10, 16 and 17 in the heavy chain (FIG. 1D) and by clones

10 and 17 in the light chain (FIG. 1E). IPs were performed on 2 different occasions with similar results.

[0024] FIG. 2 shows the importance of SHM in antigen recognition by human monoclonal antibodies to PAD4. (FIG. 2A) Antibody binding to PAD3/PAD4 was addressed by IP of radiolabeled substrates using mutated (SHM) and germline (GL) reverted anti-PAD4 antibodies (3.4 nM antibody/assay). Antibodies 10GL and 17GL are the same antibody (10/17GL). (FIG. 2B) Anti-PAD4 antibodies and their germline variants were titrated from 0.4 pM to 685 nM against purified PAD4 by ELISA. (FIG. 2C) Half maximal effective concentration (EC_{50}) for anti-PAD4 binding were calculated from B (SD, standard deviation). (FIG. 2D) The heavy and light chain (IgH and IgL, respectively) of SHM and GL reverted anti-PAD4 antibodies were combined to generate monoclonal antibodies (lanes 1-4). Irrelevant (IR) IgH and IgL from an antibody with no reactivity to PADs were used to produce anti-PAD4 hybrid antibodies (lanes 5-8). Antibody binding to PAD3/PAD4 was addressed by IP (3.4 nM antibody/assay) using radiolabeled substrates. The experiments in FIG. 2A and FIG. 2D were performed on two separate occasions with similar results.

[0025] FIG. 3 shows the mutated and germline human anti-PAD4 antibodies are not polyreactive and have no cross-reactivity to citrullinated antigens. (FIG. 3A) The specificity of mutated and GL anti-PAD4 monoclonal antibodies against ssDNA, dsDNA, LPS, insulin or cardiolipin was addressed by ELISA. The assays were performed in duplicate using serial dilutions of monoclonal antibody (stock 6.8 μ M) or human SLE serum as positive control. (FIG. 3B) Reactivity of SHM and GL anti-PAD4 monoclonal antibodies to cyclic citrullinated peptide 3 (CCP3) was addressed by ELISA. The assays were performed in duplicate.

[0026] FIG. 4 shows the functional characterization of mutated (SHM) and germline reverted (GL) anti-PAD4 monoclonal antibodies. (FIG. 4A and FIG. 4B) Histone H3 (H3) was citrullinated with PAD4 at 0.2 mM (A) or 0.5 mM (B) calcium in presence of mutated or GL anti-PAD4 antibodies (0.14 μ M) (lanes 4-14). Clone 16 also included the analysis of antibodies containing mutated heavy chain (IgH) combined with GL light chain (IgL-GL) (lane 9) or an irrelevant IgL (IgL-IR) (lane 10), both with reactivity to PAD3 and PAD4. As controls, H3 and PAD4 were incubated without (lane 1) or with calcium (lanes 2 and 3) in the absence (lane 1 and 2) or presence (lane 3) of an irrelevant antibody with no reactivity to PADs (clone 119). IgH and H3 were detected by Ponceau S staining. PAD4 and citrullinated H3 (cit-H3) were detected by

immunoblotting. The experiments were performed on three separate occasions with similar results. (FIG. 4C) Competition assays among different anti-PAD4 monoclonal antibodies were performed by measuring murinized antibody binding to PAD4 following incubation with increasing amounts of competing (human) antibodies (murinized:human antibody ratio from 1:0 to 1:100). The “% Binding” represents the amount of murinized antibody bound to PAD4 in relation to the amount of antibody binding observed in the absence of competing antibody. The data are from the average of 2 independent assays.

[0027] FIG. 5 shows that agonistic antibodies partially restore the activity of calcium-binding site 5 (Ca5) mutant PAD4. Histone H3 (H3) was incubated with wild type (WT) or PAD4 mutants at calcium-binding sites Ca3 and Ca5 (D¹⁶⁵A), Ca5 (D¹⁶⁸A), Ca3 and Ca4 (D¹⁷⁹A) or Ca4 (D³⁸⁸A) in the presence of 10 mM CaCl₂, with or without anti-PAD4 monoclonal antibodies. After 2 hrs at 37°C, PAD4 and citrullinated H3 (Cit-H3) were detected by immunoblotting. IgH and H3 were detected by Ponceau S staining as loading control. The experiments were performed on two separate occasions with similar results.

[0028] FIG. 6 shows the genetic characteristics and functional features of RA-derived monoclonal antibodies to PAD4. Antibodies lineage trees were generated by comparison of Ig VH sequences. Monoclonal antibodies were derived from 2 patients with RA (RA-1 and RA-3). Clonally related antibodies were identified based on sequences derived from a single germline rearrangement characterized by the same V, D and J gene usage, CDR3 length and sequence. The numbers beside the arrows represent the number of mutations acquired in the heavy (VHSHM) and light (VLSHM) chain. The germline and mutated antibody reactivities are specified, as well as their capacity to enhance PAD4 activity.

[0029] FIG. 7 shows FACS analysis of mWasabi-PAD4⁺ memory B cells. B cells pre-enriched by negative selection were stained with antibodies against human CD3, CD14, CD19, CD20, CD38, CD27 and IgM, as well as mWasabi-PAD4. The gating strategy to identify CD3⁻CD14⁻CD19⁺CD20⁺CD27⁺CD38⁻IgM⁻ mWasabi-PAD4⁺ cells is shown from one healthy control and one representative patient with RA. In this patient, mWasabi-PAD4⁺ memory B cells represent 0.11% of the total memory B cells.

[0030] FIG. 8 shows germline reverted anti-PAD4 monoclonal antibodies have agonistic effect at high concentration. (FIG. 8A and FIG. 8B) Histone H3 (H3) was citrullinated with PAD4 at 1 mM calcium in presence of mutated (0.14 nM) or increasing amounts of germline reverted (0.14

μM , 0.46 μM or 1.4 μM) anti-PAD4 antibodies. As controls, H3 and PAD4 were incubated without (lane 1) or with calcium (lane 2) in the absence (lanes 1 and 2) or presence (FIG. 8B, lanes 3-5) of increasing amounts of an irrelevant antibody with no reactivity to PADs (clone 119). IgH and H3 were detected by Ponceau S staining. PAD4 and citrullinated histone H3 (cit-H3) were detected by immunoblotting. The experiments were performed on two separate occasions with similar results.

[0031] FIG. 9 shows mutated, and germline reverted anti-PAD4 antibodies have no effect on PAD4 activity at high calcium concentrations. Histone H3 (H3) was citrullinated with PAD4 at 5 mM calcium in presence of mutated or germline anti-PAD4 antibodies. As controls, H3 and PAD4 were incubated without (lane 1) or with calcium (lanes 2) in the absence (lane 1 and 2) or presence (lane 3) of an irrelevant antibody with no reactivity to PADs (clone 119). IgH and H3 were detected by Ponceau S staining. PAD4 and citrullinated H3 (cit-H3) were detected by immunoblotting. The experiments were performed on two separate occasions with similar results.

[0032] FIG. 10 shows binding efficiency of murinized anti-PAD4 antibodies. (FIG. 10A) Murinized anti-PAD4 antibodies were titrated from 0.4 pM to 685 nM against purified PAD4 by ELISA. (FIG. 10B) Half maximal effective concentration (EC_{50}) for anti-PAD4 binding were calculated from A.

[0033] FIG. 11 shows characterization of immunodominant regions recognized by anti-PAD4 monoclonal antibodies. (FIG. 11A) PAD4 comprises an N-terminal domain (NTD, residues 1 to 300), further divided into subdomain 1 (NT1, amino acids 1 to 132) and subdomain 2 (NT2, amino acids 132 to 300), and a C-terminal domain (CTD, amino acids 301 to 663). Truncated proteins generated for IP assays included the NTD, both subdomains, the CTD and NT2 plus the CTD (NT2+C). (FIG. 11B and FIG. 11C) IP of IVTT-radiolabeled full length PAD4 (only in FIG. 11B) and truncated proteins (NTD, NT1, NT2, CTD and NT2+C) using anti-PAD4 monoclonal antibodies. Immune complexes were formed by incubating the IVTT products with antibodies at 3.4 nM for 2 hrs (FIG. 11B) or at 34 nM overnight (FIG. 11C). IVTT products directly resolved by SDS-PAGE are shown to demonstrate loading. The experiments were performed on two separate occasions with similar results.

[0034] FIG. 12A shows the amino acid sequences of the monoclonal antibodies of the present disclosure.

[0035] FIG. 12B shows the amino acid sequences of the monoclonal antibodies of the present disclosure.

DETAILED DESCRIPTION

[0036] Disclosed herein are compositions comprising monoclonal antibodies as well as compositions comprising said antibodies, methods of using said antibodies to develop and screen for new treatments (e.g., therapeutic agents) for autoimmune disease and kits containing said antibodies and compositions.

[0037] Section headings as used in this section and the entire disclosure herein are merely for organizational purposes and are not intended to be limiting.

1. Definitions

[0038] The terms “comprise(s),” “include(s),” “having,” “has,” “can,” “contain(s),” and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms, or words that do not preclude the possibility of additional acts or structures. As used herein, comprising a certain sequence or a certain SEQ ID NO usually implies that at least one copy of said sequence is present in recited peptide or polynucleotide. However, two or more copies are also contemplated. The singular forms “a,” “and,” and “the” include plural references unless the context clearly dictates otherwise. The present disclosure also contemplates other embodiments “comprising,” “consisting of,” and “consisting essentially of,” the embodiments or elements presented herein, whether explicitly set forth or not.

[0039] For the recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the number 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[0040] An “Affinity” as used herein refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., a binding protein, such as, an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (KD). Affinity can be measured by common methods known in the art. For example, the affinity of an antibody for an

antigen or epitope of interest can be measured using any art-recognized assay. Such methods include, for example, fluorescence activated cell sorting (FACS), separable beads (e.g., magnetic beads), antigen panning, and/or ELISA (see, e.g., Janeway et al. (eds.), *Immunobiology*, 5th ed., Garland Publishing, New York, N.Y., 2001).

[0041] An “Antibody” and “antibodies” as used herein refers to monoclonal antibodies, chimeric antibodies, multispecific antibodies, human antibodies, humanized antibodies (fully or partially humanized), animal antibodies such as, but not limited to, a bird (for example, a duck or a goose), a shark, a whale, and a mammal, including a non-primate (for example, a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, etc.) or a non-human primate (for example, a monkey, a chimpanzee, etc.), recombinant antibodies, chimeric antibodies, single-chain Fvs (“scFv”), single chain antibodies, single domain antibodies, Fab fragments, F(ab') fragments, F(ab')₂ fragments, disulfide-linked Fvs (“sdFv”), and anti-idiotypic (“anti-Id”) antibodies, dual-domain antibodies, dual variable domain (DVD) or triple variable domain (TVD) antibodies (dual-variable domain immunoglobulins and methods for making them are described in Wu, C., *et al.*, *Nature Biotechnology*, 25(11):1290-1297 (2007) and PCT International Application WO 2001/058956, the contents of each of which are herein incorporated by reference), and functionally active epitope-binding fragments of any of the above. Antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, namely, molecules that contain an analyte-binding site. Immunoglobulin molecules can be of any type (for example, IgG, IgE, IgM, IgD, IgA, and IgY), class (for example, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), or subclass.

[0042] An “Antibody fragment” as used herein refers to a portion of an intact antibody comprising the antigen-binding site or variable region. The portion does not include the constant heavy chain domains (*i.e.*, CH2, CH3, or CH4, depending on the antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include, but are not limited to, Fab fragments, Fab' fragments, Fab'-SH fragments, F(ab')₂ fragments, Fd fragments, Fv fragments, diabodies, single-chain Fv (scFv) molecules, single-chain polypeptides containing only one light chain variable domain, single-chain polypeptides containing the three CDRs of the light-chain variable domain, single-chain polypeptides containing only one heavy chain variable region, and single-chain polypeptides containing the three CDRs of the heavy chain variable region.

[0043] An “Autoimmune disease” as used herein refers to disorders associated with an undesired immune response in a subject. Autoimmune diseases fall in two broad categories: organ-specific and systemic. Autoimmune diseases include, without limitation, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), type I diabetes mellitus, type II diabetes mellitus, multiple sclerosis (MS), neuromyelitis optica, immune-mediated infertility such as premature ovarian failure, scleroderma, Sjogren's disease, vitiligo, alopecia (baldness), polyglandular, Grave's disease, hypothyroidism, polymyositis, pemphigus vulgaris, pemphigus foliaceus, inflammatory bowel disease including Crohn's disease and ulcerative colitis, autoimmune hepatitis including that associated with hepatitis B virus (HBV) and hepatitis C virus (HCV), hypopituitarism, graft-versus-host disease (GvHD), myocarditis, Addison's disease, autoimmune skin diseases, uveitis, pernicious anemia, and hypoparathyroidism.

[0044] Autoimmune diseases may also include, without limitation, Hashimoto's thyroiditis, Type I and Type II autoimmune polyglandular syndromes, paraneoplastic pemphigus, bullous pemphigoid, dermatitis herpetiformis, linear IgA disease, epidermolysis bullosa acquisita, erythema nodosa, pemphigoid gestationis, cicatricial pemphigoid, mixed essential cryoglobulinemia, chronic bullous disease of childhood, hemolytic anemia, thrombocytopenic purpura, Goodpasture's syndrome, autoimmune neutropenia, myasthenia gravis, Eaton-Lambert myasthenic syndrome, stiff-man syndrome, acute disseminated encephalomyelitis, Guillain-Barre syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, multifocal motor neuropathy with conduction block, chronic neuropathy with monoclonal gammopathy, opsonoclonus-myoelonus syndrome, cerebellar degeneration, encephalomyelitis, retinopathy, primary biliary sclerosis, sclerosing cholangitis, gluten-sensitive enteropathy, ankylosing spondylitis, reactive arthritides, polymyositis/dermatomyositis, mixed connective tissue disease, Bechet's syndrome, psoriasis, polyarteritis nodosa, allergic angitis and granulomatosis (Churg-Strauss disease), polyangiitis overlap syndrome, hypersensitivity vasculitis, Wegener's granulomatosis, temporal arteritis, Takayasu's arteritis, Kawasaki's disease, isolated vasculitis of the central nervous system, thromboangiitis obliterans, sarcoidosis, glomerulonephritis, and cryopathies. These conditions are well known in the medical arts and are described, for example, in *Harrison's Principles of Internal Medicine*, 14th ed., Fauci A S et al., eds., New York: McGraw-Hill, 1998.

[0045] A “Binding protein” as used herein refers to a monomeric or multimeric protein that binds to and forms a complex with a binding partner, such as, for example, a polypeptide, an antigen, a chemical compound or other molecule, or a substrate of any kind. A binding protein specifically binds a binding partner. Binding proteins include antibodies, as well as antigen-binding fragments thereof and other various forms and derivatives thereof as are known in the art and described herein below, and other molecules comprising one or more antigen-binding domains that bind to an antigen molecule or a particular site (epitope) on the antigen molecule. Accordingly, a binding protein includes, but is not limited to, an antibody a tetrameric immunoglobulin, an IgG molecule, an IgG1 molecule, a monoclonal antibody, a chimeric antibody, a CDR-grafted antibody, a humanized antibody, a human antibody, an affinity matured antibody, and fragments of any such antibodies that retain the ability to bind to an antigen.

[0046] A “Bispecific antibody” as used herein refers to a full-length antibody that is generated by quadroma technology (see Milstein *et al.*, *Nature*, 305(5934): 537-540 (1983)), by chemical conjugation of two different monoclonal antibodies (see, Staerz *et al.*, *Nature*, 314(6012): 628-631 (1985)), or by knob-into-hole or similar approaches, which introduce mutations in the Fc region (see Holliger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90(14): 6444-6448 (1993)), resulting in multiple different immunoglobulin species of which only one is the functional bispecific antibody. A bispecific antibody binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). By this definition, a bispecific antibody has two distinct antigen-binding arms (in both specificity and CDR sequences) and is monovalent for each antigen to which it binds to. Bispecific antibodies can be made using the techniques as described in Brinkmann *et al.*, *MABS*, 9(2):182-212 (2017), the contents of which are herein incorporated by reference.

[0047] A “CDR” as used herein refers to the “complementarity determining region” within an antibody variable sequence. There are three CDRs in each of the variable regions of the heavy chain and the light chain. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted “CDR1”, “CDR2”, and “CDR3”, for each of the variable regions. The term “CDR set” as used herein refers to a group of three CDRs that occur in a single variable region that binds the antigen. An antigen-binding site, therefore, may include six CDRs, comprising the CDR set from each of a heavy and a light chain variable region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2, or CDR3) may be referred to as a “molecular recognition

unit". Crystallographic analyses of antigen-antibody complexes have demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units may be primarily responsible for the specificity of an antigen-binding site. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

[0048] The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as "Kabat CDRs". Chothia and coworkers (Chothia and Lesk, *J. Mol. Biol.*, 196: 901-917 (1987); and Chothia *et al.*, *Nature*, 342: 877-883 (1989)) found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as "L1", "L2", and "L3", or "H1", "H2", and "H3", where the "L" and the "H" designate the light chain and the heavy chain regions, respectively. These regions may be referred to as "Chothia CDRs", which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been described by Padlan, *FASEB J.*, 9: 133-139 (1995), and MacCallum, *J. Mol. Biol.*, 262(5): 732-745 (1996). Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat- or Chothia-defined CDRs.

[0049] A "chimeric antibody" as used herein refers to a molecule in which different portions of the antibody are derived from different immunoglobulin molecules such as antibodies having a variable region derived from a non-human antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, 1985, *Science* 229:1202; Oi *et al.*, 1986, *BioTechniques* 4:214; Gillies *et al.*, 1989, *J. Immunol. Methods* 125:191-202; and U.S. Pat. Nos. 6,311,415, 5,807,715, 4,816,567, and 4,816,397. Chimeric antibodies comprising one or more CDRs from a non-human species and framework

regions from a human immunoglobulin molecule can be produced using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; International Publication No. WO 91/09967; and U.S. Pat. Nos. 5,225,539, 5,530,101, and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, 1991, *Molecular Immunology* 28(4/5):489-498; Studnicka et al., 1994, *Protein Engineering* 7:805; and Roguska et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:969), and chain shuffling (U.S. Pat. No. 5,565,332).

[0050] A “Derivative” as used herein refers to an antibody or antigen-binding fragment thereof that immunospecifically binds to an antigen, but which comprises, one, two, three, four, five or more amino acid substitutions, additions, deletions or modifications relative to a “parental” (or wild-type) molecule. Such amino acid substitutions or additions may introduce naturally occurring (i.e., DNA-encoded) or non-naturally occurring amino acid residues. In some aspects, “derivative” encompasses, for example, chimeric or humanized variants of any of antibodies 1.3, 4.5 or 7.8, as well as variants having altered CH1, hinge, CH2, CH3 or CH4 regions, so as to form, for example antibodies, etc., having variant Fc regions that exhibit enhanced or impaired effector or binding characteristics. In other aspects, “derivative” additionally encompasses non-amino acid modifications, for example, amino acids that may be glycosylated (e.g., have altered mannose, 2-N-acetylglucosamine, galactose, fucose, glucose, sialic acid, 5-N-acetylneuraminic acid, 5-glycolneuraminic acid, etc. content), acetylated, pegylated, phosphorylated, amidated, derivatized by known protecting/blocking groups, proteolytic cleavage, linked to a cellular ligand or other protein, etc. In some embodiments, the altered carbohydrate modifications modulate one or more of the following: solubilization of the antibody, facilitation of subcellular transport and secretion of the antibody, promotion of antibody assembly, conformational integrity, and antibody-mediated effector function. In a specific embodiment the altered carbohydrate modifications enhance antibody mediated effector function relative to the antibody lacking the carbohydrate modification. Carbohydrate modifications that lead to altered antibody mediated effector function are well known in the art (for example, see Shields, R. L. et al. (2002) “*Lack Of Fucose On Human IgG N-Linked Oligosaccharide Improves Binding To Human Fcγγ RIII And Antibody-Dependent Cellular Toxicity*,” *J. Biol. Chem.* 277(30): 26733-26740; Davies J. et al. (2001) “*Expression Of GnTIII In A Recombinant Anti-CD20 CHO Production Cell Line: Expression Of Antibodies With Altered Glycoforms Leads To An Increase In ADCC Through Higher Affinity For FC γγ RIII*,”

Biotechnology & Bioengineering 74(4): 288-294). Methods of altering carbohydrate contents are known to those skilled in the art, see, e.g., Wallick, S. C. et al. (1988) “*Glycosylation Of A VH Residue Of A Monoclonal Antibody Against Alpha (1----6) Dextran Increases Its Affinity For Antigen*,” J. Exp. Med. 168(3): 1099-1109; Tao, M. H. et al. (1989) “*Studies Of Aglycosylated Chimeric Mouse-Human IgG. Role Of Carbohydrate In The Structure And Effector Functions Mediated By The Human IgG Constant Region*,” J. Immunol. 143(8): 2595-2601; Routledge, E. G. et al. (1995) “*The Effect Of Aglycosylation On The Immunogenicity Of A Humanized Therapeutic CD3 Monoclonal Antibody*,” Transplantation 60(8):847-53; Elliott, S. et al. (2003) “*Enhancement Of Therapeutic Protein In Vivo Activities Through Glycoengineering*,” Nature Biotechnol. 21:414-21; Shields, R. L. et al. (2002) “*Lack Of Fucose On Human IgG N-Linked Oligosaccharide Improves Binding To Human Fcγγ RIII And Antibody-Dependent Cellular Toxicity*,” J. Biol. Chem. 277(30): 26733-26740).

[0051] A “Dual-specific antibody” as used herein to refer to a full-length antibody that can bind two different antigens (or epitopes) in each of its two binding arms (a pair of HC/LC) (see PCT publication WO 02/02773). Accordingly, a dual-specific binding protein has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen to which it binds.

[0052] A “Dual variable domain” as used herein to refer to two or more antigen binding sites on a binding protein, which may be divalent (two antigen binding sites), tetravalent (four antigen binding sites), or multivalent binding proteins. DVDs may be monospecific, *i.e.*, capable of binding one antigen (or one specific epitope), or multispecific, *i.e.*, capable of binding two or more antigens (*i.e.*, two or more epitopes of the same target antigen molecule or two or more epitopes of different target antigens). A preferred DVD binding protein comprises two heavy chain DVD polypeptides and two light chain DVD polypeptides and is referred to as a “DVD immunoglobulin” or “DVD-Ig.” Such a DVD-Ig binding protein is thus tetrameric and reminiscent of an IgG molecule but provides more antigen binding sites than an IgG molecule. Thus, each half of a tetrameric DVD-Ig molecule is reminiscent of one half of an IgG molecule and comprises a heavy chain DVD polypeptide and a light chain DVD polypeptide, but unlike a pair of heavy and light chains of an IgG molecule that provides a single antigen binding domain, a pair of heavy and light chains of a DVD-Ig provide two or more antigen binding sites.

[0053] Each antigen binding site of a DVD-Ig binding protein may be derived from a donor ("parental") monoclonal antibody and thus comprises a heavy chain variable domain (VH) and a light chain variable domain (VL) with a total of six CDRs involved in antigen binding per antigen binding site. Accordingly, a DVD-Ig binding protein that binds two different epitopes (*i.e.*, two different epitopes of two different antigen molecules or two different epitopes of the same antigen molecule) comprises an antigen binding site derived from a first parental monoclonal antibody and an antigen binding site of a second parental monoclonal antibody.

[0054] A description of the design, expression, and characterization of DVD-Ig binding molecules is provided in PCT Publication No. WO 2007/024715, U.S. Patent No. 7,612,181, and Wu *et al.*, *Nature Biotech.*, 25: 1290-1297 (2007). A preferred example of such DVD-Ig molecules comprises a heavy chain that comprises the structural formula VD1-(X1)_n-VD2-C-(X2)_n, wherein VD1 is a first heavy chain variable domain, VD2 is a second heavy chain variable domain, C is a heavy chain constant domain, X1 is a linker with the proviso that it is not CH1, X2 is an Fc region, and n is 0 or 1, but preferably 1; and a light chain that comprises the structural formula VD1-(X1)_n-VD2-C-(X2)_n, wherein VD1 is a first light chain variable domain, VD2 is a second light chain variable domain, C is a light chain constant domain, X1 is a linker with the proviso that it is not CH1, and X2 does not comprise an Fc region; and n is 0 or 1, but preferably 1. Such a DVD-Ig may comprise two such heavy chains and two such light chains, wherein each chain comprises variable domains linked in tandem without an intervening constant region between variable regions, wherein a heavy chain and a light chain associate to form tandem functional antigen binding sites, and a pair of heavy and light chains may associate with another pair of heavy and light chains to form a tetrameric binding protein with four functional antigen binding sites. In another example, a DVD-Ig molecule may comprise heavy and light chains that each comprise three variable domains (VD1, VD2, VD3) linked in tandem without an intervening constant region between variable domains, wherein a pair of heavy and light chains may associate to form three antigen binding sites, and wherein a pair of heavy and light chains may associate with another pair of heavy and light chains to form a tetrameric binding protein with six antigen binding sites.

[0055] In some aspects, a DVD-Ig binding protein not only binds the same target molecules bound by its parental monoclonal antibodies, but also possesses one or more desirable properties of one or more of its parental monoclonal antibodies. Preferably, such an additional property is

an antibody parameter of one or more of the parental monoclonal antibodies. Antibody parameters that may be contributed to a DVD-Ig binding protein from one or more of its parental monoclonal antibodies include, but are not limited to, antigen specificity, antigen affinity, potency, biological function, epitope recognition, protein stability, protein solubility, production efficiency, immunogenicity, pharmacokinetics, bioavailability, tissue cross reactivity, and orthologous antigen binding.

[0056] A “Fragment antigen-binding fragment” or “Fab fragment” as used herein refers to a fragment of an antibody that binds to antigens and that contains one antigen-binding site, one complete light chain, and part of one heavy chain. A Fab is a monovalent fragment consisting of the VL, VH, CL and CH1 domains. A Fab is composed of one constant and one variable domain of each of the heavy and the light chain. The variable domain contains the paratope (the antigen-binding site), comprising a set of complementarity determining regions, at the amino terminal end of the monomer. Each arm of the Y thus binds an epitope on the antigen. Fab fragments can be generated such as has been described in the art, e.g., using the enzyme papain, which can be used to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment, or can be produced by recombinant means.

[0057] A “F(ab')₂ fragment” as used herein refers to antibodies generated by pepsin digestion of whole IgG antibodies to remove most of the Fc region while leaving intact some of the hinge region. F(ab')₂ fragments have two antigen-binding F(ab) portions linked together by disulfide bonds, and therefore are divalent with a molecular weight of about 110 kDa. Divalent antibody fragments (F(ab')₂ fragments) are smaller than whole IgG molecules and enable a better penetration into tissue thus facilitating better antigen recognition in immunohistochemistry. The use of F(ab')₂ fragments also avoids unspecific binding to Fc receptor on live cells or to Protein A/G. F(ab')₂ fragments can both bind and precipitate antigens.

[0058] A “Framework” (FR) or “Framework sequence” as used herein may mean the remaining sequences of a variable region minus the CDRs. Because the exact definition of a CDR sequence can be determined by different systems (for example, see above), the meaning of a framework sequence is subject to correspondingly different interpretations. The six CDRs (CDR-L1, -L2, and -L3 of light chain and CDR-H1, -H2, and -H3 of heavy chain) also divide the framework regions on the light chain and the heavy chain into four sub-regions (FR1, FR2, FR3, and FR4) on each chain, in which CDR1 is positioned between FR1 and FR2, CDR2 between

FR2 and FR3, and CDR3 between FR3 and FR4. Without specifying the particular sub-regions as FR1, FR2, FR3, or FR4, a framework region, as referred by others, represents the combined FRs within the variable region of a single, naturally occurring immunoglobulin chain. As used herein, a FR represents one of the four sub-regions, and FRs represents two or more of the four sub-regions constituting a framework region.

[0059] Human heavy chain and light chain FR sequences are known in the art that can be used as heavy chain and light chain "acceptor" framework sequences (or simply, "acceptor" sequences) to humanize a non-human antibody using techniques known in the art. In one embodiment, human heavy chain and light chain acceptor sequences are selected from the framework sequences listed in publicly available databases such as V-base or in the international ImMunoGeneTics® (IMGT®) information system,

[0060] A "Functional antigen binding site" as used herein may mean a site on a binding protein (*e.g.*, an antibody) that is capable of binding a target antigen. The antigen binding affinity of the antigen binding site may not be as strong as the parent binding protein, *e.g.*, parent antibody, from which the antigen binding site is derived, but the ability to bind antigen must be measurable using any one of a variety of methods known for evaluating protein, *e.g.*, antibody, binding to an antigen. Moreover, the antigen binding affinity of each of the antigen binding sites of a multivalent protein, *e.g.*, multivalent antibody, herein need not be quantitatively the same.

[0061] A "human antibody" as used herein refers to antibodies developed in a transgenic animal (*e.g.*, mouse or rat) that have been genetically engineered with the human immunoglobulin locus (in contrast to humanized mAbs which are initially generated in wild type animal (*e.g.*, mouse or rat) with a native genome bearing the mouse immunoglobulin locus). Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences (see U.S. Pat. Nos. 4,444,887 and 4,716,111; and International Publication Nos. WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741). Human antibodies can be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic

stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized using conventional methodologies with a selected antigen, e.g., all or a portion of any of the polypeptides of antibodies A-E, described herein. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology (see, e.g., U.S. Pat. No. 5,916,771). The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93, which is incorporated herein by reference in its entirety). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International Publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923, 5,625,126, 5,633,425, 5,569,825, 5,661,016, 5,545,806, 5,814,318, and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.) and Medarex (Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[0062] A “humanized antibody” as used herein refers to an immunoglobulin comprising a human framework region and one or more CDR's from a non-human (usually a transgenic animal such as a mouse or rat) immunoglobulin. The non-human immunoglobulin providing the CDR's is called the “donor” and the human immunoglobulin providing the framework is called the “acceptor.” Constant regions need not be present, but if they are, they must be substantially identical to human immunoglobulin constant regions, i.e., at least about 85-90%, preferably about 95% or more identical. Hence, all parts of a humanized immunoglobulin, except possibly the CDR's, are substantially identical to corresponding parts of natural human immunoglobulin

sequences. A humanized antibody is an antibody comprising a humanized light chain and a humanized heavy chain immunoglobulin. For example, a humanized antibody would not encompass a typical chimeric antibody, because, e.g., the entire variable region of a chimeric antibody is non-human. One says that the donor antibody has been "humanized," by the process of "humanization," because the resultant humanized antibody is expected to bind to the same antigen as the donor antibody that provides the CDR's. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit, or a non-human primate having the desired specificity, affinity, and capacity. In some instances, Framework Region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin that immunospecifically binds to an Fc RIIIB polypeptide, that has been altered by the introduction of amino acid residue substitutions, deletions, or additions (i.e., mutations).

Humanized antibodies and methods for making such antibodies are described in, for example, European Patent Nos. EP 239,400, EP 592,106, and EP 519,596; International Publication Nos. WO 91/09967 and WO 93/17105; U.S. Pat. Nos. 5,225,539, 5,530,101, 5,565,332, 5,585,089, 5,766,886, and 6,407,213; and Padlan, 1991, *Molecular Immunology* 28(4/5):489-498; Studnicka et al., 1994, *Protein Engineering* 7(6):805-814; Roguska et al., 1994, *PNAS* 91:969-973; Tan et al., 2002, *J. Immunol.* 169:1119-1125; Caldas et al., 2000, *Protein Eng.* 13:353-360; Morea et al., 2000, *Methods* 20:267-79; Baca et al., 1997, *J. Biol. Chem.* 272:10678-10684; Roguska et al., 1996, *Protein Eng.* 9:895-904; Couto et al., 1995, *Cancer Res.* 55 (23 Supp):5973s-5977s; Couto et al., 1995, *Cancer Res.* 55:1717-22; Sandhu, 1994, *Gene* 150:409-10; Pedersen et al., 1994, *J. Mol. Biol.* 235:959-973; Jones et al., 1986, *Nature* 321:522-525; Reichmann et al., 1988, *Nature* 332:323-329; and Presta, 1992, *Curr. Op. Struct. Biol.* 2:593-596.

[0063] The terms “Identical” or “identity,” as used herein in the context of two or more polypeptide or polynucleotide sequences, can mean that the sequences have a specified percentage of residues that are the same over a specified region. The percentage can be calculated by optimally aligning the two sequences, comparing the two sequences over the specified region, determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the specified region, and multiplying the result by 100 to yield the percentage of sequence identity. In cases where the two sequences are of different lengths or the alignment produces one or more staggered ends and the specified region of comparison includes only a single sequence, the residues of the single sequence are included in the denominator but not the numerator of the calculation.

[0064] The terms “immunogen” and “antigen” are used interchangeably herein and refer to any molecule, compound, or substance that induces an immune response in an animal (e.g., a mammal). An “immune response” can entail, for example, antibody production and/or the activation of immune effector cells. An antigen in the context of the disclosure can comprise any subunit, fragment, or epitope of any proteinaceous or non-proteinaceous (e.g., carbohydrate or lipid) molecule that provokes an immune response in a mammal. By “epitope” is meant a sequence of an antigen that is recognized by a binding protein, an antibody or an antigen receptor. Epitopes also are referred to in the art as “antigenic determinants.” In certain embodiments, an epitope is a region of an antigen that is specifically bound by an antibody. In certain embodiments, an epitope may include chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl, or sulfonyl groups. In certain embodiments, an epitope may have specific three-dimensional structural characteristics (e.g., a “conformational” epitope) and/or specific charge characteristics. The antigen can be a protein or peptide of viral, bacterial, parasitic, fungal, protozoan, prion, cellular, or extracellular origin, which provokes an immune response in a mammal, preferably leading to protective immunity.

[0065] An “Isolated polynucleotide” as used herein may mean a polynucleotide (e.g., of genomic, cDNA, or synthetic origin, or a combination thereof) that, by virtue of its origin, the isolated polynucleotide is not associated with all or a portion of a polynucleotide with which the “isolated polynucleotide” is found in nature; is operably linked to a polynucleotide that it is not linked to in nature; or does not occur in nature as part of a larger sequence.

[0066] A “Monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen (e.g., although cross-reactivity or shared reactivity may occur). Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The monoclonal antibodies herein specifically include “chimeric” antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological. Methods for producing monoclonal antibodies are well known in the art. For example, monoclonal antibodies can be produced by: (1) fusion of sensitized lymphocytes and myelomas from different sources to produce continuous antibody-producing cell lines; (2) *in vitro* viral transformation of sensitized lymphocytes to form continuous antibody-producing cells; or (3) hybrid fusion of sensitized lymphocytes and continuous B lymphocyte cell lines (R.K Nakamura, “Monoclonal Antibodies: Methods and Clinical Lab Applications”, *Clin. Physiol. Biochem*, 1(2-5):160-72 (1983)).

[0067] A “Multivalent binding protein” as used herein to refer to a binding protein comprising two or more antigen binding sites (also referred to herein as “antigen binding domains”). A multivalent binding protein is preferably engineered to have three or more antigen binding sites and is generally not a naturally occurring antibody. The term “multispecific binding protein” refers to a binding protein that can bind two or more related or unrelated targets, including a binding protein capable of binding two or more different epitopes of the same target molecule.

[0068] A “peptide” or “polypeptide” as used herein refers to a linked sequence of two or more amino acids linked by peptide bonds. Peptides and polypeptides include proteins such as binding proteins, receptors, and antibodies. The terms “polypeptide” and “protein” are used interchangeably herein.

[0069] As used herein, the terms “providing,” “administering,” and “introducing” are used interchangeably herein and refer to the placement of the nanocomplexes and/or compositions of the present disclosure into a subject by a method or route which results in at least partial localization to a desired site.

[0070] A “Recombinant antibody” and “recombinant antibodies” as used herein refers to antibodies prepared by one or more steps, including cloning nucleic acid sequences encoding all or a part of one or more monoclonal antibodies into an appropriate expression vector by recombinant techniques and subsequently expressing the antibody in an appropriate host cell. The terms include, but are not limited to, recombinantly produced monoclonal antibodies, chimeric antibodies, humanized antibodies (fully or partially humanized), multi-specific or multi-valent structures formed from antibody fragments, bifunctional antibodies, heteroconjugate Abs, DVD-Ig@s, and other antibodies as described in herein (Dual-variable domain immunoglobulins and methods for making them are described in Wu, C., et al., *Nature Biotechnology*, 25:1290-1297 (2007)). The term “bifunctional antibody,” as used herein, refers to an antibody that comprises a first arm having a specificity for one antigenic site and a second arm having a specificity for a different antigenic site, *i.e.*, the bifunctional antibodies have a dual specificity.

[0071] As used herein, when an antibody or other entity (e.g., antigen binding domain) “specifically recognizes” or “specifically binds” an antigen or epitope, it preferentially recognizes the antigen in a complex mixture of proteins and/or macromolecules, and binds the antigen or epitope with affinity which is substantially higher than to other entities not displaying the antigen or epitope. In this regard, “affinity which is substantially higher” means affinity that is high enough to enable detection of an antigen or epitope which is distinguished from entities using a desired assay or measurement apparatus. Typically, it means binding affinity having a binding constant (K_a) of at least 10^7 M^{-1} (e.g., $>10^7 \text{ M}^{-1}$, $>10^8 \text{ M}^{-1}$, $>10^9 \text{ M}^{-1}$, $>10^{10} \text{ M}^{-1}$, $>10^{11} \text{ M}^{-1}$, $>10^{12} \text{ M}^{-1}$, $>10^{13} \text{ M}^{-1}$, etc.). In certain such embodiments, an antibody is capable of binding different antigens so long as the different antigens comprise that particular epitope. In certain instances, for example, homologous proteins from different species may comprise the same epitope.

[0072] A “subject” or “patient” may be human or non-human and may include, for example, animal strains or species used as “model systems” for research purposes, such a mouse model as

described herein. Likewise, patient may include either adults or juveniles (e.g., children). Moreover, patient may mean any living organism, preferably a mammal (c.g., human or non-human) that may benefit from the administration of compositions contemplated herein. Examples of mammals include, but are not limited to, any member of the Mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. In one embodiment, the mammal is a human.

[0073] As used herein, “treat,” “treating,” and the like means a slowing, stopping, or reversing of progression of a disease or disorder when provided a peptide or composition described herein to an appropriate subject. The term also includes a reversing of the progression of such a disease or disorder to a point of eliminating or greatly reducing the disease. As such, “treating” means an application or administration of the peptides or compositions described herein to a subject, where the subject has a disease or a symptom of a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease or symptoms of the disease.

[0074] A “Variant” as used herein, refers to and/or describes a peptide or polypeptide that differs in amino acid sequence by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity. Representative examples of “biological activity” include the ability to be bound by a specific antibody or to promote an immune response. Variant is also used herein to describe a protein with an amino acid sequence that is substantially identical to a referenced protein with an amino acid sequence that retains at least one biological activity. A conservative substitution of an amino acid, *i.e.*, replacing an amino acid with a different amino acid of similar properties (e.g., hydrophilicity, degree, and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art. Kyte *et al.*, *J. Mol. Biol.* 157:105-132 (1982). The hydropathic index of an amino acid is based on a consideration of its hydrophobicity and charge. It is known in the art that amino acids of similar hydropathic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydropathic indexes of ± 2 are substituted. The

hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity. U.S. Patent No. 4,554,101, incorporated fully herein by reference.

Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. Substitutions may be performed with amino acids having hydrophilicity values within ± 2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties.

[0075] Unless otherwise defined herein, scientific, and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. The meaning and scope of the terms should be clear; in the event, however of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0076] Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the present disclosure. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

DETAILED DESCRIPTION

Antibodies

[0077] In one aspect, the present invention relates to one or more monoclonal antibodies that can be used to enhance or increase PAD4 in a subject (e.g., a human, a mouse, a rat, a rabbit, a dog, a cat, etc.). In one aspect, the monoclonal antibodies are human monoclonal antibodies,

humanized monoclonal antibodies, or chimeric antibodies having the following heavy and light chain variable sequences:

[0078] Clone 16 (Antibody A)

[0079] 16 heavy chain

[0080] QVQLVESGAIEVKKPGSSVKVSKSSGGTFNNYAVVWVRQAPGQGPEWMGGI
IPMFDTPHYAQKFQGRVTITADEDTSTAYMELNSLRSDDTAVYYCATSYFRQWLPRDW
GQGTLVTVSSA (SEQ ID NO:1).

[0081] 16 kappa chain (light chain)

[0082] EIVMTQSPSSLSASVGDRVTITCRASQGIRNELGWYQQKPGKAPRLLIYAASSL
QSGVPSRFSGSGSGTDFTLTIGSLQPEDFATYYCLQDNNYPRTFGQGTKVDIK (SEQ ID
NO:2).

[0083] Clone 10 (Antibody B)

[0084] 10 heavy chain

[0085] EVQLVQSGAIEVKKPGSSVKVSKLASGGTFNNYAVSWVRQAPGQGLEWMGGI
IPMFDAIKYPQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCATSYFRQWLTWDWG
QGTLVTVSSA (SEQ ID NO:3).

[0086] 10 kappa chain (light chain)

[0087] EIVLTQSPATLSLSPGQRATLSCGASQPVSSNYLAWFQHKPGLAPRLLIYDTSIR
ATGVPRFRFSGSGSGTDFTLTISGLEPEDFAVYYCQQYGGSPQITFGGGTKVDIK (SEQ ID
NO:4).

[0088] Clone 17 (Antibody C)

[0089] 17 heavy chain

[0090] EVQLLESQAIEVKKPGSSVKVSKSSGGTFNNYAVVWVRQAPGQGPEWMGGII
PMFDTPHYAQKFQGRVTITADEDTSTAYMELNSLRSDDTAVYYCATSYFRQWLPRDW
GQGTLVTVSSA (SEQ ID NO:5).

[0091] 17 kappa chain (light chain)

[0092] EIVLTQSPATLSLSPGQRATLSCGASQSVSSNYLAWYQQKPGAPRLLIYDASI
RATGIPDRFRGSGSGTDFTLTISGLEPEDFAVYYCHQYATAPQITFGGGTKVEIK (SEQ ID
NO:6).

[0093] Clone 102 (Antibody D)

[0094] 102 heavy chain

[0095] QVQLVESGGGLVQPGGSLRLSCAASGFTFSRYTMNWVRQAPGKGLEWVSYIS
SSSSIIHYADSVKGRFTISRDNAKKSLYLQMNSLRAEDTAVYYCASSEVVPATRYFDYW
QGTLTVSSA (SEQ ID NO:7).

[0096] 102 lambda chain (light chain)

[0097] SYELTQPPSVSVAPGQTARITCGGNNIGTKSVHWYQQRPGRAPVLVYDDSD
RPSGIPERYSGSNSGNTATLTINRVEAGDEADYYCQVWDGISDHLEVFGGGTKLTVLGQ
PKAAPSVTL (SEQ ID NO:8).

[0098] **Clone 104 (Antibody E)**

[0099] 104 heavy chain

[0100] EVQLVESGPGLVKPSETLSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYIYY
GSTNYPNPSLKSRVSVSDTSKNQFSLRLSSVTAADTAVYYCAGHFRSSSSAKEYFHHWG
QGTLTVSSA (SEQ ID NO:9).

[0101] 104 kappa chain

[0102] DIQLTQSPSSVSASVGDRVTITCRASQDISTWLAWYQQKPGKAPNLLIFAASSL
QSGVPSRFRSGDGSGLTFTLTISLQPEDFATYYCQQASSFPYTFGQGTKVEIK (SEQ ID
NO:10).

[0103] In another aspect, the present disclosure relates to monoclonal antibodies having the
below SEQ ID NOS, as well as any antigen binding fragments thereof comprising:

[0104] A) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least
96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:1 and a
light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at
least 98%, at least 99% or at least 100% identity to SEQ ID NO:2;

[0105] B) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least
96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:3 and a
light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at
least 98%, at least 99% or at least 100% identity to SEQ ID NO:4;

[0106] C) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least
96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:5 and a
light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at
least 98%, at least 99% or at least 100% identity to SEQ ID NO:6;

[00107] D) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:7 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:8; or

[00108] E) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:9 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:10.

[00109] In another aspect, the present invention relates to monoclonal antibodies having the below SEQ ID NOS. as well as any antigen binding fragments thereof comprising:

[00110] A) a heavy variable chain comprising SEQ ID NO:1 and a light variable chain comprising SEQ ID NO:2;

[00111] B) a heavy variable chain comprising SEQ ID NO:3 and a light variable chain comprising SEQ ID NO:4;

[00112] C) a heavy variable chain comprising SEQ ID NO:5 and a light variable chain comprising SEQ ID NO:6;

[00113] D) a heavy variable chain comprising SEQ ID NO:7 and a light variable chain comprising SEQ ID NO:8; or

[00114] E) a heavy variable chain comprising SEQ ID NO:9 and a light variable chain comprising SEQ ID NO:10.

[00115] In one aspect, of the monoclonal antibodies of the present disclosure are a human antibody, a humanized antibody, a chimeric antibody or any derivative or variant thereof. In other aspects, the monoclonal antibodies are bispecific antibodies or any derivative or variant thereof. In some embodiments, the antibody is a fragment selected from the group consisting of Fab, Fab-C, Fab'-SH, Fv, scFv, and (Fab')₂ fragments.

[00116] In another aspect, the monoclonal antibodies of the present disclosure are human IgG antibodies.

[00117] The antibodies of the present disclosure may be produced by any method known in the art useful for the production of polypeptides, e.g., *in vitro* synthesis, recombinant DNA production, and the like. In some aspects, any of antibodies A-E can be produced by recombinant DNA technology. Specifically, any of antibodies A-E may be produced using recombinant

immunoglobulin expression technology. The recombinant production of immunoglobulin molecules, including humanized antibodies are described in U.S. Pat. No. 4,816,397, U.S. Pat. Nos. 6,331,415 and 4,816,567, U.K. patent GB 2,188,638, and U.K. patent GB 2,209,757. Techniques for the recombinant expression of immunoglobulins, including humanized immunoglobulins, can also be found, in Goeddel et al., *Gene Expression Technology Methods in Enzymology* Vol. 185 Academic Press (1991), and Borreback, *Antibody Engineering*, W. H. Freeman (1992). Additional information concerning the generation, design and expression of recombinant antibodies can be found in Mayforth, *Designing Antibodies*, Academic Press, San Diego (1993).

[00118] Recombinant chimeric antibodies of any of antibodies A-E can also be produced using techniques known in the art. For example, an exemplary process for the production of the recombinant chimeric antibodies for any of antibodies A-E can include the following: a) constructing, by conventional molecular biology methods, an expression vector that encodes and expresses an antibody heavy chain in which the CDRs and variable region of any of monoclonal antibodies A-E are fused to an Fc region derived from a human immunoglobulin, thereby producing a vector for the expression of a chimeric antibody heavy chain; b) constructing, by conventional molecular biology methods, an expression vector that encodes and expresses an antibody light chain of any of monoclonal antibodies A-E, thereby producing a vector for the expression of chimeric antibody light chain; c) transferring the expression vectors to a host cell by conventional molecular biology methods to produce a transfected host cell for the expression of chimeric antibodies; and d) culturing the transfected cell by conventional cell culture techniques so as to produce chimeric antibodies.

[00119] Recombinant humanized antibodies of any of antibodies A-E can also be produced using techniques known in the art. For example, an exemplary process for the production of recombinant humanized antibodies of any of antibodies A-E can include the following: a) constructing, by conventional molecular biology methods, an expression vector that encodes and expresses a heavy chain of any of antibodies A-E in which the CDRs and a minimal portion of the variable region framework that are required to retain donor antibody binding specificity are derived from a non-human immunoglobulin, such as any of monoclonal antibodies A-E, and the remainder of the antibody is derived from a human immunoglobulin, thereby producing a vector for the expression of a humanized antibody heavy chain; b)

constructing, by conventional molecular biology methods, an expression vector that encodes and expresses an antibody light chain in which the CDRs and a minimal portion of the variable region framework that are required to retain donor antibody binding specificity are derived from a non-human immunoglobulin, such as any of monoclonal antibodies A-E, and the remainder of the antibody is derived from a human immunoglobulin, thereby producing a vector for the expression of humanized antibody light chain; c) transferring the expression vectors to a host cell by conventional molecular biology methods to produce a transfected host cell for the expression of humanized antibodies; and d) culturing the transfected cell by conventional cell culture techniques so as to produce humanized antibodies.

[00120] With respect to either of the above-described methods, host cells may be co-transfected with such expression vectors, which may contain different selectable markers but, with the exception of the heavy and light chain coding sequences, are preferably identical. This procedure provides for equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA or both. The host cell used to express any of the recombinant monoclonal antibodies A-E, can be either a bacterial cell such as *Escherichia coli*, or more preferably a eukaryotic cell (e.g., a Chinese hamster ovary (CHO) cell or a HEK-293 cell). The choice of expression vector is dependent upon the choice of host cell, and may be selected so as to have the desired expression and regulatory characteristics in the selected host cell. Other cell lines that may be used include, but are not limited to, CHO-K1, NSO, and PER.C6 (Crucell, Leiden, Netherlands).

[00121] Any of the above-described antibodies can be used to generate antiidiotype antibodies using techniques well known to those skilled in the art (see, e.g., Greenspan, N. S. et al. (1989) "*Idiotypes: Structure and Immunogenicity*," FASEB J. 7:437-444; and Nisioff, A. (1991) "*Idiotypes: Concepts and Applications*," J. Immunol. 147(8):2429-2438).

[00122] The binding properties of any of the above antibodies can, if desired, be further improved by screening for variants that exhibit such desired characteristics. For example, such antibodies can be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains, such as Fab and Fv or disulfide-bond

stabilized Fv, expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage, including fd and M13. The antigen binding domains are expressed as a recombinantly fused protein to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the immunoglobulins, or fragments thereof, of the present disclosure include those disclosed in Brinkman, U. et al. (1995) "*Phage Display Of Disulfide-Stabilized Fv Fragments*," *J. Immunol. Methods*, 182:41-50, 1995; Ames, R. S. et al. (1995) "*Conversion Of Murine Fabs Isolated From A Combinatorial Phage Display Library To Full Length Immunoglobulins*," *J. Immunol. Methods*, 184:177-186; Kettleborough, C. A. et al. (1994) "*Isolation Of Tumor Cell-Specific Single-Chain Fv From Immunized Mice Using Phage-Antibody Libraries And The Reconstruction Of Whole Antibodies From These Antibody Fragments*," *Eur. J. Immunol.*, 24:952-958, 1994; Persic, L. et al. (1997) "*An Integrated Vector System For The Eukaryotic Expression Of Antibodies Or Their Fragments After Selection From Phage Display Libraries*," *Gene*, 187:9-18; Burton, D. R. et al. (1994) "*Human Antibodies From Combinatorial Libraries*," *Adv. Immunol.* 57:191-280; PCT Publications WO 92/001047; WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Pat. Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108.

[00123] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including humanized antibodies, or any other desired fragments, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')₂ fragments can also be employed using methods known in the art (such as those disclosed in PCT Publication WO 92/22324; Mullinax, R. L. et al. (1992) "*Expression Of A Heterodimeric Fab Antibody Protein In One Cloning Step*," *BioTechniques*, 12(6):864-869; and Sawai et al. (1995) "*Direct Production Of The Fab Fragment Derived From The Sperm Immobilizing Antibody Using Polymerase Chain Reaction And cDNA Expression Vectors*," *Am. J. Reprod. Immunol.* 34:26-34; and Better, M. et al. (1988) "*Escherichia coli Secretion Of An Active*

Chimeric Antibody Fragment,” Science 240:1041-1043). Examples of techniques which can be used to produce single-chain Fvs, and antibodies include those described in U.S. Pat. Nos. 4,946,778 and 5,258,498; Huston, J. S. et al. (1991) “*Protein Engineering Of Single-Chain Fv Analogs And Fusion Proteins*,” Methods in Enzymology 203:46-88; Shu, L. et al., “*Secretion Of A Single-Gene-Encoded Immunoglobulin From Myeloma Cells*,” Proc. Natl. Acad. Sci. (USA) 90:7995-7999; and Skerra, A. et al. (1988) “*Assembly Of A Functional Immunoglobulin Fv Fragment In Escherichia coli*,” Science 240:1038-1040.

[00124] Phage display technology can be used to increase the affinity of any of antibodies A-E. This technique would be useful in obtaining high affinity antibodies that could be used in the disclosed combinatorial methods. This technology, referred to as affinity maturation, employs mutagenesis or CDR walking and re-selection using such receptors or ligands (or their extracellular domains) or an antigenic fragment thereof to identify antibodies that bind with higher affinity to the antigen when compared with the initial or parental antibody (See, e.g., Glaser, S. M. et al. (1992) “*Antibody Engineering By Codon-Based Mutagenesis In A Filamentous Phage Vector System*,” J. Immunol. 149:3903-3913). Mutagenizing entire codons rather than single nucleotides results in a semi-randomized repertoire of amino acid mutations. Libraries can be constructed consisting of a pool of variant clones each of which differs by a single amino acid alteration in a single CDR and which contain variants representing each possible amino acid substitution for each CDR residue. Mutants with increased binding affinity for the antigen can be screened by contacting the immobilized mutants with labeled antigen. Any screening method known in the art can be used to identify mutant antibodies with increased avidity to the antigen (e.g., ELISA) (see, e.g., Wu, H. et al. (1998) “*Stepwise In Vitro Affinity Maturation Of Vitaxin, An Alphav Beta3-Specific Humanized Mab*,” Proc. Natl. Acad. Sci. (USA) 95(11):6037-6042; Yelton, D. E. et al. (1995) “*Affinity Maturation Of The BR96 Anti-Carcinoma Antibody By Codon-Based Mutagenesis*,” J. Immunol. 155:1994-2004). CDR walking which randomizes the light chain may be used possible (see, Schier et al. (1996) “*Isolation Of Picomolar Affinity Anti-C-ErbB-2 Single-Chain Fv By Molecular Evolution Of The Complementarity Determining Regions In The Center Of The Antibody Binding Site*,” J. Mol. Biol. 263:551-567).

[00125] Also contemplated herein is the use of random mutagenesis to identify improved CDRs. Phage display technology can alternatively be used to increase (or decrease) CDR

affinity. This technology, referred to as affinity maturation, employs mutagenesis or “CDR walking” and re-selection uses the target antigen or an antigenic fragment thereof to identify antibodies having CDRs that bind with higher (or lower) affinity to the antigen when compared with the initial or parental antibody (see, e.g., Glaser, S. M. et al. (1992) “*Antibody Engineering By Codon-Based Mutagenesis In A Filamentous Phage Vector System*,” J. Immunol. 149:3903-3913). Mutagenizing entire codons rather than single nucleotides results in a semi-randomized repertoire of amino acid mutations. Libraries can be constructed consisting of a pool of variant clones each of which differs by a single amino acid alteration in a single CDR and which contain variants representing each possible amino acid substitution for each CDR residue. Mutants with increased (or decreased) binding affinity for the antigen can be screened by contacting the immobilized mutants with labeled antigen. Any screening method known in the art can be used to identify mutant antibodies with increased (or decreased) avidity to the antigen (e.g., ELISA) (see, Wu, H. et al. (1998) “*Stepwise In Vitro Affinity Maturation Of Vitaxin, An Alphav Beta3-Specific Humanized Mab*,” Proc. Natl. Acad. Sci. (USA) 95(11):6037-6042; Yelton, D. E. et al. (1995) “*Affinity Maturation Of The BR96 Anti-Carcinoma Antibody By Codon-Based Mutagenesis*,” J. Immunol. 155:1994-2004). CDR walking which randomizes the light chain may be used possible (see, Schier et al. (1996) “*Isolation Of Picomolar Affinity Anti-C-ErbB-2 Single-Chain Fv By Molecular Evolution Of The Complementarity Determining Regions In The Center Of The Antibody Binding Site*,” J. Mol. Biol. 263:551-567).

[00126] Methods for accomplishing such affinity maturation are described for example in: Krause, J. C. et al. (2011) “*An Insertion Mutation That Distorts Antibody Binding Site Architecture Enhances Function Of A Human Antibody*,” MBio. 2(1) pii: e00345-10. doi: 10.1128/mBio.00345-10; Kuan, C. T. et al. (2010) “*Affinity-Matured Anti-Glycoprotein NMB Recombinant Immunotoxins Targeting Malignant Gliomas And Melanomas*,” Int. J. Cancer 10.1002/ijc.25645; Hackel, B. J. et al. (2010) “*Stability And CDR Composition Biases Enrich Binder Functionality Landscapes*,” J. Mol. Biol. 401(1):84-96; Montgomery, D. L. et al. (2009) “*Affinity Maturation And Characterization Of A Human Monoclonal Antibody Against HIV-1 gp41*,” MAbs 1(5):462-474; Gustchina, E. et al. (2009) “*Affinity Maturation By Targeted Diversification Of The CDR-H2 Loop Of A Monoclonal Fab Derived From A Synthetic Naïve Human Antibody Library And Directed Against The Internal Trimeric Coiled-Coil Of Gp41 Yields A Set Of Fabs With Improved HIV-1 Neutralization Potency And Breadth*,”

Virology 393(1):112-119; Finlay, W. J. et al. (2009) “*Affinity Maturation Of A Humanized Rat Antibody For Anti-RAGE Therapy: Comprehensive Mutagenesis Reveals A High Level Of Mutational Plasticity Both Inside And Outside The Complementarity-Determining Regions*,” J. Mol. Biol. 388(3):541-558; Bostrom, J. et al. (2009) “*Improving Antibody Binding Affinity And Specificity For Therapeutic Development*,” Methods Mol. Biol. 525:353-376; Steidl, S. et al. (2008) “*In Vitro Affinity Maturation Of Human GM-CSF Antibodies By Targeted CDR-Diversification*,” Mol. Immunol. 46(1):135-144; and Barderas, R. et al. (2008) “Affinity maturation of antibodies assisted by in silico modeling,” Proc. Natl. Acad. Sci. (USA) 105(26):9029-9034.

[00127] The disclosure particularly contemplates the production and use of derivatives of any of the above-described antibodies and their antigen-binding fragments. In some embodiments, a humanized antibody is a derivative. Such a humanized antibody comprises amino acid residue substitutions, deletions or additions in one or more non-human CDRs. The humanized antibody derivative may have substantially the same binding, better binding, or worse binding when compared to a non-derivative humanized antibody. In specific embodiments, one, two, three, four, or five amino acid residues of the CDR have been substituted, deleted or added (i.e., mutated). A derivative antibody or antibody fragment may be modified by chemical modifications using techniques known to those of skill in the art, including, but not limited to, specific chemical cleavage, acetylation, formulation, metabolic synthesis of tunicamycin, etc. In one embodiment, an antibody derivative will possess a similar or identical function as the parental antibody. In another embodiment, an antibody derivative will exhibit an altered activity relative to the parental antibody. For example, a derivative antibody (or fragment thereof) can bind to its epitope more tightly or be more resistant to proteolysis than the parental antibody.

[00128] Derivatized antibodies may be used to alter the half-lives (e.g., serum half-lives) of parental antibodies in a mammal, preferably a human. Preferably such alteration will result in a half-life of greater than 15 days, preferably greater than 20 days, greater than 25 days, greater than 30 days, greater than 35 days, greater than 40 days, greater than 45 days, greater than 2 months, greater than 3 months, greater than 4 months, or greater than 5 months. The increased half-lives of the humanized antibodies of the present disclosure or fragments thereof in a mammal, preferably a human, results in a higher serum titer of said antibodies or antibody fragments in the mammal, and thus, reduces the frequency of the administration of said

antibodies or antibody fragments and/or reduces the concentration of said antibodies or antibody fragments to be administered. Antibodies or fragments thereof having increased *in vivo* half-lives can be generated by techniques known to those of skill in the art. For example, antibodies or fragments thereof with increased *in vivo* half-lives can be generated by modifying (e.g., substituting, deleting or adding) amino acid residues identified as involved in the interaction between the Fc domain and the FcRn receptor. The humanized antibodies of any of antibodies A-E can be engineered to increase biological half-lives (see, e.g. U.S. Pat. No. 6,277,375).

[00129] Antibodies or fragments thereof with increased *in vivo* half-lives can be generated by attaching to said antibodies or antibody fragments polymer molecules such as high molecular weight polyethyleneglycol (PEG). PEG can be attached to said antibodies or antibody fragments with or without a multifunctional linker either through site-specific conjugation of the PEG to the N- or C-terminus of said antibodies or antibody fragments or via epsilon-amino groups present on lysine residues. Linear or branched polymer derivatization that results in minimal loss of biological activity will be used. The degree of conjugation will be closely monitored by SDS-PAGE and mass spectrometry to ensure proper conjugation of PEG molecules to the antibodies. Unreacted PEG can be separated from antibody-PEG conjugates by, e.g., size exclusion or ion-exchange chromatography. Alternatively, bispecific antibodies can be made using the techniques as described in Brinkmann et al., *MABS*, 9(2):182-212 (2017), the contents of which are herein incorporated by reference.

Compositions

[00130] In another aspect, the present invention comprises a monoclonal antibody comprising:

[00131] A) a heavy variable chain comprising SEQ ID NO:1 and a light variable chain comprising SEQ ID NO:2;

[00132] B) a heavy variable chain comprising SEQ ID NO:3 and a light variable chain comprising SEQ ID NO:4;

[00133] C) a heavy variable chain comprising SEQ ID NO:5 and a light variable chain comprising SEQ ID NO:6;

[00134] D) a heavy variable chain comprising SEQ ID NO:7 and a light variable chain comprising SEQ ID NO:8; or

[00135] E) a heavy variable chain comprising SEQ ID NO:9 and a light variable chain comprising SEQ ID NO:10 and at least one excipient or pharmaceutically acceptable carrier.

[00136] In another aspect, the present disclosure relates to monoclonal antibodies having the below SEQ ID NOS, as well as any antigen binding fragments thereof comprising:

[00137] A) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:1 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:2;

[00138] B) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:3 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:4;

[00139] C) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:5 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:6;

[00140] D) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:7 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:8; or

[00141] E) a heavy variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:9 and a light variable chain having at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% identity to SEQ ID NO:10 and at least one pharmaceutically acceptable carrier.

[00142] The choice of pharmaceutically acceptable excipient or pharmaceutically acceptable carrier will depend on factors including, but not limited to, the particular mode of administration, the effect of the excipient on solubility and stability, and the nature of the dosage form. The compositions of the present invention will be readily apparent to those skilled in the art. Techniques and formulations may be found, for example, in Remington's Pharmaceutical Sciences, 19th Edition (Mack Publishing Company, 1995).

[00143] The term "pharmaceutically acceptable carrier," as used herein, means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material, surfactant, cyclodextrins or

formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are sugars such as, but not limited to, lactose, glucose and sucrose; starches such as, but not limited to, corn starch and potato starch; cellulose and its derivatives such as, but not limited to, sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as, but not limited to, cocoa butter and suppository waxes; oils such as, but not limited to, peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; surfactants such as, but not limited to, cremophor EL, cremophor RH 60, Solutol HS 15 and polysorbate 80; cyclodextrins such as, but not limited to, alpha-CD, beta-CD, gamma-CD, HP-beta-CD, SBE-beta-CD; glycols; such as propylene glycol; esters such as, but not limited to, ethyl oleate and ethyl laurate; agar; buffering agents such as, but not limited to, magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as, but not limited to, sodium lauryl sulfate and magnesium stearate, as well as releasing agents, coating agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

[00144] The route by which the disclosed antibodies are administered, and the form of the composition will dictate the type of carrier to be used. The composition may be in a variety of forms, suitable, for example, for systemic administration (e.g., oral, rectal, nasal, sublingual, buccal, implants, or parenteral injections) or topical administration (e.g., dermal, pulmonary, nasal, aural, ocular, liposome delivery systems, or iontophoresis). In some aspects, the composition may be inhaled.

Methods of Use

[00145] Also disclosed herein are methods of using the disclosed monoclonal antibodies and compositions. In some aspects, the monoclonal antibodies described herein can be used to screen and/or develop new therapies or therapeutic agents that inhibit PAD4. These new therapies or therapeutic agents could be used to develop said agents for use in treating one or more autoimmune disease or any other diseases that result from or are caused by increased levels of PAD4 in a subject. In some aspects, the autoimmune disease is rheumatoid arthritis.

[00146] In one aspect, the monoclonal antibodies herein can be used to develop animal models, such as, for example, mouse, rat, rabbit, goat, dog, cat, etc. More specifically, the antibodies of

the present disclosure can be administered to an animal to increase the amount or level of PAD4 in the animal. Once the levels of PAD4 in the animal are increased, the animal can be administered one more therapeutic agents to screen for new therapies. Specifically, the animal can be screened and evaluated to determine whether the therapeutic agent decreased the level of PAD4, increased the level of PAD4, or if the level of PAD4 remained the same in the animal. Therapeutic agents that reduced of the level of PAD4 (e.g., inhibit PAD4) in the animal can be selected for further evaluation, study and/or testing. The therapeutic agents that can be tested can be small molecules, biologics, antibody-drug conjugates, a peptide, a microRNA, a short interfering RNA, or any combinations thereof.

Kits

[00147] Also disclosed herein are kits comprising one or more of the antibodies (e.g., compositions) as disclosed herein. The kits can also comprise other agents (e.g., pharmaceutical agents) and/or products co-packaged, co-formulated, and/or co-delivered with other components. The kits can also comprise instructions for using the components of the kit. The instructions are relevant materials or methodologies pertaining to the kit. The materials may include any combination of the following: background information, list of components, brief or detailed protocols for using the monoclonal antibodies, troubleshooting, references, technical support, and any other related documents. Instructions can be supplied with the kit or as a separate member component, either as a paper form or an electronic form which may be supplied on computer readable memory device or downloaded from an internet website, or as recorded presentation.

[00148] It is understood that the disclosed kits can be employed in connection with the disclosed methods. The kit may further contain containers or devices for use with the methods disclosed herein. For example, the kits can comprise delivery devices (e.g., syringes).

[00149] The kits provided herein are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Individual member components of the kits may be physically packaged together or separately.

[00150] The present disclosure has multiple aspects, illustrated by the following non-limiting examples.

EXAMPLES

Example 1: Preparation of Antibodies

[00151] PADs cloning and expression. Plasmids encoding human PAD2, PAD3, PAD4 and *P. gingivalis* PAD were previously described^{34,35}. DNA sequences encoding specific PAD4 domains were generated by PCR using PAD4 plasmid as template and then cloned into pcDNA3.1 (Invitrogen). Plasmids encoding N-terminal His₆-T7-tagged PAD4 calcium-binding site mutants were generated by site-directed mutagenesis using PAD4 cloned into pET28a(+) (Novagen) as template. cDNA for the monomeric green fluorescent protein mWasabi (Allele Biotech) was cloned in frame with PAD4 into pET28a(+), generating a N-terminal His₆-T7-mWasabi-tagged PAD4 fusion protein. Plasmids were used to generate [³⁵S]methionine-labeled proteins by *in vitro* coupled transcription/translation (IVTT) (Promega). N-terminal His₆-T7-tagged proteins were expressed in BL21-Gold (DE3) cells (Agilent) and purified using Ni-NTA agarose (Qiagen).

[00152] Single anti-PAD4 memory B cell sorting. B cells were enriched from whole blood using RosetteSep human B cell enrichment cocktail (Stemcell Technologies) according to the manufacturer's instructions and further stained with LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Thermo Fisher Scientific), PE-CF594 mouse anti-human CD19, BV421 mouse anti-human CD20, BV605 mouse anti-human CD14, APC-H7 mouse anti-human CD27, PE-CyTM7 mouse anti-human CD38, APC mouse anti-human IgM, BV510 mouse anti-human CD3 (BD Biosciences) and mWasabi-PAD4. Single cells CD3⁻CD14⁻CD19⁺CD20⁺CD27⁺CD38⁻IgM⁻ with high binding to mWasabi-PAD4 were sorted into 96-well PCR plates containing 10 µl/well of 10 mM Tris pH 8.0 and 8 U RNasin Ribonuclease Inhibitor (Promega).

[00153] Cloning and Ig sequence analysis. Single cell cDNA was generated using SuperScript VILO Master Mix (Thermo Fisher Scientific). IgH and IgL (κ or λ) variable regions were amplified using the cDNA by nested PCR, using previously described primers and PCR conditions.³⁶ Restriction sites were introduced using gene-specific primers during the second round of PCR and used to clone the products into expression vectors containing human Igγ1, Igκ, or Igλ constant regions (kindly provided by Eric Meffre, Yale University School of Medicine, New Haven, CT). Variable regions were analyzed by Sanger sequencing. V(D)J gene usage, CDR3s and SHM were determined using Ig BLAST and JOINSOLVER.³⁷

[00154] Reversion of SHM sequences to germline. The V(D)J germline sequences with the lowest number of mismatch nucleotides compared to mutated sequences were obtained using IgBLAST and synthesized as DNA strings containing cloning restriction sites using the GeneArt

Gene Synthesis service (Thermo Fisher Scientific). After restriction digestion, the DNA strings were cloned into expression vectors containing human Ig γ 1, Ig κ , or Ig λ constant regions.

[00155] Antibody production. Antibodies were produced by co-transfecting plasmids encoding IgH and IgL from single B cells into suspension cultured Expi293 cells (Thermo Fisher Scientific). Supernatants were collected after 6 days of culture, and antibodies were purified using HiTrap Protein G HP (GE Healthcare Life Sciences) on an ÄKTA Prime Chromatography system (Amersham Biosciences).

[00156] Immunoprecipitation (IP). IVTT-radiolabelled proteins were diluted in IP buffer (1%NP-40/PBS) and incubated with 3.4 or 34 nM of purified monoclonal antibody as indicated in the figure legends. After 1 hr at room temperature (RT), immune complexes were purified using Protein A agarose beads (Pierce). Samples were washed in IP buffer and immunoprecipitates visualized by SDS-PAGE/sodium salicylate autoradiography.

[00157] Citrullination assay. Monoclonal antibodies (0.14 μ M, 0.46 μ M or 1.4 μ M), 0.09 μ M PAD4 (wild type or mutants) and 2.2 μ M histone H3.1 (New England Biolabs) were mixed in 100 mM Tris pH 7.5/1 mM dithiothreitol (DTT) and increasing amounts of calcium. After incubation for 2 hrs at 37°C, the mixtures were boiled in SDS sample buffer, separated by electrophoresis, transferred to nitrocellulose, stained with Ponceau S and analyzed by immunoblotting with antibodies to citrullinated histone H3 (citrulline R2 + R8 + R17, Abcam) and PAD4 (Sigma).

[00158] ELISA assay for polyreactivity and anti-CCP3. Antibodies were screened for reactivity against previously defined polyreactive antigens, including ssDNA, dsDNA, cardiolipin, insulin and LPS. ssDNA or dsDNA (Sigma) at 5 μ g/mL in 10 mM 1-methylimidazole/50 mM N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide were coated on covalink plates (ThermoFisher) for 5 hrs at 50°C. Human recombinant insulin (ThermoFisher) at 5 μ g/mL in PBS was coated on Maxisorp plates (ThermoFisher), LPS (Invivogen) at 10 μ g/mL in 50 mM bicarbonate buffer pH 9.6 was coated on Medisorp plates (ThermoFisher) and cardiolipin (Sigma) at 10 μ g/mL diluted in pure ethanol was coated on Polysorp plates (ThermoFisher) at 4°C overnight. Plates were blocked in 3% bovine serum albumin (BSA) in PBS at RT for 1 hr and washed in PBS/0.05% tween/1 mM EDTA. Monoclonal antibodies or SLE serum were diluted in 1% BSA/PBS/0.05% tween and assayed in duplicate. HRP-conjugated anti-human IgG at 100 ng/mL was used as a secondary antibody. The reactivity of

anti-PAD4 antibodies to citrullinated antigen was determined using the QUANTA Lite® CCP3 IgG assay (Inova Diagnostics).

[00159] Anti-PAD4 ELISA assay. Maxisorp plates (ThermoFisher) were coated with 66 nM human recombinant PAD4 in 0.1 M carbonate buffer pH 9.6. Plates were blocked with 3% BSA/PBS. Antibodies were diluted in 1% BSA/PBS/0.05% tween and assayed in duplicate. After antibody incubation for 1 hr, the plate was washed and HRP-conjugated anti-human IgG or HRP-conjugated anti-mouse IgG at 100 ng/mL was used as a secondary antibody. The EC₅₀ values were calculated from linear regressions. The average EC₅₀ was determined from two independent experiments.

[00160] Cross-competition ELISA. Human anti-PAD4 monoclonal antibodies were murinized by cloning the variable region into the pFUSE-mIgG2a-Fc vector (InvivoGen). Human recombinant PAD4 was coated on ELISA plates at 66 nM in 0.1 M carbonate buffer pH 9.6 and the plates blocked with 3% BSA in PBS. After washing, the plates were incubated in the absence or presence of human anti-PAD4 monoclonal antibodies (competing antibody, from 0.25 nM to 548 nM) in 1% BSA/PBS/0.05% tween. After 1 hour at RT, murinized antibodies (5.5 nM) were directly added into the wells and the plate was further incubated for 1 hour at RT. After washing, HRP-conjugated anti-mouse IgG was used to detect murinized antibodies. The percentage of remaining antibody bound to PAD4 was determined according to the amount of murinized antibody detected in the absence of competing antibody, which was defined as 100% binding. Murinized clone 16 was not included in the assay because it had no yield.

Example 2: Use of PAD4-tagged with the monomeric fluorescent protein mWasabi and single cell cloning technology to produce monoclonal antibodies.

Materials and Methods

[00161] Patients. All samples were collected after obtaining signed informed consent in accordance with the Johns Hopkins Institutional Review Board. Patients with RA were recruited from the Johns Hopkins Arthritis Center and screened for anti-PAD4 and anti-PAD3/4XR antibodies in serum by immunoprecipitation (IP) as previously described.¹⁷ Peripheral blood anti-PAD4 single memory B cells were purified from three patients who had with high titer anti-PAD4 and anti-PAD3/4XR antibodies were used to generate monoclonal antibodies as per Example 1.

Results

[00162] Sequence analysis of anti-PAD4 antibodies cloned from single memory B cells.

Single anti-PAD4 memory B cells were isolated by flow cytometry using recombinant PAD4 tagged with the monomeric fluorescent protein mWasabi (FIG. 7). In total, 44 single memory B cells with high binding to mWasabi-PAD4 were collected from three patients with RA. Heavy chain (IgH) and corresponding light chain (IgL) variable gene sequences were amplified from 32 cells and used to generate IgG1 monoclonal antibodies. The antibodies were screened by IP (Fig. 1A), identifying five monoclonal antibodies with specificity to PAD4. Clones 10, 16 and 17 derived from patient #1 were cross-reactive with PAD3, while clones 102 and 104 from patient #3 only recognized PAD4 (anti-PAD4-only antibodies). No antibodies to PAD4 were isolated from patient #2. None of the anti-PAD4 antibodies recognized PAD2 or the PAD from *Porphyromonas gingivalis* (PPAD) (Fig. 1B).

[00163] Based on the IgH V-D-J usage, complementarity-determining region 3 (CDR3) sequence, CDR3 length and the presence of common mutations, monoclonals 10, 16 and 17 were determined to be clonally related (Fig. 1C). The number of somatic hypermutations (SHM) shared by the IgH of antibodies 10, 16 and 17 are shown in FIG. 1D. Clones 16 and 17 had the highest similarity with 28 common mutations. Antibody 10 showed 24 mutations, 7 of which were shared by clones 16 and 17. Clones 10 and 17 also shared a related V κ gene rearrangement (IGKV3D-20/IGKJ4), similar CDR3 sequence, and 5 mutations (FIG. 1C and FIG. 1E), confirming that these were derived from the same B cell precursor. However, clone 16 used a different V κ gene rearrangement (IGKV1-06/IGKJ1) and had a different CDR3 sequence, suggesting the possibility of light chain replacement^{19,20}. Antibodies 102 and 104 were not clonally related (FIG. 1C). The enrichment of SHM in all antibodies suggests that they originated from antigen experienced cells.

[00164] Anti-PAD4 autoantibodies arise from autoreactive precursors. To determine whether anti-PAD3 and PAD4 reactivity was due to SHM, we reverted the IgH and IgL chain genes of the antibodies to their germline form and antigen binding was addressed by IP. Binding to PAD3 and PAD4 by clones 10 and 17, which are the same antibody when reverted to germline, was not affected after germline reversion (FIG. 2A). Similarly, clone 104 retained PAD4 reactivity, albeit less, in its germline form (FIG. 2A). In contrast, reversion of mutations in clone 16 and 102 led to a complete loss of PAD binding (FIG. 2A). Since IP is not sensitive enough to detect low affinity antibodies or quantitatively compare antibody binding, the binding

efficiency of mutated and germline antibodies were additionally compared by ELISA (FIG. 2B and FIG. 2C). In contrast to IP, all mutated and germline antibodies showed reactivity to PAD4 when compared with control IgG₁, suggesting that these antibodies were derived from B cell precursors for which reactivity to PAD4 resulted from V(D)J rearrangements. Except for mutated and germline clone 104, which have similar antigen binding, reversion to germline importantly decreased the binding of the other antibodies to PAD4, supporting that their reactivity was enhanced through affinity maturation.

[00165] Heavy and light chain requirements for PAD binding. While the IgH of antibody 16 is clonally related to antibody 10 and 17, the use of a different light chain suggests that this antibody underwent IgL replacement^{19,20}. Nevertheless, it is interesting that this antibody retained binding to PAD4, suggesting that the light chain replacement was insufficient to change its autoreactivity. The study of autoantibodies to DNA has shown that antigen binding can be mediated primarily by the IgH regardless of the IgL²¹. To better understand the independent roles of the mutated heavy and light chains in antigen binding, monoclonals were generated that combined mutated IgH or IgL with either their corresponding germline light and heavy chains or irrelevant chains from a monoclonal with no reactivity to PADs.

[00166] Similar to the germline forms of antibodies 10, 17 and 104, reversion to germline of either IgL or IgH retained antigen binding (FIG. 2D). However, hybrid antibodies 10, 17 and 104 that used an irrelevant IgH or IgL completely lost efficient antigen binding (FIG. 2D), suggesting that their reactivity was entirely dependent on the combination of their heavy and light chains either as germline or mutated forms. Antibody 102 lost efficient PAD4 binding when either the heavy or light chain was reverted to germline (FIG. 2D) or replaced with an irrelevant IgH or IgL (FIG. 2D), demonstrating that this antibody required both mutated IgH and IgL for efficient binding to PAD4.

[00167] While clone 16 containing mutated IgL and germline IgH showed no binding to PADs (FIG. 2D), mutated IgH retained reactivity to PAD3 and PAD4 when combined with any light chain (FIG. 2D), suggesting that antigen binding by this clone is largely dependent on mutations in the IgH. This may explain why replacement of the IgL in clone 16 was insufficient to silence antibody reactivity to PADs.

[00168] Mutated and germline reverted anti-PAD4 antibodies are not polyreactive and are not cross-reactive to citrullinated antigens. V(D)J recombination can generate harmful B

cells expressing autoreactive antibodies. It is estimated that 55-75% of early immature B cells from healthy donors are polyreactive to self-antigens and lipopolysaccharide (LPS)²². While most of these cells are removed at discrete checkpoints during B cell development, their abnormal survival has been linked to autoimmune diseases⁴. Since anti-PAD4 antibodies reverted to their germline form retained antigen binding, we addressed whether their reactivity to PAD4 may result from polyreactive recognition. In contrast to antibodies present in the serum from a patient with systemic lupus erythematosus (SLE) that were polyreactive, both mutated and reverted anti-PAD4 antibodies showed no polyreactivity to self-antigens (ssDNA, dsDNA, insulin and cardiolipin) or LPS (FIG. 3A). Similarly, the monoclonal antibodies showed no reactivity to cyclic citrullinated peptides (CCPs) (FIG. 3B), confirming that they were developed independently of ACPAs.

[00169] Functional characterization of human anti-PAD4 monoclonal antibodies.

Autoantibodies that enhance PAD4 activity are strongly associated with more erosive arthritis and interstitial lung disease (ILD) in patients with RA^{17,23,24}. These antibodies were studied as polyclonal IgG purified from the serum of patients with RA, so the functional effects of individual antibody specificities could not be determined. The monoclonal anti-PAD4 antibodies developed here were studied for their individual capacity to activate PAD4 over a physiologic range of calcium concentrations in synovial fluid (0.2 to 1.0 mM), which are suboptimal to fully activate the enzyme *in vitro*¹⁷. Using 0.2 mM calcium and enzymatic reactions in the linear range to citrullinate histone H3, a physiologic target of PAD4²⁵, clone 102 demonstrated the highest agonistic activity (FIG. 4A, lanes 12), followed by clones 16 and 104 (FIG. 4A, lanes 8 and 14, respectively).

[00170] At 0.5 and 1.0 mM calcium, the enhancing PAD4 activity by clones 16, 102 and 104 became more prominent, with clones 10 and 17 also showing some functional activity (FIG. 4B and FIG. 8). Importantly, while the germline reversions conserved antigen recognition (FIG. 2B), these monoclonal antibodies failed to enhance PAD4 activity at 0.2, 0.5 or 1.0 mM calcium (FIG. 4A and B, and FIG. 8). Similarly, antibodies containing mutated IgH from clone 16 combined with its corresponding germline IgL or an irrelevant IgL (Fig. 4A and FIG. 4B), both of which retained PAD reactivity (FIG. 2A), were also unable to activate the enzyme. At non-physiologic saturating calcium concentrations (i.e. 5 mM), PAD4 was fully active independently of any antibody (FIG. 9).

[00171] Since the monoclonal antibodies reverted to germline retained some PAD4 reactivity at high concentrations (FIG. 2B), we tested their ability to activate PAD4 using increasing amounts of antibody (FIG. 8). Unlike mutated anti-PAD4 antibodies, the germline versions required at least 10 times more antibody to induce even minimal PAD4 activity. Together, these data demonstrate that, while antigen experience is not necessary to produce anti-PAD4 autoantibodies, affinity maturation is required for the generation of efficient agonistic antibodies to PAD4.

[00172] Functional antibodies target different epitopes in PAD4. While agonistic anti-PAD4 antibodies were initially recognized by their cross-reactivity with PAD3¹⁷, it is noteworthy that functional autoantibodies derived from anti-PAD3/4XR positive patients were identified that only had reactivity to PAD4. This indicates that more than one epitope may be targeted to activate the enzyme. To address whether agonistic anti-PAD4 antibodies target distinct or overlapping epitopes, the antibodies were murinized and their binding to PAD4 was addressed by ELISA in the presence of increasing amounts of competing human anti-PAD4 antibodies (FIG. 4C). With the exception of antibody 16, the rest of the clones were efficiently generated as murine antibodies, which retained similar PAD4 reactivity as their human counterparts (FIG. 10). Antigen binding by the clonally related antibodies 10 and 17 was completely blocked by one another, supporting that they target the same epitope (FIG. 4C). Analysis of the other antibodies, however, is more complex because their considerable differences in binding efficiency (FIG. 2C). Human clone 16, for which binding efficiency is lower than its clonally related antibodies 10 and 17, required 4-10 times more antibody to efficiently block these clones (FIG. 4C). Antibody 104, which has the highest relative affinity, completely blocked clone 102 and partially blocked clones 10 and 17 at high concentrations, but was not significantly blocked by any of other anti-PAD4 antibodies (FIG. 4C). Clone 102 partially blocked antibodies 10 and 17, but was only significantly blocked by clone 104 (FIG. 4C). Based on the distinct recognition of PAD3 (FIG. 1A), at least 2 epitopes are likely to be targeted by antibodies that enhance PAD4 activity. Nevertheless, it is possible that antibody 102 may recognized a third epitope that partially overlaps with clones 10, 16 and 17 (PAD3/4XR antibodies) and clone 104 (anti-PAD4-only antibody).

[00173] To gain further insights into the structural basis of the interaction between the monoclonal antibodies and PAD4, an attempt was made to map the regions targeted by the

antibodies using PAD4 deletion mutants. PAD4 consists of a C-terminal domain and an N-terminal domain that is divided into subdomains 1 and 2 (FIG. 11A)²⁶. Under standard conditions used to IP PAD4, none of the antibodies showed binding to any domain (FIG. 11B). When the antibody concentration and the incubation time were increased in the IP assays (FIG. 11C), clone 104 showed reactivity to the N-terminal domain and subdomain 1. However, the other antibodies still failed to react with any subdomain of PAD4 (Fig. FIG.11C). These findings strongly suggest that the epitope targeted by the majority of functional anti-PAD4 antibodies is structural in nature, requiring folding of the complete molecule.

[00174] Agonistic autoantibodies enhance PAD4 activity by regulating the enzyme at calcium-binding site 5. Since agonistic anti-PAD4 antibodies work by lowering the calcium requirement for PAD4 activity, we explored potential sites of interaction using PAD4 mutants that lack the ability to bind calcium at specific sites. PAD4 has 5 calcium-binding sites designated Ca1-Ca5. Ca1 and Ca2 in the C-terminal domain are required for catalysis, while Ca3-Ca5 are likely relevant to regulate enzyme activity²⁶. Aspartic acid residues that coordinate calcium-binding at Ca3 and Ca5 (D¹⁶⁵), Ca3 and Ca4 (D¹⁷⁹), only Ca4 (D³⁸⁸) and only Ca5 (D¹⁶⁸) were changed to alanine.²⁷ Then, the PAD4 mutants were used to test the functional activity of the antibodies. None of the PAD4 mutants were active in the absence of anti-PAD4 antibodies (FIG. 5). However, the activity of the D¹⁶⁸, a mutant (Ca5), was partially rescued by antibody clones 10, 16, 102 and 104 (FIG. 5). Interestingly, the enhancing effect of the monoclonal antibodies on the mutant D¹⁶⁸A was proportional to their activity on wild type PAD4 (i.e. 102 > 104 = 16 > 10) (FIG. 4B and FIG. 5). Together, these data suggest that functional anti-PAD4 antibodies decrease the calcium requirement of PAD4 by regulating the enzyme at Ca5. Although clones 16, 102 and 104 showed some minimal effect on the activity of the mutant D³⁸⁸A, Ca5 appears to be the most important site of interaction.

Discussion

[00175] Analysis of recombinant antibodies cloned from single new emigrant and mature naïve RA B cells provided initial evidence of defective central and peripheral checkpoints in B cell tolerance in patients with RA^{4,18}. Despite the considerable understanding of ACPA generation gained through the study of recombinant antibodies cloned from single RA B cells^{3,11}, monoclonal antibodies to PAD4 have not previously been identified. The antibodies identified in this study have unique features that provide novel insights into the understanding of the humoral

autoimmune response in RA. In contrast to ACPAs and other antibodies cloned from patients with RA^{3,11,13,14}, the antibodies identified in this study are not polyclonal and antigen recognition was independent of SHM. All clones appear to have emerged from anti-PAD4 B cell precursors (FIG. 6), which likely bypassed early tolerance checkpoints. Nevertheless, these results demonstrate that affinity maturation is essential for the antibodies to gain efficient agonistic effect against PAD4.

[00176] While polyclonal RA sera containing agonistic antibodies to PAD4 were initially identified by their cross-reactivity with PAD3, the cloning of individual antibodies from these patients demonstrated that the functional effect on PAD4 is not driven by antibodies binding to a single epitope. Instead, antibodies targeting different sites on the enzyme, including at least one epitope with structural similarity to PAD3, was able to enhance PAD4 activity.

[00177] In PAD4, Ca5 is positioned near the surface of subdomain 2 at the interface between the N- and C-terminal domains in a region shown to be important for full activation of the related enzyme PAD2^{26,33}. This location may allow Ca5 to be accessible to functional autoantibody binding and potentially to other regulatory molecules. Although pharmacologic PAD inhibition has been focused on blocking the catalytic site in the enzyme, the finding that autoantibodies can modulate PAD4 activity by regulating Ca5 offers an additional target for inhibitor development. Molecules designed to interact with Ca5 could potentially block PAD4 hyperactivation with a limited effect on physiologic functions that require lower enzyme activity. Similarly, such molecules may be useful to counteract PAD4 interactions with agonistic autoantibodies in patients with RA. In addition to mechanistic insights, the analysis of patient-derived monoclonal antibodies, as employed here, offers the opportunity to address pathogenic pathways in erosive RA and to define approaches to offset the consequences of such damage.

[00178] It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods of the present disclosure described herein are readily applicable and appreciable, and may be made using suitable equivalents without departing from the scope of the present disclosure or the aspects and embodiments disclosed herein. Having now described the present disclosure in detail, the same will be more clearly understood by reference to the following examples, which are merely intended only to illustrate some aspects and embodiments of the disclosure, and should not be viewed as limiting to the scope of the disclosure. The

disclosures of all journal references, U.S. patents, and publications referred to herein are hereby incorporated by reference in their entireties.

[00179] The present disclosure has multiple aspects, illustrated by the non-limiting examples described herein.

[00180] It is understood that the foregoing detailed description and accompanying examples are merely illustrative and are not to be taken as limitations upon the scope of the present disclosure, which is defined solely by the appended claims and their equivalents.

[00181] Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to the chemical structures, substituents, derivatives, intermediates, syntheses, compositions, formulations, or methods of use of the present disclosure, may be made without departing from the spirit and scope thereof.

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What is claimed is:

1. A monoclonal antibody comprising:
 - a. a heavy variable chain comprising SEQ ID NO:1 and a light variable chain comprising SEQ ID NO:2;
 - b. a heavy variable chain comprising SEQ ID NO:3 and a light variable chain comprising SEQ ID NO:4;
 - c. a heavy variable chain comprising SEQ ID NO:5 and a light variable chain comprising SEQ ID NO:6;
 - d. a heavy variable chain comprising SEQ ID NO:7 and a light variable chain comprising SEQ ID NO:8; or
 - e. a heavy variable chain comprising SEQ ID NO:9 and a light variable chain comprising SEQ ID NO:10.
2. The monoclonal antibody of claim 1, wherein the antibody is a human monoclonal antibody.
3. A composition comprising at least one monoclonal antibody of claim 1.
4. The composition of claim 3, wherein the composition further comprises an excipient or at least one pharmaceutically acceptable carrier.
5. A kit comprising the composition of claim 3.
6. A method of increasing the level of PAD4 in a subject, the method comprising administering an effective amount of a composition of claim 3 to a subject.
7. A method of screening for an agent that inhibits PAD4, the method comprising:
 - a. administering to an animal at least one monoclonal antibody of claim 1 or composition of claim 3 to an animal to increase the level of PAD4 in the animal;
 - b. administering at least one agent to the animal having an increased level of PAD4; and
 - c. determining if the level of PAD4 in the animal has decreased, increased, or remained the unchanged after administration of the agent to the animal.

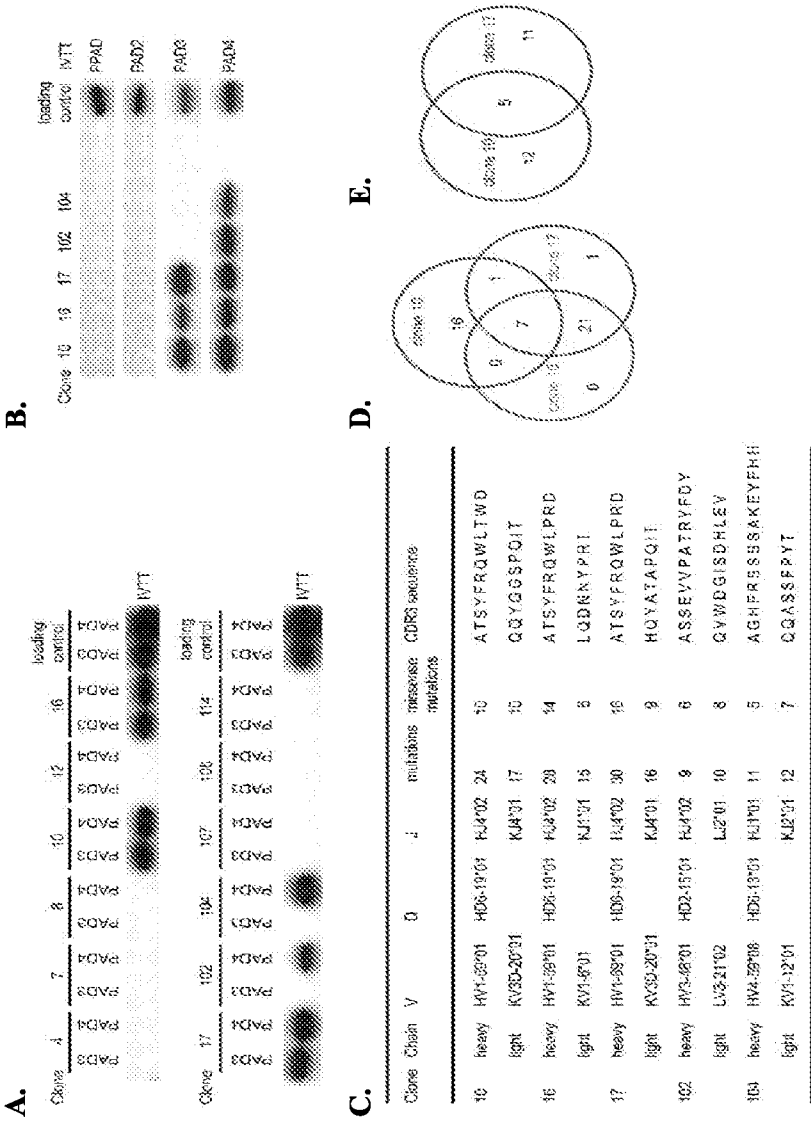


FIG. 1A-E

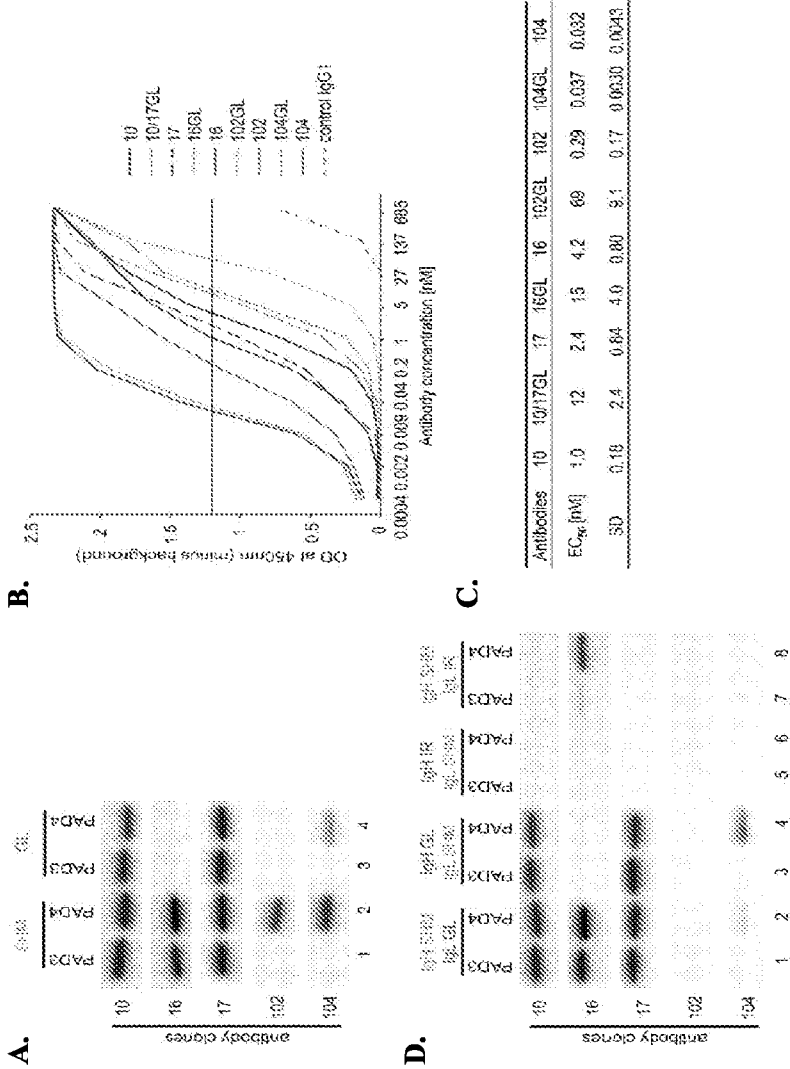


FIG. 2A-D

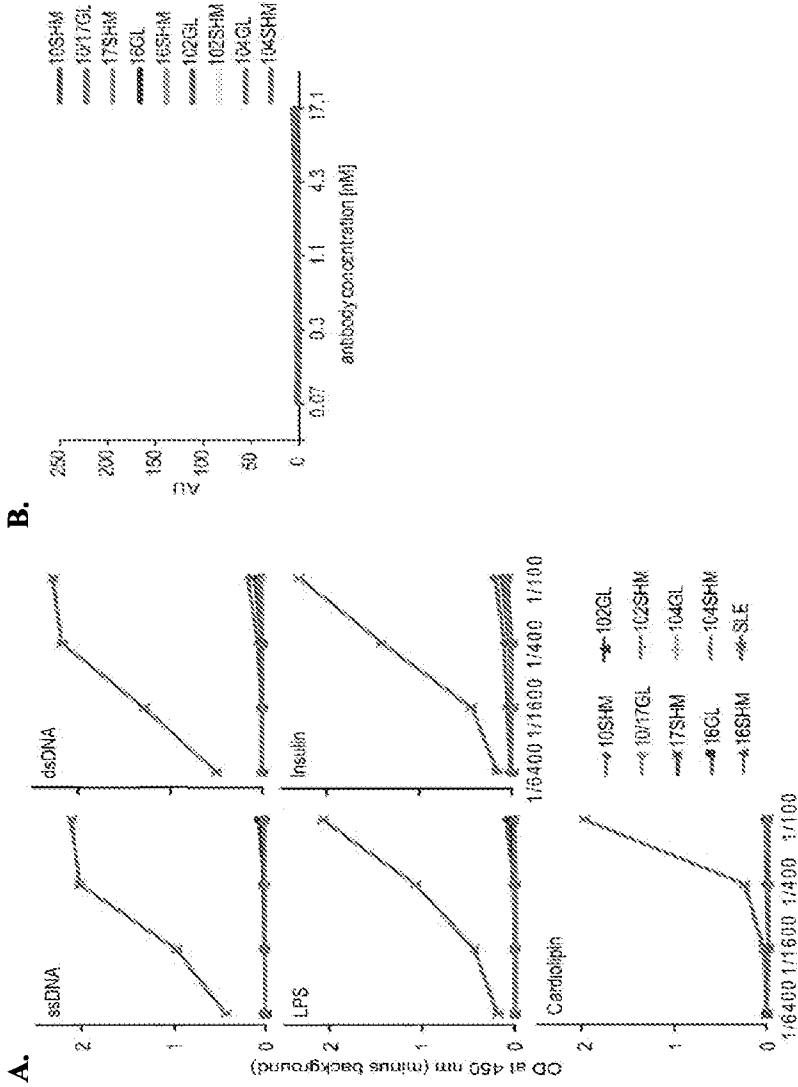


FIG. 3A-B

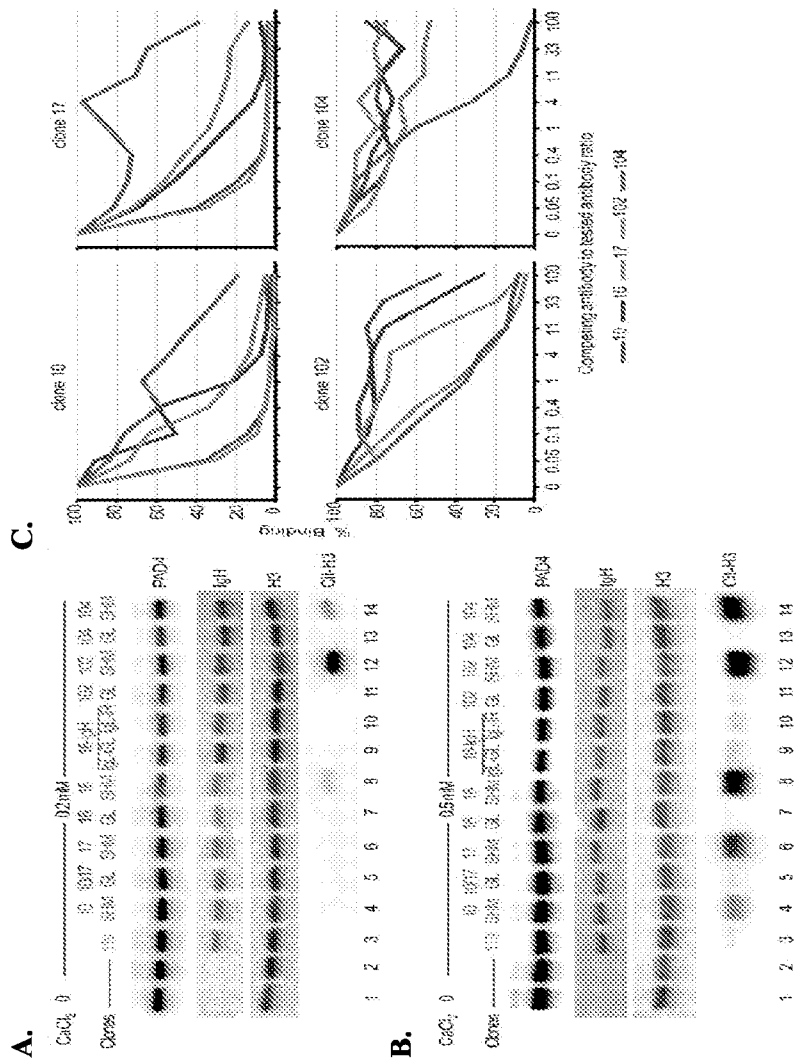


FIG. 4A-C

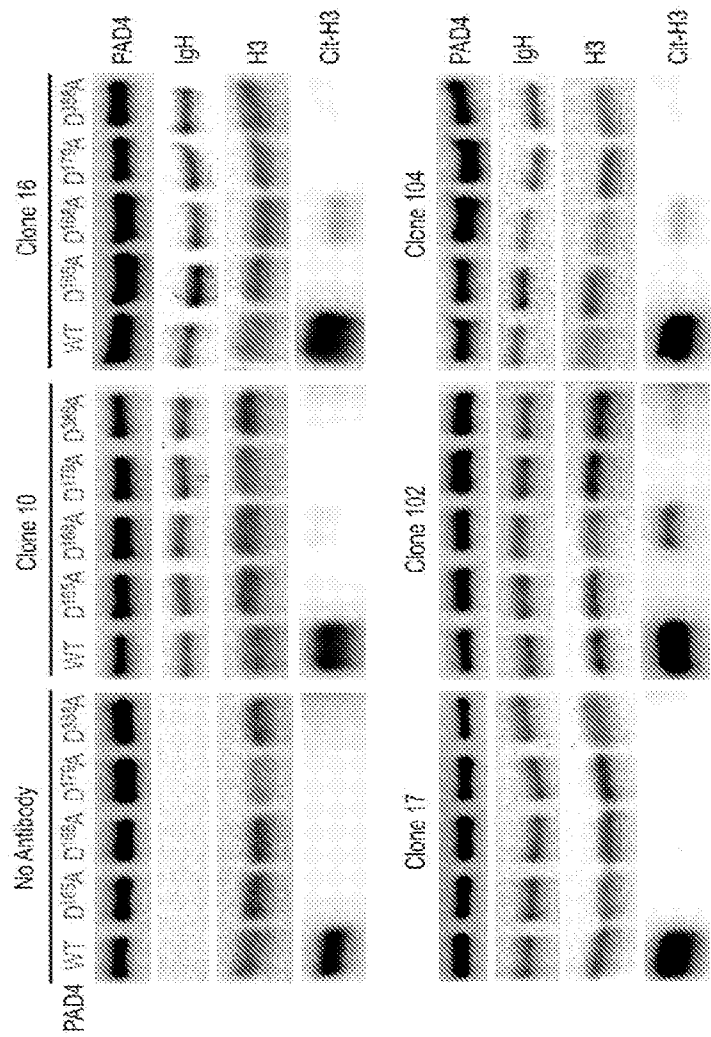


FIG. 5

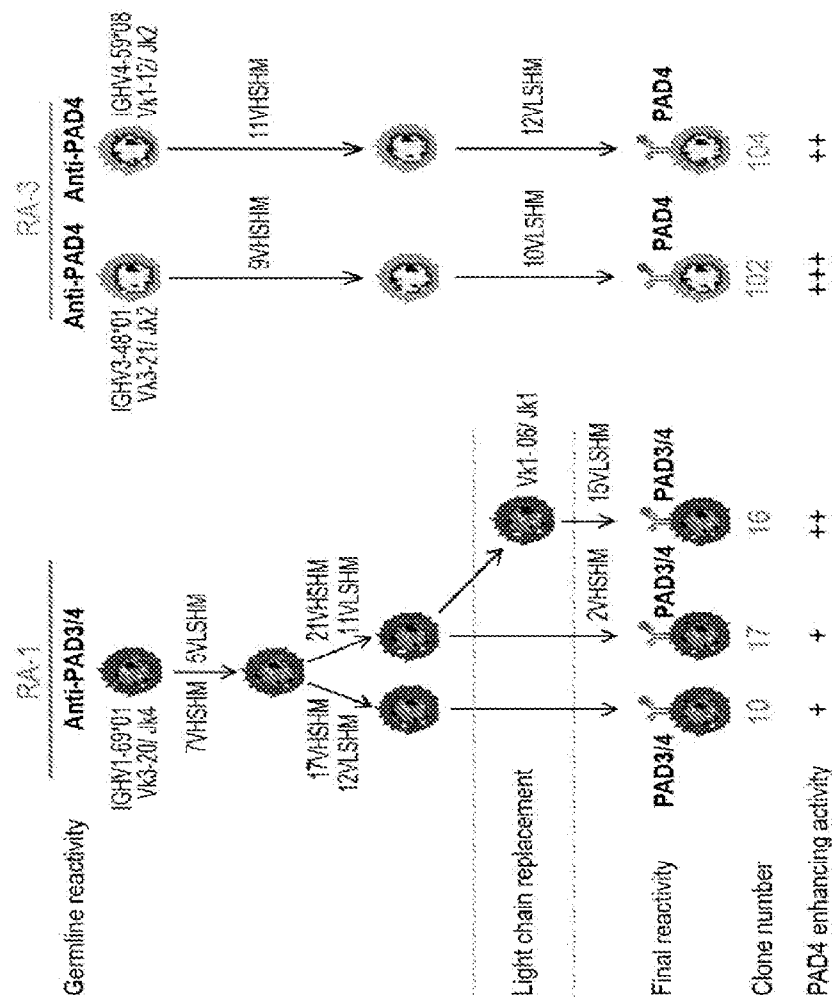


FIG. 6

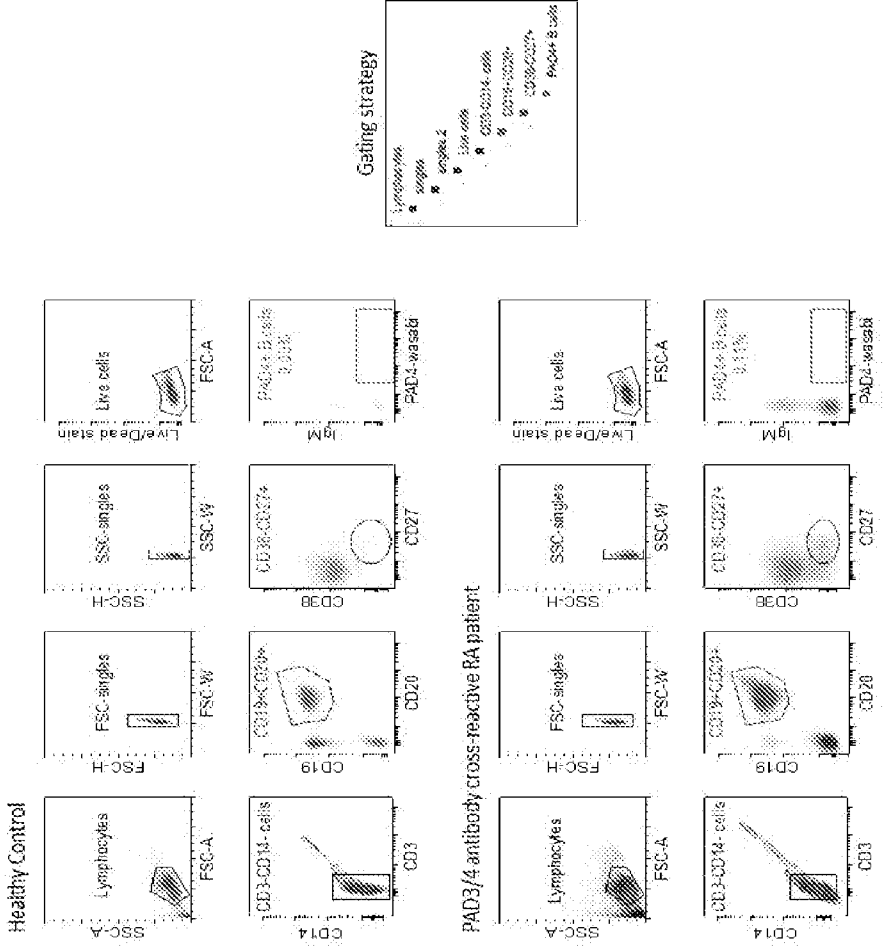


FIG. 7

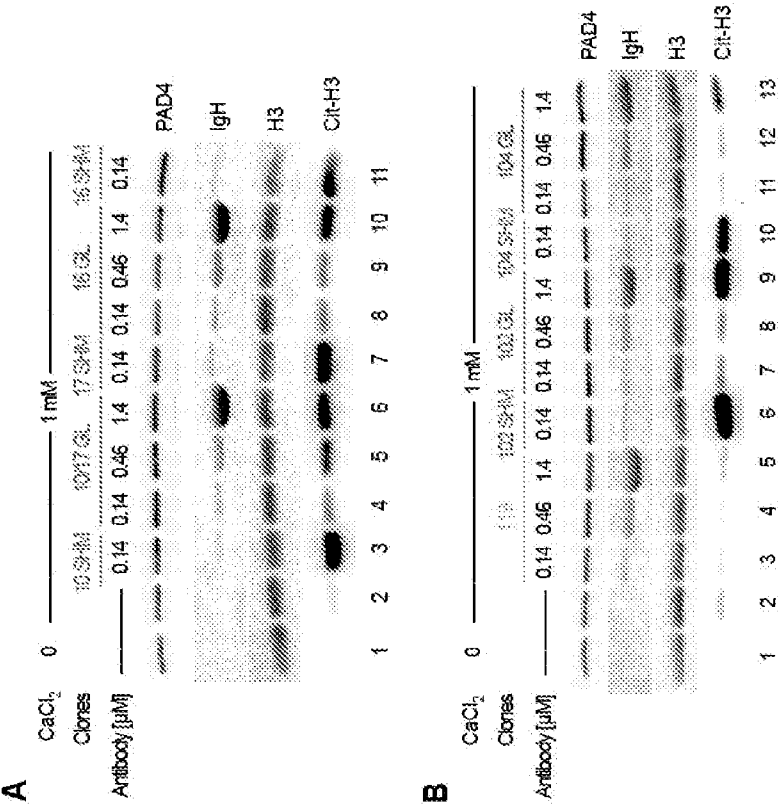


FIG. 8A-B

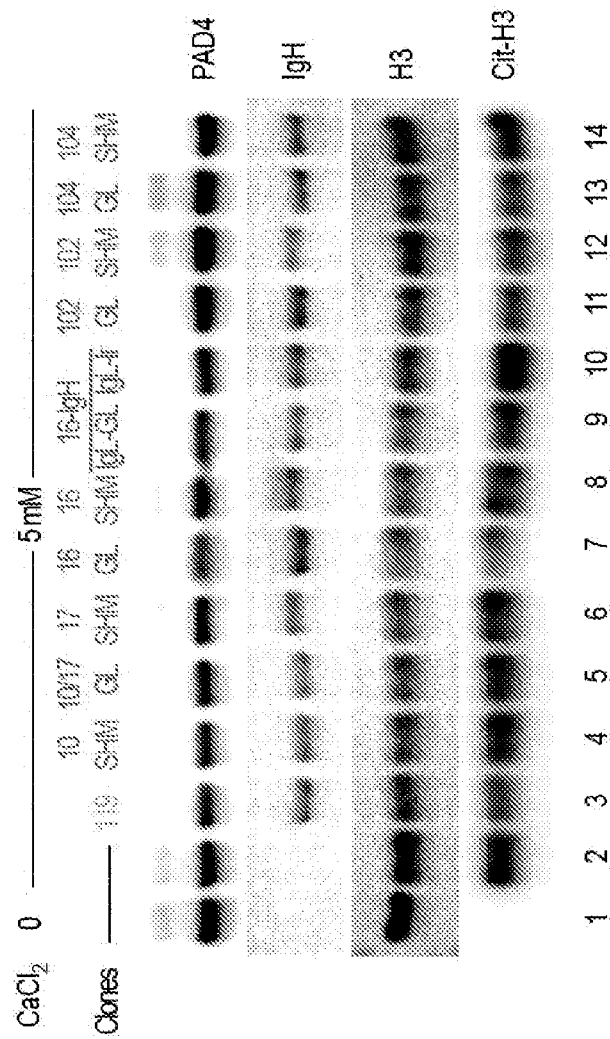


FIG. 9

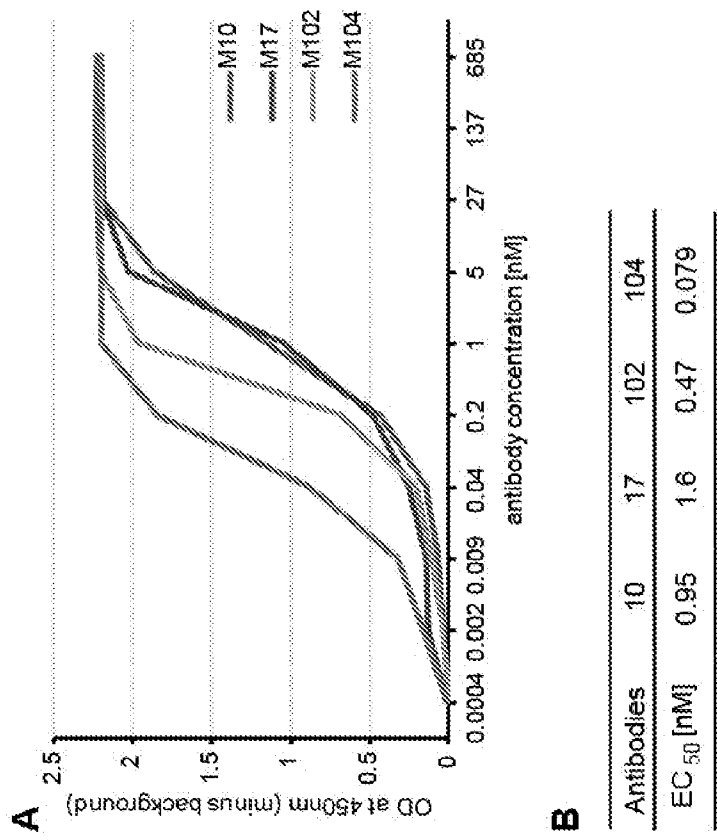


FIG. 10A-B

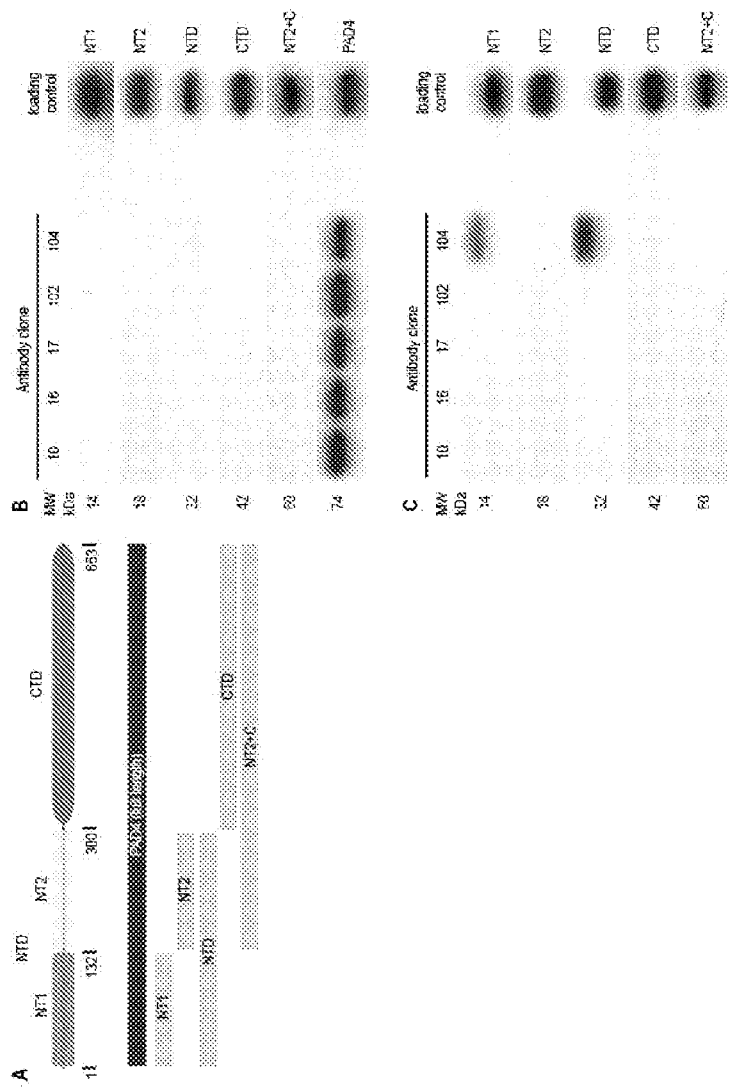


FIG. 11A-B

A.

Clone 16

16 heavy chain

QVQLVESGAELVKKPGSSVKYSCKSSGGTNNYAVVWVRQAPGQGEWMGGHPMEDTP
HYAQKFQGRVTHADEDTSTAYMELNSLRSDDTAVYYCAISYERQWLPRDWGQGLV
TVSSA

16 kappa chain

EIVMTQSPSSLASVGDRTVITCRASQGRNELGWYQQKPGKAPRLITYAASLSQGPS
REFSGSGTDFTLTISGLQPEDFATYYCLQDNNYPRTFGQGTKVDIK

Clone 10

10 heavy chain

EVQLVQSGAEVKKPGSSVKYSLASGGTNNYAVSWVRQAPGQGLEWMGGHPMEDIA
NYPQKFQGRVTHADETSTAYMELNSLRSEDTAVYYCATSYTRQWLTDWVGQGLVT
VSSA

10 kappa chain

EIVLTQSPATLSLSPGQRATLSCGASQPVSSNYLAWFQHKPGLAPRLITYDSIRATGVFD
REFSGSGTDFTLTISGLEPEDEAVYYCQQYGGSPQHFGGGTKVDIK

Clone 17

17 heavy chain

EVQLVESGAELVKKPGSSVKYSKSSGGTNNYAVVWVRQAPGQGEWMGGHPMEDTP
HYAQKFQGRVTHADEDTSTAYMELNSLRSDDTAVYYCATSYERQWLPRDWGQGLV
TVSSA

17 kappa chain

EIVLTQSPATLSLSPGQRATLSCGASQSVSSNYLAWYQQKPGAPRLITYDSIRATGIPD
REFSGSGTDFTLTISGLEPEDEAVYYCHQYATAPQITGGGINKVEIK

FIG. 12A

B.

Clone 102

102 heavy chain

QVQLVESGGGLVQPGGSLRLSCAASGFTESRYTMNWVRQAPGKGLEWVSYISSSSIH
YADSVKGRFTISRDNAKKSLEYQMNSLRAEDTAVYVCASSEVVPATRYFDYWGQGTL
VTVSSA

102 lambda chain

SYELTQPPSVSVAPGQTARITCGGNIGTKSVHWYQQRPGRAPVLVYDDSDRPSGIPE
RYSGSNSGNTATLTINRVEAGDEADYVCQVWDGSDHLEVFGGGKLTVLGQPKAAPSS
VTL

Clone 104

104 heavy chain

EVQLVESGPGLVKPSEILSLTCTVSGGSISSYYWSWIRQPPGKGLEWIGYIYSGSTNYN
PSLKSRVSIISVDTSKNQFSRLSSVTAAADTAVYCACGHRSSSSSAKEYFHHWGQGTLVT
VSSA

104 kappa chain

DIQLTQSPSSVSASVGDRVTITCRASQDISTWLAWYQQKPKAPNLLIFAAASSLQSGVPS
RFGDGSGLDFLTITSSIQPEDEATYYCQQAS

FIG. 12B