

COMPOSITIONS AND METHODS OF REDUCING IMMUNOGENICITY AND IMPROVING STABILITY OF ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of priority to U.S. Provisional Patent Application No. 63/538,598, filed on September 15, 2023, the content of which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

10 This application contains a Sequence Listing that has been submitted electronically as an XML file named 50887-0046WO1_ST26_SL.XML.” The XML file, created on August 30, 2024, is 35,169 bytes in size. The material in the XML file is hereby incorporated by reference in its entirety.

BACKGROUND

15 Antibodies are natural proteins that the vertebrate immune system forms in response to foreign substances (antigens), primarily for defense against infection. For over a century, antibodies have been induced in animals under artificial conditions and harvested for use in therapy or diagnosis of disease conditions, or for biological research. Each individual antibody producing-cell produces a single type of antibody with a chemically defined composition, 20 however, antibodies obtained directly from animal serum in response to antigen inoculation actually comprise an ensemble of non-identical molecules (i.e., polyclonal antibodies) made from an ensemble of individual antibody-producing cells.

 The utility of rodent (specifically murine) monoclonal antibodies in human therapy is limited because of problems associated with their non-human origin, in particular their 25 immunogenicity in a human host. In order to minimize the human immune response against therapeutic antibody drugs, monoclonal antibody technology has evolved from full mouse antibodies to chimeric antibodies (mouse variable domains grafted on a human IgG backbone), and to humanized antibodies (mouse CDRs grafted on a human IgG backbone). Humanization of mouse monoclonal antibodies was initially achieved by combining mouse variable domains 30 with human constant domains, creating so called chimeric antibodies having about 70% of

human content. A further degree of humanization was subsequently achieved by grafting the complementarity-determining regions (CDRs) of mouse monoclonal antibodies onto human framework regions of the variable antibody domains of human antibodies. In addition, several amino acid residues present in those framework regions were identified as interacting with the CDRs or antigen and were back mutated in the humanized antibody to improve binding. (Almagro et al., *Frontiers Bioscience* 13:1619-1633 (2008)).

Immunogenicity is the result of a complex series of responses to a substance that is perceived as foreign, and may include production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, hypersensitivity responses, and anaphylaxis. Several factors can contribute to protein immunogenicity, including but not limited to protein sequence, route and frequency of administration, and patient population.

Several humanization methods known in the art, primarily consisting of introducing mutations in regions of the antibody sequence predicted to be immunogenic. However, there are drawback to these methods, including a reduction in the stability of the resulting antibody. Often, the introduction of a deimmunizing mutation during the humanization process impacts antibody conformation (e.g., by disrupting the network of molecular interactions that hold the antibody in the correct conformation for antigen recognition). For example, a deimmunizing mutation might interfere with the intrachain interactions between the light chain and heavy chain, thereby causing significant loss of molecular stability. Loss of stability, in turn, can lead to a number of problems, including low expression yield, reduced processability, shorter shelf-life, and unacceptable pharmacokinetic parameters. As there is a need to develop improved humanized antibodies that have reduced immunogenicity and/or improved stability, there is a need for methods of reducing immunogenicity and/or increasing the stability of humanized antibodies.

SUMMARY

The present disclosure relates to antibodies and antigen-binding antibody fragments that reduce immunogenicity and improve stability of antibodies. Also disclosed herein are compositions comprising these antibodies or antigen-binding antibody fragments, and methods of using these antibodies and antigen-binding antibody fragments to reduce immunogenicity.

Thus, disclosed herein is a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and a light chain variable domain comprising a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain is a positively charged amino acid, and the charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain is a negatively charged amino acid.

In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid is a basic amino acid. In some instances, the negatively charged amino acid is an acidic amino acid. In some instances, the positively charged amino acid is an arginine. In some instances, the negatively charged amino acid is an aspartic acid. In some instances, the humanized antibody further comprises a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain.

In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with at least one of: an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with both the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the amino acid at an amino

acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

In some instances, the substituted phenylalanine derivative comprises a hydroxy-substituted phenyl group. In some instances, the substituted phenylalanine derivative is a tyrosine residue. In some instances, the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain is a glutamine, and/or wherein the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine. In some instances, the humanized antibody further comprises at least one of following: a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of following: an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of following: a serine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and a serine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light

chain. In some instances, the humanized antibody further comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

Also disclosed herein is a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a substituted
5 phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain. In some instances, the humanized antibody further comprises a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain, and a light chain
10 variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain.

In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with at least one of: the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the
15 polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with both: the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human
20 IgG1 light chain, and the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

In some instances, the substituted phenylalanine derivative comprises a hydroxy-substituted phenyl group. In some instances, the substituted phenylalanine derivative is a tyrosine. In some instances, the polar uncharged amino acid residue at an amino acid position
25 corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine. In some instances, the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain variable domain, is a glutamine. In some instances, the humanized antibody further comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid
30 position 44 of a human IgG1 heavy chain. In some instances, the humanized antibody further

comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and
5 the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge.

In some instances, the positively charged amino acid is a basic amino acid. In some instances, the negatively charged amino acid is an acidic amino acid. In some instances, the positively charged amino acid is an arginine. In some instances, the negatively charged amino
10 acid is an aspartic acid. In some instances, the humanized antibody further comprises at least one of following: a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position
15 78 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of following: an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light
25 chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

Also disclosed herein is a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a positively
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charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position
5 corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light
10 chain. In some instances, the light chain variable comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

15 In some instances, the light chain variable domain comprises each of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged residue at an amino acid position corresponding to Chothia canonical amino acid position 100 of a
20 human IgG1 light chain.

In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some
25 instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain is an arginine. In some instances, the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain is an aspartic acid.

In some instances, the humanized antibody further comprises one or both of: an alanine
30 residue at an amino acid position corresponding to Chothia canonical amino acid position 49 of a

human IgG1 heavy chain; and an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In another embodiment, discloses herein is a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the light chain variable domain comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprising each of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the humanized antibody further comprises one or both of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises a positively charged amino acid at an

amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain.

In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, is an arginine. In some instances, the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain, is an aspartic acid. In some instances, the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain, is a serine.

In yet another embodiment, disclosed herein is a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the light chain variable domain comprises an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprising each of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position

corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the humanized antibody further comprises one or both of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain.

In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, is an arginine. In some instances, the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain, is an aspartic acid. In some instances, the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain is a serine.

In some instances, the humanized antibody further comprises at least one of following: a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to

Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

5 In some instances, the humanized antibody further comprises a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain.

 In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, forms
10 a hydrogen bond interaction with at least one of an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

 In some instances, the substituted phenylalanine derivative at an amino acid position
15 corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond interaction with both the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

20 In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, comprises a hydroxy-substituted phenyl group. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, is a tyrosine. In some instances, the amino acid at an amino acid
25 position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, is a glutamine. In some instances, the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain, is a glutamine.

 In some instances, the antibody is an antigen-binding antibody fragment. In some instances, the antigen-binding antibody fragment is a Fab fragment, a Fab'₂ fragment, or a single
30 chain Fv. In some instances, the humanized antibody is a full antibody, a chimeric antibody, a veneered antibody, a CDR-grafted antibody, or a recombinant antibody. In some instances, the

humanized antibody is a human IgG antibody. In some instances, the human IgG antibody has a human IgG1 isotype.

In some instances, the antibody is conjugated to a therapeutic, cytotoxic, cytostatic, immunomodulatory, neurotrophic, or neuroprotective agent.

5 In some instances, the heavy chain variable domain is fused to a heavy chain constant region and the light chain variable domain is fused to a light chain constant region. In some instances, the heavy chain constant region is a mutant form of a natural human heavy chain constant region which has reduced binding to an Fcγ receptor relative to a natural heavy chain constant region. In some instances, the heavy chain constant region is of IgG1 isotype. In some
10 instances, the antibody has at least one mutation in a constant region.

In some instances, the at least one mutation reduces complement fixation or activation by the constant region. In some instances, the at least one mutation is at one or more positions of: 241, 264, 265, 270, 296, 297, 318, 320, 322, 329, and 331 by EU numbering. In some instances, the humanized antibody has an alanine at positions 318, 320, and 322 by EU numbering. In some
15 instances, the humanized antibody has increased thermal stability as compared to a reference antibody. In some instances, the increased thermal stability comprises an increase in expression yield compared to the reference antibody. In some instances, the increased thermal stability comprises an increase in expression yield of at least 100 mg/L compared to the reference antibody. In some instances, the increased thermal stability comprises an increase in melting
20 point as compared to the reference antibody. In some instances, the increase in melting point is an increase of at least 2°C compared to the reference antibody. In some instances, the increase in melting point is an increase of at least 5°C compared to the reference antibody. In some instances, the humanized antibody has a melting temperature of 55°C or greater. In some instances, the humanized antibody has a melting temperature of 65°C or greater. In some
25 instances, the humanized antibody has a melting temperature of 70°C or greater. In some instances, the melting temperature is determined by differential scanning calorimetry.

In some instances, the humanized antibody has a decreased immunogenicity score as compared to a reference antibody. In some instances, the immunogenicity score is determined using Formula I: $\text{Ig Score} = (-\text{KDHI} + 4.5) \times P_{\text{t (average)}} \times \text{Tail Bonus (Formula I)}$. In some
30 instances, the reference antibody comprises the same sequence as the humanized antibody without the one or more amino acid substitutions.

Also disclosed herein is a pharmaceutical composition comprising any one of the humanized antibodies disclosed herein and a pharmaceutically acceptable carrier.

Also disclosed herein is a nucleic acid encoding the heavy chain variable domain and/or the light chain variable domain of an antibodies disclosed herein.

5 Also disclosed herein is a vector comprising a nucleic acid encoding a heavy chain variable domain and a light chain variable domain operably linked to one or more regulatory sequences to effect expression in a mammalian cell of the humanized antibodies disclosed herein. In some instances, the one or more regulatory sequences comprises one or more of a enhancer, ribosome binding site, transcription termination signal, and promoter, optionally,
10 wherein the promoter is a eukaryotic promoter. In some instances, the nucleic acid is codon-optimized for expression in a host cell.

Also disclosed herein is a host cell transformed with any vector described herein. Also disclosed herein is a host cell comprising any nucleic acid described herein.

Also disclosed herein is a method of reducing immunogenicity of a parent antibody by
15 introducing amino acid substitution(s) into the parent antibody to produce a modified antibody comprising: (i) a heavy chain variable domain comprising one or more of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an alanine at an amino acid
20 position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; and a phenylalanine at an
25 amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and/or (ii) a light chain variable domain comprising one or more of: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid
30 position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain;

an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

Also disclosed herein is a method of increasing thermal stability of an antibody by introducing amino acid substitution(s) into a parent antibody to produce a modified antibody comprising: (i) a heavy chain variable domain comprising one or more of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; and a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and/or (ii) a light chain variable domain comprising one or more of: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

Also disclosed herein is a method of reducing immunogenicity of a parent antibody by introducing a first amino acid substitution and a second amino acid substitution into the parent antibody to generate a modified antibody, wherein the first amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and the second amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; wherein the modified antibody has a reduction in immunogenicity compared to the parent antibody. In some instances, the method further comprises introducing a third amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; and a fourth amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the method further comprises introducing at least one of the following amino acid substitutions: an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 77 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; and an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the modified antibody has a melting temperature of 65°C or greater.

Also disclosed herein is a method of increasing thermal stability of a parent antibody by introducing a first amino acid substitution and a second amino acid substitution into the parent antibody to generate a modified antibody, wherein the first amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and the second amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; wherein the modified antibody has an increase in melting temperature of 2°C or greater compared to the parent antibody as measured by differential scanning calorimetry.

In some instances, the method further comprises introducing a third amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino

acid position 91 of a human IgG1 heavy chain; and a fourth amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the method further comprises introducing at least one of the following amino acid substitutions: an amino acid substitution at an amino acid

5 corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 77 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 78 of a

10 human IgG1 heavy chain; and an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the modified antibody has increase in melting temperature of 5°C or greater compared to the parent antibody as measured by differential scanning calorimetry. In some instances, the modified antibody has a melting temperature of 65°C or greater.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A-1C show Chothia canonical amino acid sequences for heavy chain variable regions disclosed herein. VH/VL interfaces are shown in shaded gray, and Vernier zone residues are shown with stippled shading.

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FIGs. 2A-2C show Chothia canonical amino acid sequences for light chain variable regions disclosed herein. VH/VL interfaces are shown in shaded gray, and Vernier zone residues are shown with stippled shading.

DETAILED DESCRIPTION

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A. Definitions

The term “antibody” includes intact antibodies and binding fragments thereof. Typically, fragments compete with the intact antibody from which they were derived for specific binding to the target including separate heavy chains, light chains Fab, Fab', F(ab')₂, F(ab)c, Dabs, nanobodies, and Fv. Fragments can be produced by recombinant DNA techniques, or by

30 enzymatic or chemical separation of intact immunoglobulins. The term “antibody” also includes a bispecific antibody and/or a humanized antibody. A bispecific or bifunctional antibody is an

artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites (*see, e.g.*, Songsivilai and Lachmann, *Clin. Exp. Immunol.*, 79:315-321 (1990); Kostelny et al., *J. Immunol.*, 148:1547-53 (1992)).

Neutral amino acid residue refers to an alanine residue, an asparagine residue, an
5 isoleucine residue, a glycine residue, a glutamine residue, a cysteine residue, a threonine residue, a serine residue, a tyrosine residue, a phenylalanine residue, a proline residue, a valine residue, a methionine residue, a leucine residue, and a tryptophan residue. The nine amino acids that have hydrophobic side chains are glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), phenylalanine (Phe), methionine (Met), and tryptophan (Trp). Six amino
10 acids have side chains that are polar but not charged: serine (Ser), threonine (Thr), cysteine (Cys), asparagine (Asn), glutamine (Gln), and tyrosine (Tyr). Three amino acids have basic side chains at neutral pH: arginine (Arg), lysine (Lys), and histidine (His). Two amino acids have acidic side chains at neutral pH: aspartic acid or aspartate (Asp) and glutamic acid or glutamate (Glu).

15 Specific binding of an antibody to its target antigen means an affinity and/or avidity of at least 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , or 10^{12} M^{-1} . Specific binding is detectably higher in magnitude and distinguishable from non-specific binding occurring to at least one unrelated target. Specific binding can be the result of formation of bonds between particular functional groups or particular spatial fit (*e.g.*, lock and key type) whereas nonspecific binding is usually
20 the result of van der Waals forces. Specific binding does not however necessarily imply that an antibody binds one and only one target.

The basic antibody structural unit is a tetramer of subunits. Each tetramer includes two identical pairs of polypeptide chains, each pair having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The amino-terminal portion of each chain includes a variable
25 region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. This variable region is initially expressed linked to a cleavable signal peptide. The variable region without the signal peptide is sometimes referred to as a mature variable region. Thus, for example, a light chain mature variable region means a light chain variable region without the light chain signal peptide. The carboxy-terminal portion of each chain defines a constant region
30 primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 or more amino acids. *See generally, Fundamental Immunology*, Paul, W., ed., 2nd ed. Raven Press, N.Y., 1989, Ch. 7 (incorporated by reference in its entirety for all purposes).

An immunoglobulin light or heavy chain variable region (also referred to herein as a "light chain variable domain" ("VL domain") or "heavy chain variable domain" ("VH domain"), respectively) consists of a "framework" region interrupted by three "complementarity determining regions" or "CDRs." The framework regions serve to align the CDRs for specific binding to an epitope of an antigen. The CDRs include the amino acid residues of an antibody that are primarily responsible for antigen binding. From amino-terminus to carboxyl-terminus, both VL and VH domains comprise the following framework (FR) and CDR regions: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. CDRs 1, 2, and 3 of a VL domain are also referred to herein, respectively, as CDR-L1, CDR-L2, and CDR-L3; CDRs 1, 2, and 3 of a VH domain are also referred to herein, respectively, as CDR-H1, CDR-H2, and CDR-H3. When the application discloses a VL sequence with R as the C-terminal residue, the R can alternatively be considered as being the N-terminal residue of the light chain constant region. Thus, the application should also be understood as disclosing the VL sequence without the C-terminal R.

The assignment of amino acids to each VL and VH domain is in accordance with any conventional definition of CDRs. Conventional definitions include, the Kabat definition (Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD, 1987 and 1991), the Chothia definition (Chothia & Lesk, *J. Mol. Biol.* 196:901-917, 1987; Chothia *et al.*, *Nature* 342:878-883, 1989); a composite of Chothia Kabat CDR in which CDR-H1 is a composite of Chothia and Kabat CDRs; the AbM definition used by Oxford Molecular's antibody modelling software; and, the contact definition of Martin et al (bioinfo.org.uk/abs) (*see* Table 1). Kabat provides a widely used numbering convention (Kabat numbering) in which corresponding residues between different heavy chains or between different light chains are assigned the same number. When an antibody is said to comprise CDRs by a certain definition of CDRs (e.g., Kabat) that definition specifies the minimum number of CDR residues present in the antibody (*i.e.*, the Kabat CDRs). It does not exclude that other residues falling within another

conventional CDR definition but outside the specified definition are also present. For example, an antibody comprising CDRs defined by Kabat includes among other possibilities, an antibody in which the CDRs contain Kabat CDR residues and no other CDR residues, and an antibody in which CDR H1 is a composite Chothia-Kabat CDR H1 and other CDRs contain Kabat CDR residues and no additional CDR residues based on other definitions.

Table 1. Conventional Definitions of CDRs using Kabat Numbering

Loop	Kabat	Chothia	Composite of Chothia & Kabat	AbM	Contact
L1	L24--L34	L24--L34	L24--L34	L24--L34	L30--L36
L2	L50--L56	L50--L56	L50--L56	L50--L56	L46--L55
L3	L89--L97	L89--L97	L89--L97	L89--L97	L89--L96
H1	H31--H35B	H26--H32..H34*	H26--H35B*	H26--H35B	H30--H35B
H2	H50--H65	H52--H56	H50--H65	H50--H58	H47--H58
H3	H95--H102	H95--H102	H95--H102	H95--H102	H93--H101

*CDR-H1 by Chothia can end at H32, H33, or H34 (depending on the length of the loop).

This is because the Kabat numbering scheme places insertions of extra residues at 35A and 35B, whereas Chothia numbering places them at 31A and 31B. If neither H35A nor H35B (Kabat numbering) is present, the Chothia CDR-H1 loop ends at H32. If only H35A is present, it ends at H33. If both H35A and H35B are present, it ends at H34.

The term “epitope” refers to a site on an antigen to which an antibody binds. An epitope can be formed from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of one or more proteins. Epitopes formed from contiguous amino acids (also known as linear epitopes) are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding (also known as

conformational epitopes) are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. *See, e.g.,* Epitope Mapping Protocols, in *Methods in Molecular Biology*, Vol. 66, Glenn E. Morris, Ed. (1996).

Antibodies that recognize the same or overlapping epitopes can be identified in a simple immunoassay showing the ability of one antibody to compete with the binding of another antibody to a target antigen. The epitope of an antibody can also be defined by X-ray crystallography of the antibody bound to its antigen to identify contact residues. Alternatively, two antibodies have the same epitope if all amino acid mutations in the antigen that reduce or eliminate binding of one antibody reduce or eliminate binding of the other. Two antibodies have overlapping epitopes if some amino acid mutations that reduce or eliminate binding of one antibody reduce or eliminate binding of the other.

Competition between antibodies is determined by an assay in which an antibody under test inhibits specific binding of a reference antibody to a common antigen (*see, e.g.,* Junghans *et al., Cancer Res.* 50:1495, 1990). A test antibody competes with a reference antibody if an excess of a test antibody (*e.g.,* at least 2x, 5x, 10x, 20x or 100x) inhibits binding of the reference antibody by at least 50% as measured in a competitive binding assay. Some test antibodies inhibit binding of the reference antibody by at least 75%, 90% or 99%. Antibodies identified by competition assay (competing antibodies) include antibodies binding to the same epitope as the reference antibody and antibodies binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antibody for steric hindrance to occur.

The term “pharmaceutically acceptable” means that the carrier, diluent, excipient, or auxiliary is compatible with the other ingredients of the formulation and not substantially deleterious to the recipient thereof.

The term “patient” or “subject” includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

An individual is at increased risk of a disease if the subject has at least one known risk-factor (*e.g.,* genetic, biochemical, family history, and situational exposure) placing individuals

with that risk factor at a statistically significant greater risk of developing the disease than individuals without the risk factor.

The term “biological sample” refers to a sample of biological material within or obtainable from a biological source, for example a human or mammalian subject. Such samples
5 can be organs, organelles, tissues, sections of tissues, bodily fluids, peripheral blood, blood plasma, blood serum, cells, molecules such as proteins and peptides, and any parts or combinations derived therefrom. The term biological sample can also encompass any material derived by processing the sample. Derived material can include cells or their progeny. Processing of the biological sample may involve one or more of filtration, distillation, extraction,
10 concentration, fixation, inactivation of interfering components, and the like.

The term “control sample” refers to a biological sample not known or suspected to include a tissue with no or little increased immunogenicity, or at least not known or suspect to include diseased tissues of a given type. Control samples can be obtained from individuals not afflicted with an immunogenic disease. Alternatively, control samples can be obtained from
15 patients afflicted with an immunogenic disease. Such samples can be obtained at the same time as a biological sample thought to comprise an immunogenic disease or on a different occasion. A biological sample and a control sample can both be obtained from the same tissue. Preferably, control samples consist essentially or entirely of normal, healthy tissues and can be used in comparison to a biological sample thought to comprise immunogenic disease-affected regions.
20 In some instances, the tissue in the control sample is the same type as the tissue in the biological sample. In some instances, the immunogenic disease-affected cells thought to be in the biological sample arise from the same cell type as the type of cells in the control sample.

The term “disease” refers to any abnormal condition that impairs physiological function. The term is used broadly to encompass any disorder, illness, abnormality, pathology, sickness,
25 condition, or syndrome in which physiological function is impaired, irrespective of the nature of the etiology.

“Immunogenicity” refers to the ability of a foreign molecule or substance to provoke or to stimulate an immune response in a cell or a subject. An immune response can include activation of a non-specific innate response and/or activation of an adaptive response that
30 includes a cell-mediated immune response, which is carried out by T cells, and a humoral immune response, which is controlled by activated B cells and endogenous antibodies. In some

instances, the humanized antibodies disclosed herein reduce immunogenicity in the cell or the subject.

An “immunogenicity score” refers to a value assigned to immunogenicity or reduction thereof caused by an antibody (e.g., humanized antibody) disclosed herein. Immunogenicity
5 Score is a predictor of immunogenicity. The higher the score, the greater the predicted immunogenicity. The score can be an absolute number based on predicted potential binding of the antibody (e.g., humanized antibody) within a protein sequence of an endogenous protein (e.g., a major histocompatibility class (MHC) class II protein). In some instances, immunogenicity score can be calculated using a publicly available program such as Immune
10 Epitope Database (IEDB) and EpiQuest. In some instances, an immunogenicity score is calculated using Formula I: $\text{Ig Score} = (-\text{KDHI} + 4.5) \times P_{\text{t (average)}} \times \text{Tail Bonus (Formula I)}$.

The term “symptom” refers to a subjective evidence of a disease, such as altered gait, as perceived by the subject. A “sign” refers to objective evidence of a disease as observed by a physician.

15 The term “positive response to treatment” refers to a more favorable response in an individual patient or average response in a population of patients relative to an average response in a control population not receiving treatment.

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic side chains): met, ala, val, leu, ile;
20 Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

25 Percentage sequence identities are determined with antibody sequences maximally aligned by the Kabat numbering convention. After alignment, if a subject antibody region (e.g., the entire mature variable region of a heavy or light chain) is being compared with the same region of a reference antibody, the percentage sequence identity between the subject and reference antibody regions is the number of positions occupied by the same amino acid in both
30 the subject and reference antibody region divided by the total number of aligned positions of the two regions, with gaps not counted, multiplied by 100 to convert to percentage. A “reference

antibody” as used herein refers to an antibody or fragment thereof that competes for binding with a test antibody (e.g., an antibody having one or more substitutions described herein) to the same epitope. The reference antibody does not include the substitution in its sequence and can also be referred to as a parent antibody. In some instances, the reference antibody comprises the same
5 sequence as the humanized antibody without the one or more amino acid substitutions as described herein.

Compositions or methods “comprising” or “including” one or more recited elements may include other elements not specifically recited. For example, a composition that “comprises” or “includes” an antibody may contain the antibody alone or in combination with other ingredients.

10 Designation of a range of values includes all integers within or defining the range, and all subranges defined by integers within the range.

Unless otherwise apparent from the context, the term “about” encompasses insubstantial variations, such as values within a standard margin of error of measurement (e.g., SEM) of a stated value.

15 Statistical significance means $p \leq 0.05$.

The singular forms of the articles “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a compound” or “at least one compound” can include a plurality of compounds, including mixtures thereof.

20 **B. Humanized Antibodies**

A humanized antibody is a genetically engineered antibody in which CDRs from a non-human “donor” antibody are grafted into human “acceptor” antibody sequences (see, e.g., Queen, US 5,530,101 and 5,585,089; Winter, US 5,225,539; Carter, US 6,407,213; Adair, US 5,859,205; and Foote, US 6,881,557). The acceptor antibody sequences can be, for example, a
25 mature human antibody sequence, a composite of such sequences, a consensus sequence of human antibody sequences, or a germline region sequence. Thus, a humanized antibody is an antibody having at least three, four, five or all CDRs entirely or substantially from a donor antibody and variable region framework sequences and constant regions, if present, entirely or substantially from human antibody sequences. Similarly, a humanized heavy chain has at least
30 one, two and usually all three CDRs entirely or substantially from a donor antibody heavy chain, and a heavy chain variable region framework sequence and heavy chain constant region, if

present, substantially from human heavy chain variable region framework and constant region sequences. Similarly, a humanized light chain has at least one, two and usually all three CDRs entirely or substantially from a donor antibody light chain, and a light chain variable region framework sequence and light chain constant region, if present, substantially from human light chain variable region framework and constant region sequences. Other than nanobodies and dAbs, a humanized antibody comprises a humanized heavy chain and a humanized light chain. A CDR in a humanized antibody is substantially from a corresponding CDR in a non-human antibody when at least 85%, 90%, 95% or 100% of corresponding residues (as defined by any conventional definition but preferably defined by Kabat) are identical between the respective CDRs. The variable region framework sequences of an antibody chain or the constant region of an antibody chain are substantially from a human variable region framework sequence or human constant region respectively when at least 85%, 90%, 95% or 100% of corresponding residues defined by Kabat are identical. To be classified as humanized under the 2014 World Health Organization (WHO) International non-proprietary names (INN) definition of humanized antibodies, an antibody must have at least 85% identity to human germline antibody sequences (i.e., prior to somatic hypermutation). Mixed antibodies are antibodies for which one antibody chain (e.g., heavy chain) meets the threshold but the other chain (e.g., light chain) does not meet the threshold. An antibody is classified as chimeric if neither chain meets the threshold, even though the variable framework regions for both chains were substantially human with some murine backmutations. See, Jones et al. (2016) The INNs and outs of antibody nonproprietary names, mAbs 8:1, 1-9, DOI: 10.1080/19420862.2015.1114320. See also "WHO-INN: International nonproprietary names (INN) for biological and biotechnological substances (a review)" (Internet) 2014. Available from: www.who.int/medicines/services/inn/BioRev2014.pdf), incorporated herein by reference. For the avoidance of doubt, the term "humanized" as used herein is not intended to be limited to the 2014 WHO INN definition of humanized antibodies. Some of the humanized antibodies provided herein have at least 85% sequence identity to human germline sequences and some of the humanized antibodies provided herein have less than 85% sequence identity to human germline sequences. Some of the heavy chains of the humanized antibodies provided herein have from about 60% to 100% sequence identity to human germ line sequences, such as, for example, in the range of about 60% to 69%, 70% to 79%, 80% to 84%, or 85% to 89%. Some heavy chains fall below the 2014 WHO INN

definition and have, for example, about 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, or 82%, 83%, or 84% sequence identity to human germ line sequences, while other heavy chains meet the 2014 WHO INN definition and have about 85%, 86%, 87%, 88%, 89% or greater sequence identity to human germ line sequences. Some of the light chains of the humanized antibodies provided herein have from about 60% to 100% sequence identity to human germ line sequences, such as, for example, in the range of about 80% to 84% or 85% to 89%. Some light chains fall below the 2014 WHO INN definition and have, for example, about 81%, 82%, 83% or 84% sequence identity to human germ line sequences, while other light chains meet the 2014 WHO INN definition and have about 85%, 86%, 87%, 88%, 89% or greater sequence identity to human germ line sequences. Some humanized antibodies provided herein that are "chimeric" under the 2014 WHO INN definition have heavy chains with less than 85% identity to human germ line sequences paired with light chains having less than 85% identity to human germ line sequences. Some humanized antibodies provided herein are "mixed" under the 2014 WHO INN definition, for example, having a heavy chain with at least 85% sequence identity to human germ line sequences paired with a light chain having less than 85% sequence identity to human germ line sequences, or vice versa. Some humanized antibodies provided herein meet the 2014 WHO INN definition of "humanized" and have a heavy chain with at least 85% sequence identity to human germ line sequences paired with a light chain having at least 85% sequence identity to human germ line sequences.

Although humanized antibodies often incorporate all six CDRs (defined by any conventional definition but preferably as defined by Kabat) from a mouse antibody, they can also be made with less than all CDRs (*e.g.*, at least 3, 4, or 5 CDRs) from a mouse antibody (*e.g.*, Pascalis *et al.*, *J. Immunol.* 169:3076, 2002; Vajdos *et al.*, *J. of Mol. Biol.*, 320: 415-428, 2002; Iwahashi *et al.*, *Mol. Immunol.* 36:1079-1091, 1999; Tamura *et al.*, *J. Immunol.*, 164:1432-1441, 2000).

In some antibodies only part of the CDRs, namely the subset of CDR residues required for binding, termed the SDRs, are needed to retain binding in a humanized antibody. CDR residues not contacting antigen and not in the SDRs can be identified based on previous studies (for example residues H60-H65 in CDR H2 are often not required), from regions of Kabat CDRs lying outside Chothia hypervariable loops (Chothia, *J. Mol. Biol.* 196:901, 1987), by molecular

modeling and/or empirically, or as described in Gonzales *et al.*, *Mol. Immunol.* 41: 863, 2004. In such humanized antibodies at positions in which one or more donor CDR residues is absent or in which an entire donor CDR is omitted, the amino acid occupying the position can be an amino acid occupying the corresponding position (by Kabat numbering) in the acceptor antibody
5 sequence. The number of such substitutions of acceptor for donor amino acids in the CDRs to include reflects a balance of competing considerations. Such substitutions are potentially advantageous in decreasing the number of mouse amino acids in a humanized antibody and consequently decreasing potential immunogenicity and/or for meeting the WHO INN definition of “humanized”. However, substitutions can also cause changes of affinity, and significant
10 reductions in affinity are preferably avoided. Positions for substitution within CDRs and amino acids to substitute can also be selected empirically.

The human acceptor antibody sequences can optionally be selected from among the many known human antibody sequences to provide a high degree of sequence identity (*e.g.*, 65-85% identity) between a human acceptor sequence variable region frameworks and corresponding
15 variable region frameworks of a donor antibody chain.

In some instances, disclosed herein are humanized antibodies having a salt bridge between an amino acid in the heavy chain variable domain and an amino acid in the light chain variable domain.

In some instances, one or more amino acid substitutions are introduced into the
20 humanized antibody. In some instances, the one or more amino acid substitutions are introduced into a Chothia canonical structure.

In some instances, the one or more amino acid substitutions are introduced into a VH/VL interface, including substitutions at one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) of Chothia canonical amino acid positions 35, 37, 39, 45, 47, 91, 93, 95, and/or 103 of the human IgG1 heavy chain and/or at Chothia canonical amino acid positions
25 27F, 34, 36, 38, 44, 46, 87, 89, 91, 96 and/or 98 of the human IgG1 light chain. Exemplary amino acid substitutions that can be introduced into a VH/VL interface are shown in **FIGs. 1A-1C** and **2A-2C**, and it is appreciated that any combination of substitutions at each position is contemplated herein.

30 In some instances, one or more amino acid substitutions are introduced into a Vernier zone residue, including substitutions at one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,

15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) of Chothia canonical amino acid positions 2, 27-30, 47-49, 67, 69, 71, 73, 78, 93, 94, and/or 103 of the human IgG1 heavy chain and/or at Chothia canonical amino acid positions 2, 4, 35, 36, 46-49, 64, 66, 68, 69, 71, and/or 98 of the human IgG1 light chain. Exemplary Vernier zone residue positions and substitutions are
5 shown in **FIGs. 1A-1C** and **2A-2C**, and it is appreciated that any combination of substitutions at each position is contemplated herein.

In some instances, one or more amino acid substitutions are introduced to generate a backmutation, including substitutions at one or more (e.g., 2, 3, or 4) of Chothia canonical amino acid positions 19, 44, and/or 83 of the human IgG1 heavy chain and/or at Chothia canonical
10 amino acid position 18 of the human IgG1 light chain. It is appreciated that any combination of substitutions at each position is contemplated herein.

In some instances, one or more amino acid substitutions are deimmunizing mutation(s), including substitutions at one or more (e.g., 2, 3, 4, 5, 6, 7, or 8) of Chothia canonical amino acid positions 43, 44, 49, 74, 77, and/or 91 of the human IgG1 heavy chain and/or at Chothia
15 canonical amino acid positions 3 and/or 100 of the human IgG1 light chain. It is appreciated that any combination of substitutions at each position is contemplated herein.

In some instances, the humanized antibody includes a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human
20 IgG1 heavy chain, and a light chain variable domain comprising a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some embodiments, the charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain is a positively charged
25 amino acid. In some instances, the charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain is a negatively charged amino acid. The positively and negatively charged amino acids form an interchain salt bridge. In some instances, the positively charged amino acid is a basic amino acid. In some instances, the negatively charged amino acid is an acidic amino acid. In some instances, the
30 positively charged amino acid is an arginine. In some instances, the negatively charged amino acid is an aspartic acid.

In some instances, the humanized antibody further comprises a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with at least one of: an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with both the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative comprises a hydroxy-substituted phenyl group. In some instances, the substituted phenylalanine derivative is a tyrosine residue. In some instances, the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain is a glutamine. In some instances, the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine.

In some instances, the humanized antibody further comprises at least one of following: a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of following: an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the humanized antibody further comprises at least one of following: a serine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy

chain; and a serine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain. In some instances, the humanized antibody further comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

In some instances, the heavy chain variable domain includes a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain. In some instances, the light chain variable domain includes a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the heavy chain variable domain includes a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and a light chain variable domain includes a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the humanized antibody includes a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain. In some instances, the humanized antibody further comprises a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain, and a light chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain.

In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with at least one of: the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with both: the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative comprises a hydroxy-substituted phenyl group. In some instances, the substituted phenylalanine derivative is a tyrosine. In some instances, the polar uncharged amino acid residue at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine. In some instances, the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain variable domain, is a glutamine. In some instances, the humanized antibody further comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid is a basic amino acid. In some instances, the negatively charged amino acid is an acidic amino acid. In some instances, the positively charged amino acid is an arginine. In some instances, the negatively charged amino acid is an aspartic acid.

In some instances, the humanized antibody further comprises at least one of following: a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position

103 of a human IgG1 heavy chain; a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain. In some instances, the humanized antibody further comprises at least one of
5 following: an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In some instances, the humanized antibody further comprises at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid
10 position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to
15 Chothia canonical amino acid position 92 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

In some instances, the humanized antibody includes a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a positively charged
20 amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a
25 negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the light chain variable comprises an alanine at an amino acid position
30 corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a negatively charged amino acid at an

amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprises each of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged residue at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain is an arginine. In some instances, the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain is an aspartic acid. In some instances, the humanized antibody further comprises one or both of: an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

In some instances, the humanized antibody includes a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the light chain variable domain comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a

negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprising each of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the humanized antibody further comprises one or both of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid residue at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, is an arginine. In some instances, the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain, is an aspartic acid. In some instances, the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain, is a serine.

In some instances, the humanized antibody includes a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position

corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain. In some instances, the light chain variable domain comprises an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain. In some instances, the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain. In some instances, the light chain variable domain comprising each of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

In some instances, the humanized antibody further comprises one or both of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain. In some instances, the heavy chain variable region comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge. In some instances, the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, is an arginine. In some instances, the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain, is an aspartic acid. In some

instances, the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain is a serine.

In some instances, the humanized antibody further comprises at least one (e.g., two or three) of following: a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain. In some instances, the humanized antibody further comprises at least one (e.g., two, three, or four) of the following: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

In some instances, the humanized antibody further comprises a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, forms a hydrogen bond interaction with at least one of an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond interaction with both the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain. In some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain comprises a hydroxy-substituted phenyl group. In

some instances, the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, is a tyrosine.

In some instances, the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain is a glutamine. In some instances, the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine.

In some instances, the antibody is an antigen-binding antibody fragment. In some instances, the antigen-binding antibody fragment is a Fab fragment, a Fab₂ fragment, or a single chain Fv. In some instances, the humanized antibody is a full antibody, a chimeric antibody, a veneered antibody, a CDR-grafted antibody, or a recombinant antibody.

The isotype of the antibody from which the Fc region is originated is not particularly limited, and an Fc region originated from an IgG1, IgG2, IgG3, or IgG4 monoclonal antibody can be appropriately used. In some instances, the humanized antibody is a human IgG antibody. In some instances, the human IgG antibody has a human IgG1 isotype. In some instances, the antibody is conjugated to a therapeutic, cytotoxic, cytostatic, immunomodulatory, neurotrophic, or neuroprotective agent.

In some instances, the heavy chain variable domain is fused to a heavy chain constant region and the light chain variable domain is fused to a light chain constant region. In some instances, the heavy chain constant region is a mutant form of a natural human heavy chain constant region which has reduced binding to an Fcγ receptor relative to the natural heavy chain constant region. In some instances, the heavy chain constant region is of IgG1 isotype. In some instances, the antibody has at least one mutation in a constant region. In some instances, the at least one mutation reduces complement fixation or activation by the constant region. In some instances, the at least one mutation is at one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11) positions of: 241, 264, 265, 270, 296, 297, 318, 320, 322, 329, and 331 by EU numbering. In some instances, the humanized antibody has an alanine at positions 318, 320, and 322 by EU numbering.

Additional representative heavy and light chain variable domain sequences are provided in Tables 2 and 3, respectively.

Table 2. Heavy Chain Variable Domain Sequences

SEQ ID NO	Name	Sequence
1	m13D3-VH	EVQLVESGGGLVQPGGSLKLSAASGFTFSNYFMSWVRQTPEKRLEWVAYIST GGDSANYADNVKGRFTISRDNAKNTLYLQMNSLMSED TAMYFCARQTYYSY GGFPYWGQGT LVT VSA
2	IGHV3-48*03	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKGLEWVSYS SSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARYFDYWGQ GTLTVSS
3	AEX28899	EVQLVESGGGLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKGL EWSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRAED TAVYYCARRNYDSSGGYGHWGQGT LVT VSS
4	h13D3VHv1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMSWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNSLYLQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
5	h13D3VHv2	EVQLVESGGGLVQPGGSLKLSAASGFTFSNYFMSWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNSLYLQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
6	h13D3VHv3	EVQLVESGGGLVQPGGSLKLSAASGFTFSNYFMSWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNSLYAQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
7	h13D3VHv4	EVQLVESGGGLVQPGGSLKLSAASGFTFSNYFMSWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTLYAQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
8	h13D3VHv5	EVQLVESGGGLVQPGGSLKLSAASGFTFSNYFMSWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTAYAQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
9	h13D3VHv6	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMSWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTAYAQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
10	h13D3VHv7	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTAYAQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
11	h13D3VHv8	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTLYGQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
12	h13D3VHv9	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTGYLQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
13	h13D3VHv10	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGKGL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTLYLQMNSGRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
14	h13D3VHv11	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGKRL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTGYLQMNSLMAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS
15	h13D3VHv12	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGERL EWWAYISTGGDSANYADNVKGRFTISRDNAKNTGYLQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGT LVT VSS

16	h13D3VHv13	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMGWVRQAPGEDL EWWAYISTGGDSANYADNVKGRFTISRDNKNTGYLQMNSLRAED TAVYFCARQTYYSYGGFPYWGQGLTVTVSS
17	h13D3VHvd1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMSWVRQAPGKGL EWWSYISTGGDSANYADNVKGRFTISRDNKNSLYLQMNSLRAED TAVYYCARQTYYSYGGFPYWGQGLTVTVSS
18	h13D3VHvd2	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMSWVRQAPGKGL EWWSYISTGGDSANYADNVKGRFTISRDNKNTAYLQMNSLRAED TAVYYCARQTYYSYGGFPYWGQGLTVTVSS
19	h13D3VHvd3	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMSWVRQAPGKRL EWWAYISTGGDSANYADNVKGRFTISRDNKNSLYLQMNSLRAED TAVYYCARQTYYSYGGFPYWGQGLTVTVSS
20	h13D3VHvd4	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYFMSWVRQAPGKRL EWWSYISTGGDSANYADNVKGRFTISRDNKNTAYLQMNSLRAED TAVYYCARQTYYSYGGFPYWGQGLTVTVSS

Table 3. Light Chain Variable Domain Sequences

SEQ ID NO	Name	Sequence
21	m13D3-VL	DVVMQTQSPLSLPVS LGDQASISCRSSQSLVHSNGKTYLHWYQQKP GQSPKLLIYKVS DRYSGVSDRFSGSGSGTDFTLKISRVEDLGV YFCSQSLHVP LTFGAGTKLELK
22	IGKV2-30*02	DVVMQTQSPLSLPVT LGQPASISCRSSQSLVHSDGNTYLNWFQQR GQSPRLLIYKVS NRDSGVPDRFSGSGSGTDFTLKISRVEAEDVGV YYCMQGT HWPYTFGQGTKLEIK
23	ABC66863	DIVMTQSPLSLPVT LGQPASISCRSSQSLVSDGNTYLNWFQQR GQSPRLLIYKVS NRDSGVPDRFSGSGSGTDFTLKISRVEAEDVGV YYCMQGT HRPLTFGGG TKVEIK
24	h13D3VLv1	DVVMQTQSPLSLPVT LGQPASISCRSSQSLVHSNGKTYLHWYQQRP GQSPRLLIYKVS DRYSGVDPDRFSGSGSGTDFTLKISRVEAEDVGV YFCSQSLHVP LTFGGG TKVEIK
25	h13D3VLv2	DVVMQTQSPLSLPVT LGQQASISCRSSQSLVHSNGKTYLHWYQQRP GQSPRLLIYKVS DRYSGVDPDRFSGSGSGTDFTLKISRVEAEDVGV YFCSQSLHVP LTFGGG TKVEIK
26	h13D3VLv3	DVVMQTQSPSSLPVT LGQQASISCRSSQSLVHSNGKTYLHWYQQRP GQSPRLLIYKVS DRYSGVDPDRFSGSGSGTDFTLKISRVESEDVGV YFCSQSLHVP LTFGGG TKVEIK
27	h13D3VLv4	DVVMQTQSPSSLPVT LGQQASISCRSSQSLVHSNGKTYLHWYQQRP GQSPRLLIYKVS DRYSGVDPDRFSGSGSGTDFTLKISRVESEDVGV YFCSQSGHVPLTFGGG TKVEIK
28	h13D3VLv5	DVVMQTQSPSSLPVT LGQPASISCRSSQSLVHSNGKTYLHWYQQRP GQSPRLLIYKVS DRYSGVDPDRFSGSGSGTDFTLKISRVESEDVGV YFCSQSGHVPLTFGGG TKVEIK
29	h13D3VLv6	DVVMQTQSPSSLPVT LGQPASISCRSSQSLVHSNGKTYLHWYQQRP GQSPRLLIYKVS DRYSGVDPDRFSGSGSGTDFTLKISRVESEDVGV YFCSQSLHVP LTFGGG TKVEIK

30	h13D3VLv7	DVVMTQSPSSLPVTLGQPASISCRSSQSLVHSNGKTYLHWYQQRPGQSPRLLIYKVSDRYSGVPDRFSGSGSGTDFTLKISRVESEDVGVYFCSQSLHIPLTFGGGKVEIK
31	h13D3VLv8	DVVMTQSPSSLPVTLGQPASISCRSSQSLVHSNGKTYLHWYQQRPGQSPRLLIYKVSDRYSGVPDRFSGSGSGTDFTLKISRVESEDVGVYFCSQSLHAPLTFGGGKVEIK
32	h13D3VLv9	DVQMTQSPSSLPVTLGQPASISCRSSQSLVHSNGKTYLHWYQQRPGQSPRLLIYKVSDRYSGVPDRFSGSGSGTDFTLKISRVESEDVGVYFCSQSGHVPLTFGDGKVEIK
33	h13D3VLv10	DVQMTQSPSSLPVTLGQPASISCRSSQSLVHSNGKTYLHWYQQRPGQSPRLLIYKVSDRYSGVPDRFSGSGSGTDFTLKISRVESEDVGVYFCSQSGHVPLTFGRGKVEIK
34	h13D3VLv1	DVQMTQSPSLPVTLGQPASISCRSSQSLVHSNGKTYLHWYQQRPGQSPRLLIYKVSDRYSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYFCSQSLHVPLTFGDGKVEIK
35	h13D3VLv2	DVQMTQSPSLPVTLGQPASISCRSSQSLVHSNGKTYLHWYQQRPGQSPRLLIYKVSDRYSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYFCSQSAHVPLTFGDGKVEIK

In some instances, the humanized antibody has increased thermal stability as compared to a reference antibody. The increased thermal stability comprises an increase (e.g., at least a 5% increase, at least a 10% increase, at least a 15% increase, at least a 20% increase, at least a 25% increase, at least a 30% increase, at least a 35% increase, at least a 40% increase, at least a 45% increase, or at least a 50% increase, or a 1% increase to a 50% increase, a 1% increase to a 40% increase, a 1% increase to a 30% increase, a 1% increase to a 20% increase, a 1% increase to a 10% increase, a 1% increase to a 5% increase, a 5% increase to a 50% increase, a 5% increase to a 40% increase, a 5% increase to a 30% increase, a 5% increase to a 20% increase, a 5% increase to a 10% increase, a 10% increase to a 50% increase, a 10% increase to a 40% increase, a 10% increase to a 30% increase, a 10% increase to a 20% increase, a 20% increase to a 50% increase, a 20% increase to a 40% increase, a 20% increase to a 30% increase, a 30% increase to a 50% increase, a 30% increase to a 40% increase, or a 40% increase to a 50% increase) in expression yield compared to the reference antibody (e.g., a similar antibody not including one or more of the amino acid substitutions described herein). In some instances, the increased thermal stability comprises an increase in expression yield of at least 100 mg/L compared to the reference antibody.

In some instances, the increase in thermal stability comprises an increase in melting point as compared to the reference antibody (e.g., a similar antibody not including one or more of the

amino acid substitutions described herein). In some instances, the increase in melting point is an increase of at least 2 °C, at least 5 °C, or at least 10 °C as compared to the reference antibody. In some instances, the humanized antibody has a melting temperature of 55 °C or greater. In some instances, the humanized antibody has a melting temperature of 65 °C or greater. In some instances, the humanized antibody has a melting temperature of 70 °C or greater. In some embodiments, the humanized antibody has a melting temperature of about 55 °C to about 75 °C, about 55 °C to about 70 °C, about 55 °C to about 65 °C, about 55 °C to about 60 °C, about 60 °C to about 75 °C, about 60 °C to about 70 °C, about 60 °C to about 65 °C, about 65 °C to about 75 °C, about 65 °C to about 70 °C, about 70 °C to about 75 °C, or about 70 °C to about 80 °C. In some instances, the melting temperature is determined by differential scanning calorimetry.

In some instances, the humanized antibody has a decreased immunogenicity score as compared to a reference antibody. In some instances, the humanized antibody has a decreased immunogenicity as compared to a reference antibody.

15 C. Generation of Parent Antibodies

Methods for generating a parent antibody that can be used as the starting point for further humanization using any of the methods described herein, are known in the art. For example, an animal can be immunized with an antigen and optionally, a heterologous carrier and/or adjuvant. Suitable carriers that can be mixed with the antigen prior to immunization include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria (*e.g.*, CRM197), *E. coli*, cholera, or *H. pylori*, or an attenuated toxin derivative. T cell epitopes are also suitable carrier molecules. Some conjugates can be formed by linking an antigen to an immunostimulatory polymer molecule (*e.g.*, tripalmitoyl-S-glycerine cysteine (Pam₃Cys), mannan (a mannose polymer), or glucan (a β 1→2 polymer)), cytokines (*e.g.*, IL-1, IL-1 alpha and β peptides, IL-2, γ -INF, IL-10, GM-CSF), and chemokines (*e.g.*, MIP1- α and β , and RANTES). Immunogens may be linked to the carriers with or without spacers amino acids (*e.g.*, gly-gly). Additional carriers include virus-like particles. Virus-like particles (VLPs), also called pseudovirions or virus-derived particles, represent subunit structures composed of multiple copies of a viral capsid and/or envelope protein capable of self-assembly into VLPs of defined spherical symmetry in vivo. (Powilleit et al., (2007) *PLoS ONE* 2(5):e415.) Alternatively,

peptide immunogens can be linked to at least one artificial T-cell epitope capable of binding a large proportion of MHC Class II molecules., such as the pan DR epitope ("PADRE"). PADRE is described in US 5,736,142, WO 95/07707, and Alexander et al., *Immunity* 1:751-761, 1994. Active immunogens can be presented in multimeric form in which multiple copies of an immunogen and/or its carrier are presented as a single covalent molecule.

[0001] Fragments are often administered with pharmaceutically acceptable adjuvants. The adjuvant increases the titer of induced antibodies and/or the binding affinity of induced antibodies relative to the situation if the peptide were used alone. A variety of adjuvants can be used in combination with an immunogenic fragment to elicit an immune response. Some adjuvants augment the intrinsic response to an immunogen without causing conformational changes in the immunogen that affect the qualitative form of the response. Some adjuvants include aluminum salts, such as aluminum hydroxide and aluminum phosphate, 3 De-O-acylated monophosphoryl lipid A (MPL™) (see GB 2220211 (RIBI ImmunoChem Research Inc., Hamilton, Montana, now part of Corixa). Stimulon™ QS-21 is a triterpene glycoside or saponin isolated from the bark of the Quillaja Saponaria Molina tree found in South America (see Kensil et al., in *Vaccine Design: The Subunit and Adjuvant Approach* (eds. Powell & Newman, Plenum Press, NY, 1995); US 5,057,540), (Aquila BioPharmaceuticals, Framingham, MA; now Antigenics, Inc., New York, NY). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune stimulants, such as monophosphoryl lipid A (see Stoute et al., *N. Engl. J. Med.* 336:86-91 (1997)), pluronic polymers, and killed mycobacteria. Ribi adjuvants are oil-in-water emulsions. Ribi contains a metabolizable oil (squalene) emulsified with saline containing Tween 80. Ribi also contains refined mycobacterial products which act as immunostimulants and bacterial monophosphoryl lipid A. Another adjuvant is CpG (WO 98/40100). Adjuvants can be administered as a component of a therapeutic composition with an active agent or can be administered separately, before, concurrently with, or after administration of the therapeutic agent.

Peptides (and optionally a carrier fused to the peptide) can also be administered in the form of a nucleic acid encoding the peptide and expressed in situ in a subject. A nucleic acid segment encoding an immunogen is typically linked to regulatory elements, such as a promoter and enhancer that allow expression of the DNA segment in the intended target cells of a subject. For expression in blood cells, as is desirable for induction of an immune response, promoter and

enhancer elements from light or heavy chain immunoglobulin genes or the CMV major intermediate early promoter and enhancer are suitable to direct expression. The linked regulatory elements and coding sequences are often cloned into a vector. Antibodies can also be administered in the form of nucleic acids encoding the antibody heavy and/or light chains. If
5 both heavy and light chains are present, the chains are preferably linked as a single chain antibody.

The DNA can be delivered in naked form (*i.e.*, without colloidal or encapsulating materials). Alternatively, a number of viral vector systems can be used including retroviral systems (see, *e.g.*, Lawrie and Tumin, *Cur. Opin. Genet. Develop.* 3:102-109 (1993)) including
10 retrovirus derived vectors such as MMLV, HIV-1, and ALV; adenoviral vectors (see, *e.g.*, Bett et al., *J. Virol.* 67, 591-1 (1993)); adeno-associated virus vectors (see, *e.g.*, Zhou et al., *J. Exp. Med.* 179:1867 (1994)), lentiviral vectors such as those based on HIV or FIV gag sequences, viral vectors from the pox family including vaccinia virus and the avian pox viruses, viral vectors from the alpha virus genus such as those derived from Sindbis and Semliki Forest Viruses (see,
15 *e.g.*, Dubensky et al., *J. Virol.* 70:508-519 (1996)), Venezuelan equine encephalitis virus (see US 5,643,576) and rhabdoviruses, such as vesicular stomatitis virus (see WO 96/34625) and papillomaviruses (Ohe et al., *Human Gene Therapy* 6:325-333 (1995); Woo et al., WO 94/12629 and Xiao & Brandsma, *Nucleic Acids. Res.* 24:2630-2622 (1996)).

DNA encoding an immunogen, or encoding the antibody heavy and/or light chains, or a
20 vector containing the same, can be packaged into liposomes. Suitable lipids and related analogs are described by US 5,208,036, US 5,264,618, US 5,279,833, and US 5,283,185. Vectors and DNA encoding an immunogen or encoding the antibody heavy and/or light chains can also be adsorbed to or associated with particulate carriers, examples of which include polymethyl methacrylate polymers and polylactides and poly(lactide-co-glycolides), (see, *e.g.*, McGee et al.,
25 *J. Micro Encap.* 1996).

Vectors or segments therefrom encoding the antibody heavy and/or light chains can be incorporated in cells *ex vivo*, for example to cells explanted from an individual patient (*e.g.*, lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have
30 incorporated the transgenes. (See, *e.g.*, WO 2017/091512). Exemplary patient-derived cells

include patient derived induced pluripotent stem cells (iPSCs) or other types of stem cells (embryonic, hematopoietic, neural, or mesenchymal).

- A vector or segment therefrom encoding the antibody heavy and/or light chains can be introduced into any region of interest in cells *ex vivo*, such as an albumin gene or other safe harbor gene. Cells incorporating the vector can be implanted with or without prior differentiation. Cells can be implanted into a specific tissue, such as a secretory tissue or a location of pathology, or systemically, such as by infusion into the blood. For example, cells can be implanted into a secretory tissue of a patient, such as the liver, optionally with prior differentiation to cells present in that tissue, such as hepatocytes in the case of a liver.
- Expression of the antibody in the liver results in secretion of the antibody to the blood

D. Humanized Antibody Screening Assays

- Humanized antibodies can be initially screened for the intended binding specificity as described above. Active immunogens can likewise be screened for capacity to induce antibodies with such binding specificity. In this case, an active immunogen is used to immunize a laboratory animal and the resulting sera tested for the appropriate binding specificity.

Antibodies having the desired binding specificity can then be tested in cellular and animal models.

- Active immunogens can also be tested for induction of antibodies in the sera. Both passive and active immunogens can be tested for passage of antibodies across the blood brain barrier into the brain of a wild type or transgenic animal. Antibodies or fragments inducing an antibody can also be tested in non-human primates without disease or that naturally or through induction develop symptoms of diseases characterized by changes in antigen levels. Tests on an antibody or active agent are usually performed in conjunction with a control in which a parallel experiment is conduct except that the antibody or active agent is absent (*e.g.*, replaced by vehicle). Reduction, delay or inhibition of signs or symptoms disease attributable to an antibody or active agent under test can then be assessed relative to the control.

E. Patients Amenable to Treatment

Changes in antigen levels have been found in several diseases. The present regimen can also be used in treatment or prophylaxis of any of these diseases associated with the antigen specifically bound by the humanized antibodies provided herein.

5 Patients amenable to treatment include individuals at risk of disease but not showing symptoms, as well as patients presently showing symptoms. Patients at risk of disease include those having a known genetic risk of disease. Such individuals include those having relatives who have experienced this disease, and those whose risk is determined by analysis of genetic or biochemical markers.

10 In asymptomatic patients, treatment can begin at any age (*e.g.*, 10, 20, 30). Usually, however, it is not necessary to begin treatment until a patient reaches 40, 50, 60 or 70 years of age. Treatment typically entails multiple dosages over a period of time. Treatment can be monitored by assaying antibody levels over time. If the response falls, a booster dosage is indicated.

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F. Nucleic Acids, Host Cells, and Methods of Producing the Same

The disclosure further provides nucleic acids encoding any of the heavy and light chains described in Tables 2 and 3 (*e.g.*, encoding any one of SEQ ID NOs: 1-35). Optionally, such nucleic acids further encode a signal peptide and can be expressed with the signal peptide linked to the variable region. Coding sequences of nucleic acids can be operably linked with regulatory sequences to ensure expression of the coding sequences, such as a promoter, enhancer, ribosome binding site, transcription termination signal, and the like. The regulatory sequences can include a promoter, for example, a prokaryotic promoter or a eukaryotic promoter. The nucleic acids encoding heavy or light chains can be codon-optimized for expression in a host cell. The nucleic acids encoding heavy and light chains can encode a selectable gene. The nucleic acids encoding heavy and light chains can occur in isolated form or can be cloned into one or more vectors. The nucleic acids can be synthesized by, for example, solid state synthesis or PCR of overlapping oligonucleotides. Nucleic acids encoding heavy and light chains can be joined as one contiguous nucleic acid, *e.g.*, within an expression vector, or can be separate, *e.g.*, each cloned into its own expression vector.

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In some instances, the nucleic acid encodes for any of the heavy chain variable domain sequences and/or light chain variable domain humanized sequences described herein.

Also disclosed herein are vectors. In some instances, the vector includes a nucleic acid encoding for any of the heavy chain variable domain sequences disclosed herein. In some instances, the vector includes a nucleic acid encoding for any of the light chain variable domain sequences disclosed herein. A vector can be operably linked to one or more regulatory sequences to effect expression in a mammalian cell of the humanized antibody disclosed herein. In some instances, the vector can include one or more regulatory sequences that include one or more of an enhancer, a ribosome binding site, a transcription termination signal, and a promoter. A promoter can be a eukaryotic promoter. Examples of the vectors include M13 series vectors, pUC series vectors, pBR322, pBluescript, and pCR-Script. In addition to these vectors, for example, pGEM-T, pDIRECT, or pT7 can also be used for the purpose of cDNA subcloning and excision.

In some instances, the nucleic acid is codon-optimized for expression in a host cell. In the case of using eukaryotic cells as the host cells, animal cells or fungus cells can be appropriately used. Specifically, examples of the animal cells can include the following cells: (1) mammalian cells such as CHO (Chinese hamster ovary cell line), COS (monkey kidney cell line), myeloma cells (Sp2/O, NS0, etc.), BHK (baby hamster kidney cell line), HEK293 (human embryonic kidney cell line with sheared adenovirus (Ad)5 DNA), PER.C6 cell (human embryonic retinal cell line transformed with the adenovirus type 5 (Ad5) E1A and E1B genes), HeLa, and Vero (Current Protocols in Protein Science (May, 2001, Unit 5.9, Table 5.9.1)); (2) amphibian cells such as *Xenopus* oocytes; and (3) insect cells such as sf9, sf21, and Tn5.

The antibody can also be prepared using *E. coli* (mAbs 2012 Mar-Apr; 4 (2): 217-225) or yeast (WO2000023579). The antibody prepared using *E. coli* is not glycosylated. On the other hand, the antibody prepared using yeast is glycosylated.

In some instances, the DNA encoding all or part of the humanized antibody of interest is integrated to expression vectors so as to be expressed under the control of expression control regions, for example, an enhancer and a promoter. Next, host cells are transformed with the resulting expression vectors and allowed to express antibodies. In this case, appropriate hosts and expression vectors can be used in combination.

The humanized antibody can be recovered, for example, by culturing the transformed cells and then separating the antibody from within the molecule-transformed cells or from the culture solution thereof. The antibody can be separated and purified by appropriately using in combination methods such as centrifugation, ammonium sulfate fractionation, salting out, ultrafiltration, C1q, FcRn, protein A and protein G columns, affinity chromatography, ion-exchanged chromatography, and gel filtration chromatography.

For example, a production method can include the steps of: (i) preparing a library of humanized antibodies; (ii) selecting, from the prepared library, a humanized antibody having one or more substitutions as described herein; (iii) culturing a host cell comprising a nucleic acid encoding all or part of the humanized antibody selected in the step (ii); and (iv) recovering the humanized antibody from the host cell culture. The humanized antibody may be antibody fragments such as Fv, Fab, or Fab' or may be Fc region-containing antibodies.

G. Conjugated Antibodies

In some embodiments, humanized antibodies can be conjugated with other therapeutic moieties, other proteins, other antibodies, and/or detectable labels. *See* WO 03/057838; US 8,455,622. Such therapeutic moieties can be any agent that can be used to treat, combat, ameliorate, prevent, or improve an unwanted condition or disease in a patient, such as disease or disorder associated with the antigen recognized by the humanized antibody. In some embodiments, the methods further comprise detecting, measuring, and/or monitoring immunogenicity levels and/or the antigen levels in a subject.

Conjugated therapeutic moieties can include, neurotrophic agents, neuroprotective agents, radiotherapeutic agents, radioactive (radiopharmaceuticals), fluorescent, paramagnetic tracers, ultrasound contrast agents, immunomodulators, or any biologically active agents that facilitate or enhance the activity of the antibody, or modify bioavailability, and distribution in the body or within organs. A neurotrophic agent can be any agent, including chemical or proteinaceous agents, which promotes neuron maintenance, growth, or differentiation. A neuroprotective agent can be agent, including chemical or proteinaceous agents, which protects neurons from acute insult or degenerative processes. An immunomodulator can be any agent that stimulates or inhibits the development or maintenance of an immunologic response. A radiotherapeutic agent can be any molecule or compound that emits radiation.

Some such antibodies can be modified to act as immunotoxins. *See, e.g.,* U.S. Patent No. 5,194,594. For example, ricin, a cellular toxin derived from plants, can be coupled to antibodies by using the bifunctional reagents S-acetylmercaptosuccinic anhydride for the antibody and succinimidyl 3-(2-pyridyldithio)propionate for ricin. *See* Pietersz et al., *Cancer Res.*

5 48(16):4469-4476 (1998). The coupling results in loss of B-chain binding activity of ricin, while impairing neither the toxic potential of the A-chain of ricin nor the activity of the antibody. Similarly, saporin, an inhibitor of ribosomal assembly, can be coupled to antibodies via a disulfide bond between chemically inserted sulfhydryl groups. *See* Polito et al., *Leukemia* 18:1215-1222 (2004).

10 Some such antibodies can be linked to radioisotopes. Examples of radioisotopes include, for example, yttrium⁹⁰ (90Y), indium¹¹¹ (111In), ¹³¹I, ^{99m}Tc, radiosilver-111, radiosilver-199, and Bismuth²¹³. Linkage of radioisotopes to antibodies may be performed with conventional bifunctional chelates. For radiosilver-111 and radiosilver-199 linkage, sulfur-based linkers may be used. *See* Hazra et al., *Cell Biophys.* 24-25:1-7 (1994). Linkage of silver radioisotopes may
15 involve reducing the immunoglobulin with ascorbic acid. For radioisotopes such as 111In and 90Y, ibritumomab tiuxetan can be used and will react with such isotopes to form 111In-ibritumomab tiuxetan and 90Y-ibritumomab tiuxetan, respectively. *See* Witzig, *Cancer Chemother. Pharmacol.*, 48 Suppl 1:S91-S95 (2001).

Some such antibodies can be linked to other therapeutic moieties. Such therapeutic
20 moieties can be, for example, cytotoxic, cytostatic, neurotrophic, or neuroprotective. For example, antibodies can be conjugated with toxic chemotherapeutic drugs such as maytansine, geldanamycin, tubulin inhibitors such as tubulin binding agents (*e.g.*, auristatins), or minor groove binding agents such as calicheamicin. Other representative therapeutic moieties include agents known to be useful for treatment, management, or amelioration of a disease or disorder
25 associated with changes in immunogenicity.

Antibodies can also be coupled with other proteins. For example, antibodies can be coupled with Fynomers. Fynomers are small binding proteins (*e.g.*, 7 kDa) derived from the human Fyn SH3 domain. They can be stable and soluble, and they can lack cysteine residues and disulfide bonds. Fynomers can be engineered to bind to target molecules with the same
30 affinity and specificity as antibodies. They are suitable for creating multi-specific fusion proteins based on antibodies. For example, Fynomers can be fused to N-terminal and/or C-

terminal ends of antibodies to create bi- and tri-specific FynomAbs with different architectures. Fynomers can be selected using Fynomer libraries through screening technologies using FACS, Biacore, and cell-based assays that allow efficient selection of Fynomers with optimal properties. Examples of Fynomers are disclosed in Grabulovski *et al.*, *J. Biol. Chem.* 282:3196-3204 (2007);
 5 Bertschinger *et al.*, *Protein Eng. Des. Sel.* 20:57-68 (2007); Schlatter *et al.*, *MAbs.* 4:497-508 (2011); Banner *et al.*, *Acta. Crystallogr. D. Biol. Crystallogr.* 69(Pt6):1124-1137 (2013); and Brack *et al.*, *Mol. Cancer Ther.* 13:2030-2039 (2014).

The antibodies disclosed herein can also be coupled or conjugated to one or more other antibodies (*e.g.*, to form antibody heteroconjugates).

10 Antibodies can also be coupled with a detectable label. Such antibodies can be used in a subject having or being treated for a disease or disorder associated with changes in immunogenicity. Such antibodies are particularly useful for performing such determinations in subjects having or being susceptible to of a disease or disorder associated with changes in immunogenicity. Representative detectable labels that may be coupled or linked to an antibody
 15 include various enzymes, such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such streptavidin/biotin and avidin/biotin; fluorescent materials, such as umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as luminol; bioluminescent materials, such as luciferase, luciferin,
 20 and aequorin; radioactive materials, such as radiosilver-111, radiosilver-199, Bismuth²¹³, iodine (¹³¹I, ¹²⁵I, ¹²³I, ¹²¹I), carbon (¹⁴C), sulfur (³⁵S), tritium (³H), indium (¹¹⁵In, ¹¹³In, ¹¹²In, ¹¹¹In), technetium (⁹⁹Tc), thallium (²⁰¹Tl), gallium (⁶⁸Ga, ⁶⁷Ga), palladium (¹⁰³Pd), molybdenum (⁹⁹Mo), xenon (¹³³Xe), fluorine (¹⁸F), ¹⁵³Sm, ¹⁷⁷Lu, ¹⁵⁹Gd, ¹⁴⁹Pm, ¹⁴⁰La, ¹⁷⁵Yb, ¹⁶⁶Ho, ⁹⁰Y, ⁴⁷Sc, ¹⁸⁶Re, ¹⁸⁸Re, ¹⁴²Pr, ¹⁰⁵Rh, ⁹⁷Ru, ⁶⁸Ge, ⁵⁷Co, ⁶⁵Zn, ⁸⁵Sr, ³²P, ¹⁵³Gd, ¹⁶⁹Yb, ⁵¹Cr, ⁵⁴Mn, ⁷⁵Se, ¹¹³Sn, and
 25 ¹¹⁷Tin; positron emitting metals using various positron emission tomographies; nonradioactive paramagnetic metal ions; and molecules that are radiolabelled or conjugated to specific radioisotopes.

Linkage of radioisotopes to antibodies may be performed with conventional bifunction chelates. For radiosilver-111 and radiosilver-199 linkage, sulfur-based linkers may be used. *See*
 30 Hazra *et al.*, *Cell Biophys.* 24-25:1-7 (1994). Linkage of silver radioisotopes may involve reducing the immunoglobulin with ascorbic acid. For radioisotopes such as 111In and 90Y,

ibritumomab tiuxetan can be used and will react with such isotopes to form ^{111}In -ibritumomab tiuxetan and ^{90}Y -ibritumomab tiuxetan, respectively. *See Witzig, Cancer Chemother. Pharmacol.*, 48 Suppl 1:S91-S95 (2001).

Therapeutic moieties, other proteins, other antibodies, and/or detectable labels may be coupled or conjugated, directly or indirectly through an intermediate (e.g., a linker), to an antibody of the disclosure. *See*, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy," in *Monoclonal Antibodies And Cancer Therapy*, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery," in *Controlled Drug Delivery* (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review," in *Monoclonal Antibodies 84: Biological And Clinical Applications*, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy," in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985); and Thorpe et al., *Immunol. Rev.*, 62:119-58 (1982). Suitable linkers include, for example, cleavable and non-cleavable linkers. Different linkers that release the coupled therapeutic moieties, proteins, antibodies, and/or detectable labels under acidic or reducing conditions, on exposure to specific proteases, or under other defined conditions can be employed.

H. Pharmaceutical Compositions, Methods of Use, and Methods of Reducing Immunogenicity and/or Increasing Thermostability of a Parent Antibody

Disclosed herein are pharmaceutical compositions that include a humanized antibody disclosed herein and a pharmaceutically acceptable carrier. In some instances, a humanized antibody or agent for inducing an antibody or a pharmaceutical composition the same is administered to a patient susceptible to, or otherwise at risk of a disease in regimen (dose, frequency and route of administration) effective to reduce the risk, lessen the severity, or delay the onset of at least one sign or symptom of the disease (e.g., a disease associated with the antigen specifically bound by the humanized antibody). In therapeutic applications, a humanized antibody or agent to induce an antibody is administered to a patient suspected of, or already suffering from a disease (e.g., a disease associated with the antigen specifically bound by the humanized antibody) in a regimen (dose, frequency and route of administration) effective to

ameliorate or at least inhibit further deterioration of at least one sign or symptom of the disease. In particular, the regimen is preferably effective to decrease the level of antigen in the subject or treat a subject having a disease associated with the antigen specifically bound by the humanized antibody.

- 5 In some instances, disclosed herein are methods of reducing immunogenicity of a parent antibody by introducing amino acid substitution(s) into the parent antibody to produce a modified antibody. In some instances, the modified antibody includes one or more of (i) a heavy chain variable domain comprising one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14) of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; an alanine at an amino acid position
10 corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; a
15 substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; and a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and/or (ii) a light chain variable domain
20 comprising one or more of: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia
25 canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.
- 30 In some instances, disclosed herein are methods of increasing thermal stability of an antibody by introducing by introducing amino acid substitution(s) into the parent antibody to

produce a modified antibody comprising: (i) a heavy chain variable domain comprising one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14) of: a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; and a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and/or (ii) a light chain variable domain comprising one or more of: a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain; a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain; a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain; an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; and a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

Also provided herein are methods of reducing immunogenicity of a parent antibody by introducing a first amino acid substitution and a second amino acid substitution into the parent antibody to generate a modified antibody, wherein the first amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and the second amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; wherein the modified antibody has a reduction in immunogenicity. In some instances, the method further includes introducing a third amino acid substitution into the parent antibody at an amino acid corresponding to Chothia

canonical amino acid position 91 of a human IgG1 heavy chain; and a fourth amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

In some instances, the method further includes introducing at least one (e.g., 2, 3, 4, or 5) of the following amino acid substitutions: an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 77 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; and an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

In some instances, the modified antibody has a melting temperature of 65 °C or greater (e.g., 75 °C or greater, or 80 °C or greater).

In some instances, the methods described herein can further include introducing a first amino acid substitution and a second amino acid substitution into the parent antibody to generate a modified antibody, wherein the first amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and the second amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; wherein the modified antibody has increase in melting temperature of 2 °C or greater (e.g., 5 °C or greater, or 10 °C or greater) compared to the parent antibody as measured by differential scanning calorimetry. In some instances, the method further includes introducing a third amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; and a fourth amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

In some instances, the method further includes introducing at least one (e.g., 2, 3, 4, or 5) of the following amino acid substitutions into a parent antibody: an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain; an amino acid substitution at an amino acid

corresponding to Chothia canonical amino acid position 77 of a human IgG1 heavy chain; an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; and an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

5 In some instances, the modified antibody has increase in melting temperature of 5°C or greater (e.g., 10 °C or greater, or 15 °C or greater) compared to the parent antibody as measured by differential scanning calorimetry

 In some instances, the modified antibody has a melting temperature of 65°C or greater (e.g., 70 °C or greater, or 75 °C or greater). In some instances, the modified antibody has an
10 immunogenicity score of 5 or less, 4 or less, 3 or less, 2 or less, or 1 or less.

 A regimen is considered therapeutically or prophylactically effective if an individual treated patient achieves an outcome more favorable than the mean outcome in a control population of comparable patients not treated by methods of the disclosure, or if a more favorable outcome is demonstrated in treated patients versus control patients in a controlled
15 clinical trial (e.g., a phase II, phase II/III or phase III trial) at the $p < 0.05$ or 0.01 or even 0.001 level.

 Effective doses of vary depending on many different factors, such as means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic.

20 Exemplary dosage ranges for humanized antibodies are from about 0.01 to 60 mg/kg, or from about 0.1 to 3 mg/kg or 0.15-2 mg/kg or 0.15-1.5 mg/kg, of patient body weight. Antibody can be administered such doses daily, on alternative days, weekly, fortnightly, monthly, quarterly, or according to any other schedule determined by empirical analysis. An exemplary treatment entails administration in multiple dosages over a prolonged period, for example, of at
25 least six months. Additional exemplary treatment regimen entail administration once per every two weeks or once a month or once every 3 to 6 months.

 The amount of an agent for active administration varies from 0.1-500 µg per patient and more usually from 1-100 or 1-10 µg per injection for human administration. The timing of injections can vary significantly from once a day, to once a year, to once a decade. A typical
30 regimen consists of an immunization followed by booster injections at time intervals, such as 6 week intervals or two months. Another regimen consists of an immunization followed by

booster injections 1, 2 and 12 months later. Another regimen entails an injection every two months for life. Alternatively, booster injections can be on an irregular basis as indicated by monitoring of immune response.

Antibodies or agents for inducing antibodies are preferably administered via a peripheral route (*i.e.*, one in which an administered or induced antibody crosses the blood brain barrier to reach an intended site in the brain.) Routes of administration include topical, intravenous, oral, subcutaneous, intraarterial, intracranial, intrathecal, intraperitoneal, intranasal, intraocular, intradermal, or intramuscular. Some routes for administration of antibodies are intravenous and subcutaneous. Some routes for active immunization are subcutaneous and intramuscular. This type of injection is most typically performed in the arm or leg muscles. In some methods, agents are injected directly into a particular tissue where deposits have accumulated, for example intracranial injection.

Pharmaceutical compositions for parenteral administration are preferably sterile and substantially isotonic and manufactured under GMP conditions. Pharmaceutical compositions can be provided in unit dosage form (*i.e.*, the dosage for a single administration). Pharmaceutical compositions can be formulated using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries. The formulation depends on the route of administration chosen. For injection, antibodies can be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological saline or acetate buffer (to reduce discomfort at the site of injection). The solution can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, antibodies can be in lyophilized form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The present regimen can be administered in combination with another agent effective in treatment or prophylaxis of the disease being treated.

Antibodies are administered in an effective regimen meaning a dosage, route of administration and frequency of administration that delays the onset, reduces the severity, inhibits further deterioration, and/or ameliorates at least one sign or symptom of a disorder being treated. If a patient is already suffering from a disorder, the regimen can be referred to as a therapeutically effective regimen. If the patient is at elevated risk of the disorder relative to the general population but is not yet experiencing symptoms, the regimen can be referred to as a

prophylactically effective regimen. In some instances, therapeutic or prophylactic efficacy can be observed in an individual patient relative to historical controls or past experience in the same patient. In other instances, therapeutic or prophylactic efficacy can be demonstrated in a preclinical or clinical trial in a population of treated patients relative to a control population of untreated patients.

Administration can be parenteral, intravenous, oral, subcutaneous, intra-arterial, intracranial, intrathecal, intraperitoneal, topical, intranasal or intramuscular. Some antibodies can be administered into the systemic circulation by intravenous or subcutaneous administration. Intravenous administration can be, for example, by infusion over a period such as 30-90 min.

The frequency of administration depends on the half-life of the antibody in the circulation, the condition of the patient and the route of administration among other factors. The frequency can be daily, weekly, monthly, quarterly, or at irregular intervals in response to changes in the patient's condition or progression of the disorder being treated. An exemplary frequency for intravenous administration is between weekly and quarterly over a continuous cause of treatment, although more or less frequent dosing is also possible. For subcutaneous administration, an exemplary dosing frequency is daily to monthly, although more or less frequent dosing is also possible.

I. Diagnostics and Monitoring Methods

Also provided are methods of detecting the presence of an antigen specifically bound by the humanized antibody in a subject. Such methods are useful to diagnose or confirm diagnosis of diseases associated with the antigen recognized by the antibody specifically bound by the humanized antibody. The methods can also be used on asymptomatic subjects. The methods are also useful for monitoring disease progression and/or response to treatment in subjects who have been previously diagnosed with a disease associated with the antigen specifically bound by the humanized antibody.

Diagnostic reagents can be administered by intravenous injection into the body of the patient, or directly into the brain by intracranial injection or by drilling a hole through the skull. The dosage of reagent should be within the same ranges as for treatment methods. Typically, the reagent is labeled, although in some methods, the primary reagent is unlabeled and a secondary labeling agent is used to bind to the primary reagent. The choice of label depends on the means

of detection. For example, a fluorescent label is suitable for optical detection. Use of paramagnetic labels is suitable for tomographic detection without surgical intervention. Radioactive labels can also be detected using positron emission tomography (PET) or single-photon emission computed tomography (SPECT).

5 Diagnosis is performed by comparing the number, size, and/or intensity of labeled loci to corresponding base line values. The base line values can represent the mean levels in a population of normal individuals. Base line values can also represent previous levels determined in the same subject. For example, base line values can be determined in a subject before beginning treatment, and measured values thereafter compared with the base line values.

10 In some patients, diagnosis of a disease or disorder associated with changes in antigen levels may be aided by performing a PET scan. A PET scan can be performed using, for example, a conventional PET imager and auxiliary equipment. The scan typically includes one or more regions of the tissue known in general to be associated with a disease or disorder associated with changes in antigen levels and one or more regions in which to serve as controls.

15 In some methods, a PET scan is performed concurrent with or in the same patient visit as an MRI or CAT scan. An MRI or CAT scan provides more anatomical detail of the tissue than a PET scan. However, the image from a PET scan can be superimposed on an MRI or CAT scan image more precisely indicating the location of PET ligand. Some machines can perform both PET scanning and MRI or CAT scanning without the patient changing positions between the
20 scans facilitating superimposition of images.

Suitable PET ligands include radiolabeled antibodies of the disclosure. The radioisotope used can be, for example, C¹¹, N¹³, O¹⁵, F¹⁸, or I¹²³. The interval between administering the PET ligand and performing the scan can depend on the PET ligand and particularly its rate of uptake and clearing into the brain, and the half-life of its radiolabel.

25 PET scans can also be performed as a prophylactic measure in asymptomatic patients or in patients who have mild symptoms but have not yet been diagnosed with a disease or disorder associated with changes in antigen levels but are at elevated risk of developing a disease or disorder associated with the antigen specifically bound by the humanized antibody. For asymptomatic patients, scans are particularly useful for individuals considered at elevated risk of
30 a disease or disorder associated with the antigen specifically bound by the humanized antibody because of a family history, genetic or biochemical risk factors, or mature age. Prophylactic

scans can commence for example, at a patient age between 45 and 75 years. In some patients, a first scan is performed at age 50 years.

Prophylactic scans can be performed at intervals of for example, between six months and ten years, preferably between 1-5 years. In some patients, prophylactic scans are performed
5 annually.

The methods can be used to monitor a course of therapeutic and prophylactic treatment with the agents provided herein.

In some methods, a baseline measurement of antibody in the subject is made before administration, a second measurement is made soon thereafter to determine the peak antibody
10 level, and one or more further measurements are made at intervals to monitor decay of antibody levels. When the level of antibody has declined to baseline or a predetermined percentage of the peak less baseline (*e.g.*, 50%, 25% or 10%), administration of a further dose of antibody is administered. In some methods, peak or subsequent measured levels less background are compared with reference levels previously determined to constitute a beneficial prophylactic or
15 therapeutic treatment regimen in other subjects. If the measured antibody level is significantly less than a reference level (*e.g.*, less than the mean minus one or, preferably, two standard deviations of the reference value in a population of subjects benefiting from treatment) administration of an additional dose of antibody is indicated.

20 J. Kits

[0002] The disclosure further provides kits (*e.g.*, containers) comprising any of the humanized antibodies disclosed herein and related materials, such as instructions for use (*e.g.*, package insert). The instructions for use may contain, for example, instructions for administration of the humanized antibody and optionally one or more additional agents. The
25 containers of humanized antibody may be unit doses, bulk packages (*e.g.*, multi-dose packages), or sub-unit doses.

Package insert refers to instructions customarily included in commercial packages of therapeutic products that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic
30 products

Kits can also include a second container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It can also include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

5

K. Other Applications

The humanized antibodies can be used for detecting the antigen specifically bound by the humanized antibody in the context of clinical diagnosis or treatment or in research. Binding of the humanized antibodies to the biological sample can be compared to binding of the antibodies to a control sample. The control sample and the biological sample can comprise cells of the same tissue origin. Control samples and biological samples can be obtained from the same individual or different individuals and on the same occasion or on different occasions. If desired, multiple biological samples and multiple control samples are evaluated on multiple occasions to protect against random variation independent of the differences between the samples. A direct comparison can then be made between the biological sample(s) and the control sample(s) to determine whether antibody binding the biological sample(s) is increased, decreased, or the same relative to antibody binding to the control sample(s). Increased binding of the antibody to the biological sample(s) relative to the control sample(s) indicates the presence of the antigen specifically bound by the antibody in the biological sample(s). In some instances, the increased antibody binding is statistically significant. Optionally, antibody binding to the biological sample is at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, or 100-fold higher than antibody binding to the control sample.

In some instances, multiple biological samples from the patient are evaluated on multiple occasions to establish both a baseline and measure of random variation independent of treatment. A therapeutic agent is then administered in a regimen. The regimen may include multiple administrations of the agent over a period of time. Optionally, binding of the antibodies is evaluated on multiple occasions in multiple biological samples from the patient, both to establish a measure of random variation and to show a trend in response to immunotherapy. The various assessments of antibody binding to the biological samples are then compared. If only two assessments are made, a direct comparison can be made between the two assessments to determine whether antibody binding has increased, decreased, or remained the same between the

two assessments. If more than two measurements are made, the measurements can be analyzed as a time course starting before treatment with the therapeutic agent and proceeding through the course of therapy.

The antibodies can also be used as research reagents for laboratory research in detecting
5 an antigen, or fragments thereof. In such uses, antibodies can be labeled with fluorescent molecules, spin-labeled molecules, enzymes, or radioisotopes, and can be provided in the form of kit with all the necessary reagents to perform the detection assay. The antibodies can also be used to purify the antigen, or binding partners of the antigen, *e.g.*, by affinity chromatography.

All patent filings, websites, other publications, accession numbers and the like cited
10 above or below are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or
15 filing date of a priority application referring to the accession number if applicable. Likewise, if different versions of a publication, website or the like are published at different times, the version most recently published at the effective filing date of the application is meant unless otherwise indicated. Any feature, step, element, embodiment, or aspect of the disclosure can be used in combination with any other unless specifically indicated otherwise. Although the present
20 disclosure has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

EXAMPLES

25 ***Example 1: Methods of Selecting and Producing making anti-TDP43 monoclonal antibody 13D3***

Beginning with a murine sequence for monoclonal antibody 13D3 humanization, a search was made over the protein sequences in the Protein Data Bank (PDB) database (Deshpande et al, Nucleic Acids Res. 2005 Jan 1; 33(Database Issue): D233–D237) to find structures, which would
30 provide a rough structural model of 13D3. The crystal structure of an antibody fab PDB code 5BK5 (Scally, et al., Nat Commun. 2017 Nov 16;8(1):1568), a human germline antibody, was

used for both Vh and Vk (i.e., VL) structure since it had good resolution (3.0 Å) and overall sequence similarity to 13D3 Vh and Vk, retaining the same canonical structures for the loops.

The 5BK5 antibody is a human germline-derived antibody and has same canonical classes and belonging to human germline IGHV3-48'03 for heavy chain and IGKV2-30*02 for light chain. Therefore, 5BK5 variable heavy and variable light chain sequences were also used as human acceptor framework.

Accordingly, the framework regions of 5BK5 VH and 5BK5 VL were chosen as the acceptor sequences for the CDRs of 13D3. A model of the 13D3 CDRs grafted onto the respective human frameworks for VH and VL was built and used as a guidance for further backmutations. The amino acid sequences consisting of 5BK5 VH human frameworks and 13D3 CDRs were designated hu13D3VHvd1 and the amino acid sequences consisting of 5BK5 VL human framework and 13D3 VL CDRs were designated hu13D3VLvd1. Additional versions of hu13D3-VH and hu13D3-VL were designed to enable assessment of various framework residues for their contributions to antigen binding, thermostability, developability (deamination, oxidation, N-glycosylation, proteolysis and aggregation) and immunogenicity. The positions considered for mutation include those under the following groups.

- Positions that define the canonical CDR conformations (Martin (2010) Protein sequence and structure analysis of antibody variable domains. In: Kontermann R and Dübel S (eds). Antibody Engineering. Heidelberg, Germany: Springer International Publishing AG),
- Positions that are within the Vernier zone (Foote and Winter (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J Mol Biol. 224(2):487-99),
- Positions that localize to the VH/VL domain interface (summarized in Léger and Saldanha (2000) Preparation of recombinant antibodies from immune rodent spleens and the design of their humanization by CDR grafting. In: Shepherd P and Dean C (eds). Monoclonal Antibodies: A Practical Approach. Oxford, UK: Oxford University Press),
- Positions that are susceptible to post-translational modifications, such as glycosylation or pyroglutamination,
- Positions that are occupied by residues that are predicted to clash with CDRs, according to the model of 13D3 CDRs grafted onto VH and VL frameworks, and/or

- Positions that are occupied by residues that are rare among sequenced human antibodies, where either the parental mouse 13D3 residue or some other residue is much more prevalent within human antibody repertoire.

The goal of identifying mutations in the antibody sequences is to allow the antibodies to be stabilized in such a manner to allow for these improved properties such as improved T_m, improved yield, improved binding, and reduced predicted immunogenicity. Based on the above criteria, the following heavy and light variable regions were generated. *hu13D3-VH_vd1* includes the CDR-H1, H2, and H3 loops of 13D3-VH grafted onto the framework of 5BK5 VH. *hu13D3-VH_vd1* reverts all framework substitutions at positions that are key for defining the Chothia canonical classes, are part of the Vernier zone, or localize to the VH/VL domain interface or contribute to structural stability. The substitutions designed for this heavy chain included one or more of L5V, T77S, R19K, G44R, S77T, L78A, L78G, L80A, L80G, L82cG, and R83M. *hu13D3-VH_vd2* included L78A. *Hu13D3-VH_vd3* includes G44R, S49A, and S74A. *Hu13D3-VH_vd4* included various changes made in versions 1, 2 and 3 are tried in combination. For the light chain variable region, *hu13D3-VL_vd1* includes the CDR-L1, L2, and L3 loops of 13D3-VL grafted onto the framework of 5BK5 VL along with reverting all framework substitutions at positions that are key for defining the Chothia canonical classes, are part of the Vernier zone, or locate to the VH/VL domain interface. The following substitutions were introduced: V3Q, L9S, I2V, P15L, P18Q, E17Q, L38Q, K39R, R46L, A80S, L92G, V94I, V94A, Q100D, and L104V. For *hu13D3-VL_vd2*, the mutations from vld1 were introduced and also included L92A.

Complete sequences of heavy and light variable domains are provided in Tables 2 and 3.

Example 2. Binding Data of Humanized Antibodies

A binding screening assay was established to determine whether 13D3 and humanized variants thereof would selectively bind to phosphorylated TDP-43 found in cytoplasmic aggregates. To test binding affinity, a CAP Chip assay was performed in which binding to TDP43 was determined. 0.25ug/mL bio-TDP43-phospho-peptide was used and standard CAP chip protocol (Cytiva) was followed. Regeneration between each run cycle for improved baseline binding levels was introduced. Affinity was analyzed using a bivalent analyte model.

As shown in Table 4, Hu13D3 binding to pTDP43 peptide was maintained among all tested antibodies, compared to the Ch_13D3 Ab and Ch_13D3, all variants show sufficient binding with approximately 0.5-1nM Affinity (Bivalent analyte model), demonstrating that each antibody possessed affinity to TDP43. In addition, each of h13D3-Hd1Ld2, h13D3-Hd2Ld1, h13D3-Hd2Ld2, h13D3-Hd3Ld1, h13D3-Hd3Ld2, h13D3-Hd4Ld1, and h13D3-Hd4Ld2 demonstrated superior binding as shown by KD1 (M) values that are about 2 orders of magnitude better binder than antibodies made using conventional methods (e.g., h13D3-H9L7).

In a second experiment, Phospho-TDP43_392-414 binding was determined using a Biacore™ system. This assay produced a measured affinity of about 2 nM and can be used for humanization screening of 13D3 antibodies.

Table 4. Affinity Binding Data

Antibody	ka1 (1/Ms)	kd1 (1/s)	KD1(M)	RU (max)
Ch_13D3 Ab	2.45E+06	3.31E-02	1.35E-08	
Ch_13D3	1.28E+08	2.69E-01	2.10E-09	21.8
h13D3-H1L1	3.53E+08	6.70E-01	1.90E-09	27.5
h13D3-H2L3	1.92E+07	3.51E-02	1.83E-09	26.6
h13D3-H2L4	1.77E+07	6.41E-02	3.62E-09	24.2
h13D3-H3L2	6.37E+07	1.07E-01	1.67E-09	24.9
h13D3-H3L3	2.17E+07	3.91E-02	1.80E-09	25.8
h13D3-H3L4	2.22E+07	7.86E-02	3.55E-09	25.3
h13D3-H4L2	1.91E+09	3.49E+00	1.83E-09	25.7
h13D3-H4L4	1.65E+06	8.48E-03	5.13E-09	20.6
h13D3-H5L2	1.57E+07	2.78E-02	1.77E-09	20.8
h13D3-H5L3	4.82E+07	1.05E-01	2.19E-09	17.6
h13D3-H5L4	8.92E+06	2.90E-02	3.25E-09	14.5
h13D3-H5L5	4.88E+06	1.28E-02	2.62E-09	16.5
h13D3-H6L4	3.02E+09	4.40E+00	1.46E-09	21.8
h13D3-H7L5	1.01E+10	2.82E+01	2.79E-09	21
h13D3-H8L4	1.34E+08	2.59E-01	1.93E-09	16.3

h13D3-H8L5	1.14E+09	2.40E+00	2.11E-09	16.3
h13D3-H8L6	1.74E+08	1.45E-01	8.31E-10	16
h13D3-H9L4	8.81E+07	1.84E-01	2.09E-09	19.3
h13D3-H9L5	3.29E+08	7.67E-01	2.33E-09	19
h13D3-H9L6	6.21E+06	8.06E-03	1.30E-09	16.4
h13D3-H9L7	3.35E+06	5.88E-02	1.76E-08	24.2
h13D3-H9L8	1.25E+05	5.72E-03	4.57E-08	31.4
h13D3-H10L5	4.38E+05	1.03E-02	2.35E-08	25.5
h13D3-H10L6	8.43E+06	7.08E-02	8.36E-09	23
h13D3-H10L7	6.77E+09	1.14E+02	1.69E-08	20.4
h13D3-H10L8	1.01E+10	2.82E+01	2.79E-09	21
h13D3-Hd1Ld2	4.93E+06	4.69E-03	9.51E-10	
h13D3-Hd2Ld1	8.24E+06	3.95E-03	4.79E-10	
h13D3-Hd2Ld2	4.25E+06	5.48E-03	1.29E-09	
h13D3-Hd3Ld1	6.22E+06	3.75E-03	6.02E-10	
h13D3-Hd3Ld2	7.56E+06	6.88E-03	9.10E-10	
h13D3-Hd4Ld1	6.81E+06	3.64E-03	5.34E-10	
h13D3-Hd4Ld2	5.00E+06	4.44E-03	8.89E-10	

The results set forth in Table 4, above, demonstrate that antibodies comprising the humanizing mutations of the present disclosure (including, for example, h13D3-Hd1Ld2, h13D3-Hd2Ld1, h13D3-Hd2Ld2, h13D3-Hd3Ld1, h13D3-Hd3Ld2, h13D3-Hd4Ld1, and h13D3-Hd4Ld2) retain the strong binding to phospho-TDP-43. For example, as shown in Table 4, these antibodies have KD values of single digit nanomolar to sub-nanomolar values, which are similar to or lower than KD values for antibodies that lack humanizing mutations (e.g., chimeric 13D3 antibodies). Thus, antibodies comprising humanizing mutations of the present disclosure retain high-affinity binding to their target epitope.

10

Example 3. Thermal Stability and Expression Yield of Humanized Antibodies

Next, thermal stability (TM) of the humanize antibodies was evaluated. 3.33 mM/0.5 mg/mL of test concentration was added in 1xPBS Buffer pH 7.4. Temperature ranged from about 25°C -110 °C, and onset TM was determined for the CH2 region, the F'ab region, and the CH3 regions as shown in Tables 5. Expression yields for the humanized variants are also included in

5 Table 5.

Table 5. Yield and Tm Data

Antibody	Yield	TmOnset °C (CH2 region)	Tm1 °C (f'ab region)	Tm2 °C (CH3 Region)	Tm3 °C (CH3 Region)
Ch_13D3 Ab	509.19	65.54	75.86	--	--
h13D3-H1L1	467.8	65.71	76.37	--	--
h13D3-H2L2	356.62	65.48	76.31	--	--
h13D3-H2L3	390.7	65.20	76.36	--	--
h13D3-H2L4	341.85	61.23	71.03	82.36	--
h13D3-H3L2	40.97	61.49	65.48	82.78	--
h13D3-H3L3	37.96	61.69	65.68	82.83	--
h13D3H3L4	4.77	--	--	--	--
h13D3-H4L2	109.43	62.89	68.88	--	--
h13D3-H4L3	136.76	64.11	68.93	82.92	--
h13D3-H4L4	28.89	61.15	66.30	82.44	--
h13D3H5L2	5.6	--	--	--	--
h13D3H5L3	3.67	--	--	--	--
h13D3H5L4	0.21	--	--	--	--
h13D3H6L4	0.81	--	--	--	--
h13D3H5L5	1.67	--	--	--	--
h13D3H6L5	1.32	--	--	--	--
h13D3H7L5	1.3	--	--	--	--
h13D3H8L4	4.86	--	--	--	--
h13D3H8L5	5.15	--	--	--	--
h13D3-H8L6	2.07	58.54	--	63.20	--
h13D3-H9L4		57.86	64.35	69.68	82.34

h13D3-H9L5		57.84	<i>*64.5</i>	69.32	82.48
h13D3-H9L6	214.07	61.18	--	68.17	--
h13D3-H9L7		58.54	--	63.20	--
h13D3-H9L8		57.86	64.35	69.68	--
h13D3-H10L5	35.48	57.84	<i>*64.5</i>	69.32	--
h13D3-H10L6		61.18	--	68.17	--
h13D3-H10L7		58.54	--	63.20	--
h13D3-H10L8		57.86	64.35	69.68	--
PB-0344 (ref mAb)		67.00	78.15	--	--
h13D3Hd1Ld1	357.7	76.38	--	--	--
h13D3Hd1Ld2	368.5	74.38	--	--	--
h13D3Hd2Ld1	308.5	71.48	--	--	--
h13D3Hd2Ld2	296.4	70.37	--	--	--
h13D3Hd3Ld1	345.8	77.62	--	--	--
h13D3Hd3Ld2	350.0	76.07	--	--	--
h13D3Hd4Ld1	291.4	71.99	--	--	--
h13D3Hd4Ld2	261.0	70.50	--	--	--

*: estimated value

Table 5 demonstrates that several attempts at humanizing the 13D3 chimeric antibody using traditional humanization methods resulted in significantly reduced antibody stability. For example, many antibody variants (e.g., h13D3-H3L4, h13D3-H5L2, h13D3-H5L3, h13D3-H5L4, h13D3-H6L4, h13D3-H5L5, h13D3-H6L5, h13D3-H7L5, h13D3-H8L4, h13D3-H8L5, and h13D3-H8L6) exhibited extremely low expression yields, which are indicative of antibody instability. As a result of the low yields, many of these variants were not assessed for melting temperatures. In contrast, humanized antibodies comprising the humanization mutations of the present disclosure (e.g., h13D3-Hd1Ld1, h13D3-Hd1Ld2, h13D3-Hd2Ld1, h13D3-Hd2Ld2, h13D3-Hd3Ld1, h13D3-Hd3Ld2, h13D3-Hd4Ld1, and h13D3-Hd4Ld2) were expressed in high yield, demonstrating the stability of these antibodies.

The melting temperature data in Table 5 further demonstrate the stability of antibodies comprising humanization mutations of the present disclosure. Many antibody variants (e.g., h13D3-H8L6, h13D3-H9L4, h13D3-H9L5, h13D3-H9L6, h13D3-H9L7, h13D3-H9L8, h13D3-H10L5, h13D3-H10L6, h13D3-H10L7, and h13D3-H10L8) exhibited low T_m Onset values for the CH2 region (e.g., 5 to 10 degrees Celsius below the T_m Onset for PB-0344, a reference antibody), which are indicative of antibody instability. In contrast, humanized antibodies comprising the humanization mutations of the present disclosure (e.g., h13D3-Hd1Ld1, h13D3-Hd1Ld2, h13D3-Hd2Ld1, h13D3-Hd2Ld2, h13D3-Hd3Ld1, h13D3-Hd3Ld2, h13D3-Hd4Ld1, and h13D3-Hd4Ld2) demonstrated high T_m Onset values of 70 degrees Celsius or higher for the CH2 region, demonstrating the high stability of these antibodies. Thus, the data in Table 5 demonstrate that antibodies comprising humanizing mutations of the present disclosure exhibit high thermal stability, resulting in stable antibodies that can be expressed in high yield.

Example 4. Predicted Immunogenicity of Humanized Antibodies

Immunogenicity scores are predicted values of immunogenicity calculated from two different software programs: Immune Epitope Database (IEDB) and EpiQuest. IEDB immunogenicity analysis tool is hosted by the National Institute of Allergy and Infectious Diseases.

For IEDB, the immunogenicity prediction method used is based on predicted potential binding of a peptide within a protein sequence to major histocompatibility class (MHC) class II. The program identifies potential immunogenic regions within the protein sequence. The MHC class II tool predicts immunogenic regions using broad spectrum alleles (i.e., 26 reference alleles) for MHC class II in the human population. The software generates a series of 15 residue peptides which overlap in 10 residues. Each generated 15 residue peptide is predicted for binding to the 26 reference alleles.

For each peptide, a percentile rank for each of the three methods (combinatorial library, SMM_align and Sturniolo) was generated by comparing each peptide's score against the scores of five million random 15 residue peptides selected from the SWISSPROT database. The adjusted percentile rank is the percentile rank adjusted based on the frequency of peptide lengths. A low number percentile rank indicates high affinity. The median percentile rank of the three methods were then used to generate the rank for consensus method. By default, prediction result

is collapsed to show only the Percentile Rank and Adjusted Rank. The maximum median percentile rank threshold is set at 20.

The EpiQuest tool, T-Scanner, ranges cytotoxic T lymphocyte (CTL) epitopes according to their predicted immunodominance. The immunodominance of epitopes is defined as their relative strength in functional assays related to the target kill or release of respective cytokines. These parameters indicate functionality of the T-epitope. This program is designed to analyze and sort according to their immunodominance the CTL peptide epitopes eluted from target cells. Generally, only a low number of peptide epitopes that bind to MHC class I have actual functional activity. Relative strength of a CTL (T) epitope is defined by the strength of its binding to MHC class I and T cell receptor (TCR) (in the context of MHCI). The algorithm detects the structural and compositional features of the peptide epitope that allow it to elicit a high-affinity for TCR. This analysis is haplotype-specific and the EpiQuest T-scanner has matrix for analysis of HLAA2 and H2kB haplotypes-binding peptides.

Collectively, the data shown in Tables 6 show some humanized antibodies comprising humanizing mutations of the present disclosure have lower predicted immunogenicity scores relative to the chimeric 13D3 antibody and many antibodies humanized using traditional humanization strategies.

Table 6. Antibody Immunogenicity Score

Antibody	Immunogenicity Score	
	HV	VL
Ch_13D3 Ab	10	10
h13D3H1L1	9.5	10
h13D3H2L2	9.5	10
h13D3H2L3	9.5	9
h13D3H2L4	9.5	0.5
h13D4H3L2	6	10
h13D3H3L3	6	8.5
h13D3H3L4	6	0.5

Antibody	Immunogenicity Score	
	HV	VL
h13D3H7L5	1	0.5
h13D3H8L4	3.5	0.5
h13D3H8L5	3.5	0.5
h13D3H8L6	3.5	9
h13D3H9L6	1	9
h13D3H10L5	4	0.5
h13D3Hd1Ld1	2.5	4
h13D3Hd1Ld2	2.5	1

h13D3H4L2	5.5	10
h13D3H4L3	5.5	8.5
h13D3H4L4	5.5	0.5
h13D3H5L2	1	10
h13D3H5L3	1	8.5
h13D3H5L4	1	0.5
h13D3H6L4	1	0.5
h13D3H5L5	1	0.5
h13D3H6L5	1	0.5

h13D3Hd2Ld1	0.3	4
h13D3Hd2Ld2	0.3	1
h13D3Hd3-Ld1	2.5	4
h13D3Hd3-Ld2	2.5	1
h13D3Hd4-Ld1	0.1	4
h13D3Hd4-Ld2	0.1	1

The predicted immunogenicity data in Table 6 demonstrate that antibodies comprising humanization mutations of the present disclosure have significantly reduced immunogenicity compared to chimeric 13D3 antibody and many antibodies humanized using traditional humanization strategies. Many antibody variants (e.g., h13D3-H1L1, h13D3-H2L2, h13D3-H2L3, h13D3-H2L4, h13D3-H3L2, h13D3-H3L3, h13D3-H3L4, h13D3-H4L2, h13D3-H4L3, h13D3-H4L4, h13D3-H5L2, h13D3-H5L3, and h13D3-H8L6) exhibited high predicted immunogenicity values, which are indicative of actual immunogenicity in human subjects. In contrast, humanized antibodies comprising the humanization mutations of the present disclosure (e.g., h13D3-Hd1Ld2, h13D3-Hd2Ld1, h13D3-Hd2Ld2, h13D3-Hd3Ld2, h13D3-Hd4Ld1, and h13D3-Hd4Ld2) demonstrated low predicted immunogenicity values, demonstrating that these antibodies are unlikely to be immunogenic in human subjects. Collectively, the data in Tables 4, 5, and 6 demonstrate that antibodies comprising humanizing mutations of the present disclosure retain strong antigen binding, exhibit high thermal stability, can be expressed in high yield, and have significantly reduced immunogenicity, even when compared to antibodies that are humanized using traditional humanization methods.

What is claimed is:

1. A humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and a light chain variable domain comprising a charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

2. The humanized antibody of claim 1, wherein the charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain is a positively charged amino acid, and the charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain is a negatively charged amino acid.

3. The humanized antibody of claim 2, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge.

4. The humanized antibody of any one of claims 1-3, wherein the positively charged amino acid is a basic amino acid.

5. The humanized antibody of any one of claims 1-4, wherein the negatively charged amino acid is an acidic amino acid.

6. The humanized antibody of any one of claims 1-5, wherein the positively charged amino acid is an arginine.

7. The humanized antibody of any one of claims 1-6, wherein the negatively charged amino acid is an aspartic acid.

8. The humanized antibody of any one of claims 1-7, wherein the humanized antibody further comprises a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain.

9. The humanized antibody of claim 8, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with at least one of: an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

10. The humanized antibody of claim 8 or 9, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with both the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

11. The humanized antibody of any one of claim 8-10, wherein the substituted phenylalanine derivative comprises a hydroxy-substituted phenyl group.

12. The humanized antibody of claim 11, wherein the substituted phenylalanine derivative is a tyrosine residue.

13. The humanized antibody of any one of claims 9-12, wherein the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain is a glutamine, and/or wherein the amino acid at an amino acid position

corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine.

14. The humanized antibody of any one of claims 1-13, wherein the humanized antibody further comprises at least one of following:

a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain;

a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and

a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain.

15. The humanized antibody of any one of claims 1-14, wherein the humanized antibody further comprises at least one of following:

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

16. The humanized antibody of any one of claims 1-14, wherein the humanized antibody further comprises at least one of following:

a serine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and

a serine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

17. The humanized antibody of any one of claims 1-16, wherein the humanized antibody further comprises at least one of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain;

a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain;

an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

18. The humanized antibody of any one of claims 1-17, wherein the humanized antibody further comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

19. A humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain.

20. The humanized antibody of claim 19, wherein the humanized antibody further comprises a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain, and a light chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain.

21. The humanized antibody of claim 20, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with at least one of: the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38

of a human IgG1 light chain, and the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

22. The humanized antibody of claim 21, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond with both: the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

23. The humanized antibody of any one of claim 20-22, wherein the substituted phenylalanine derivative comprises a hydroxy-substituted phenyl group.

24. The humanized antibody of claim 23, wherein the substituted phenylalanine derivative is a tyrosine.

25. The humanized antibody of any one of claims 20-24, wherein the polar uncharged amino acid residue at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain is a glutamine.

26. The humanized antibody of any one of claims 20-25, wherein the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain variable domain, is a glutamine.

27. The humanized antibody of any one of claims 19-26, wherein the humanized antibody further comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain.

28. The humanized antibody of any one of claims 19-27, wherein the humanized antibody further comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

29. The humanized antibody of claim 27 or 28, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge.

30. The humanized antibody of any one of claims 27-29, wherein the positively charged amino acid is a basic amino acid.

31. The humanized antibody of any one of claims 27-30, wherein the negatively charged amino acid is an acidic amino acid.

32. The humanized antibody of any one of claim 27-31 wherein the positively charged amino acid is an arginine.

33. The humanized antibody of any one of claims 27-32, wherein the negatively charged amino acid is an aspartic acid.

34. The humanized antibody of any one of claims 19-33, wherein the humanized antibody further comprises at least one of following:

a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain;

a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain.

35. The humanized antibody of any one of claims 19-34, wherein the humanized antibody further comprises at least one of following:

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

36. The humanized antibody of any one of claims 19-35, wherein the humanized antibody further comprises at least one of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain;

a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain;

an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

37. A humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

38. The humanized antibody of claim 37, wherein the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

39. The humanized antibody of claim 37 or 38, wherein the light chain variable domain comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

40. The humanized antibody of any one of claims 37-39, wherein the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

41. The humanized antibody of any one of claims 37-40, wherein the light chain variable domain comprises each of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

a negatively charged residue at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

42. The humanized antibody of claim 40 or 41, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge.

43. The humanized antibody of any one of claims 37-42, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain is an arginine.

44. The humanized antibody of any one of claims 37-43, wherein the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain is an aspartic acid.

45. The humanized antibody of any one of claims 37-44, wherein the humanized antibody further comprises one or both of:

an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain; and

an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

46. A humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

47. The humanized antibody of claim 46, wherein the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

48. The humanized antibody of claim 46 or 47, wherein the light chain variable domain comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

49. The humanized antibody of any one of claims 46-48, wherein the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

50. The humanized antibody of any one of claims 46-49, wherein the light chain variable domain comprising each of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

51. The humanized antibody of any one of claims 46-50, wherein the humanized antibody further comprises one or both of:

a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

52. The humanized antibody of claim 51, wherein the heavy chain variable region comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

53. The humanized antibody of any one of claims 46-52, wherein the heavy chain variable region comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain.

54. The humanized antibody of any one of claims 46-53, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position

corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge.

55. The humanized antibody of any one of claims 51-54, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, is an arginine.

56. The humanized antibody of any one of claims 46-55, wherein the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain, is an aspartic acid.

57. The humanized antibody of any one of claims 46-56, wherein the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain, is a serine.

58. A humanized antibody comprising a heavy chain variable domain and a light chain variable domain, the heavy chain variable domain comprising a polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain, and a light chain variable domain comprising at least one of the following:

- a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

- an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

- a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

59. The humanized antibody of claim 58, wherein the light chain variable domain comprises a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

60. The humanized antibody of claim 58 or 59, wherein the light chain variable domain comprises an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

61. The humanized antibody of any one of claims 58-60, wherein the light chain variable domain comprises a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

62. The humanized antibody of any one of claims 58-61, wherein the light chain variable domain comprising each of the following:

- a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

- an alanine residue at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain; and

- a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain.

63. The humanized antibody of any one of claims 58-62, wherein the humanized antibody further comprises one or both of:

- a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and

- an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

64. The humanized antibody of claim 63, wherein the heavy chain variable region comprises an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain.

65. The humanized antibody of any one of claims 58-64, wherein the heavy chain variable region comprises a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain.

66. The humanized antibody of any one of claims 58-65, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, and the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain form an interchain salt bridge.

67. The humanized antibody of any one of claims 63-66, wherein the positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain, is an arginine.

68. The humanized antibody of any one of claims 58-67, wherein the negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain, is an aspartic acid.

69. The humanized antibody of any one of claims 58-68, wherein the polar uncharged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain is a serine.

70. The humanized antibody of any one of claims 37-69, wherein the humanized antibody further comprises at least one of following:

a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain;

a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain.

71. The humanized antibody of any one of claims 37-70, wherein the humanized antibody further comprises at least one of the following:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain;

a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain;

an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain; and

a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

72. The humanized antibody of any one of claims 37-71, wherein the humanized antibody further comprises a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain.

73. The humanized antibody of claim 72, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, forms a hydrogen bond interaction with at least one of an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and an amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

74. The humanized antibody of claim 71 or 72, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain forms a hydrogen bond interaction with both the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, and the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain.

75. The humanized antibody of any one of claims 72-74, wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, comprises a hydroxy-substituted phenyl group.

76. The humanized antibody of claim 75 wherein the substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain, is a tyrosine.

77. The humanized antibody of any one of claims 73-76, wherein the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 38 of a human IgG1 light chain, is a glutamine.

78. The humanized antibody of any one of claims 73-77, wherein the amino acid at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 heavy chain, is a glutamine.

79. The humanized antibody of any one of claims 1-78, wherein the antibody is an antigen-binding antibody fragment.

80. The humanized antibody of claim 79, wherein the antigen-binding antibody fragment is a Fab fragment, a Fab'₂ fragment, or a single chain Fv.

81. The humanized antibody of any one of claims 1-78, wherein the humanized antibody is a full antibody, a chimeric antibody, a veneered antibody, a CDR-grafted antibody, or a recombinant antibody.

82. The humanized antibody any one of claims 1-78 or 81, wherein the humanized antibody is a human IgG antibody.

83. The humanized antibody of claim 79, wherein the human IgG antibody has a human IgG1 isotype.

84. The humanized antibody of any one of claims 1-83, wherein the antibody is conjugated to a therapeutic, cytotoxic, cytostatic, immunomodulatory, neurotrophic, or neuroprotective agent.

85. The humanized antibody of any one of claims 1-78 or 81-83, wherein the heavy chain variable domain is fused to a heavy chain constant region and the light chain variable domain is fused to a light chain constant region.

86. The humanized antibody of claim 85, wherein the heavy chain constant region is a mutant form of a natural human heavy chain constant region which has reduced binding to an Fc γ receptor relative to a natural heavy chain constant region.

87. The humanized antibody of claim 85 or 86, wherein the heavy chain constant region is of IgG1 isotype.

88. The humanized antibody of any one of claims 1-86, wherein the antibody has at least one mutation in a constant region.

89. The humanized antibody of claim 88, wherein the at least one mutation reduces complement fixation or activation by the constant region.

90. The humanized antibody of claim 89, where the at least one mutation is at one or more positions of: 241, 264, 265, 270, 296, 297, 318, 320, 322, 329, and 331 by EU numbering.

91. The humanized antibody of claim 90, wherein the humanized antibody has an alanine at positions 318, 320, and 322 by EU numbering.

92. The humanized antibody of any one of claims 1-91, wherein the humanized antibody has increased thermal stability as compared to a reference antibody.

93. The humanized antibody of claim 92, wherein the increased thermal stability comprises an increase in expression yield compared to the reference antibody.

94. The humanized antibody of claim 93, wherein the increased thermal stability comprises an increase in expression yield of at least 100 mg/L compared to the reference antibody.

95. The humanized antibody of any one of claims 92-94, wherein the increased thermal stability comprises an increase in melting point as compared to the reference antibody.

96. The humanized antibody of claim 95, wherein the increase in melting point is an increase of at least 2°C compared to the reference antibody.

97. The humanized antibody of claim 95, wherein the increase in melting point is an increase of at least 5°C compared to the reference antibody.

98. The humanized antibody of claim 92, wherein the humanized antibody has a melting temperature of 55°C or greater.

99. The humanized antibody of claim 98, wherein the humanized antibody has a melting temperature of 65°C or greater.

100. The humanized antibody of claim 98, wherein the humanized antibody has a melting temperature of 70°C or greater.

101. The antibody of any one of claims 95-100, wherein the melting temperature is determined by differential scanning calorimetry.

102. The humanized antibody of any one of claims 1-101, wherein the humanized antibody has a decreased immunogenicity score as compared to a reference antibody.

103. The humanized antibody of claim 102, wherein the immunogenicity score is determined using Formula I:

$$\text{Ig Score} = (-\text{KDHI} + 4.5) \times P_{\text{t (average)}} \times \text{Tail Bonus (Formula I)}.$$

104. The humanized antibody of any one of claims 92-103, wherein the reference antibody comprises the same sequence as the humanized antibody without the one or more amino acid substitutions.

105. A pharmaceutical composition comprising the humanized antibody of any one of claims 1-104 and a pharmaceutically acceptable carrier.

106. A nucleic acid encoding the heavy chain variable domain and/or the light chain variable domain of an antibody of any one of claims 1-104.

107. A vector comprising a nucleic acid encoding a heavy chain variable domain and a light chain variable domain operably linked to one or more regulatory sequences to effect expression in a mammalian cell of the humanized antibody of any one of claims 1-104.

108. The vector of claim 107, wherein the one or more regulatory sequences comprises one or more of a enhancer, ribosome binding site, transcription termination signal, and promoter, optionally, wherein the promoter is a eukaryotic promoter.

109. The vector of claim 107 or 108, wherein the nucleic acid is codon-optimized for expression in a host cell.

110. A host cell transformed with the vector of any one of claims 107-109.

111. A host cell comprising the nucleic acid of claim 106.

112. A method of reducing immunogenicity of a parent antibody by introducing amino acid substitution(s) into the parent antibody to produce a modified antibody comprising:

(i) a heavy chain variable domain comprising one or more of:

a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain;

a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain;

a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain;

a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; and

a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and/or

(ii) a light chain variable domain comprising one or more of:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain;

a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain;

an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain;

a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; and

a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

113. A method of increasing thermal stability of an antibody by introducing amino acid substitution(s) into a parent antibody to produce a modified antibody comprising:

(i) a heavy chain variable domain comprising one or more of:

a positively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain;

a leucine substituted with an alanine at an amino acid position corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain;

a substituted phenylalanine derivative at an amino acid position corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain;

a tryptophan at an amino acid position corresponding to Chothia canonical amino acid position 103 of a human IgG1 heavy chain; and

a phenylalanine at an amino acid position corresponding to Chothia canonical amino acid position 100C of a human IgG1 heavy chain; and/or

(ii) a light chain variable domain comprising one or more of:

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain;

a glutamine at an amino acid position corresponding to Chothia canonical amino acid position 17 of a human IgG1 light chain;

a tyrosine at an amino acid position corresponding to Chothia canonical amino acid position 36 of a human IgG1 light chain;

an arginine at an amino acid position corresponding to Chothia canonical amino acid position 39 of a human IgG1 light chain;

an alanine at an amino acid position corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain;

a negatively charged amino acid at an amino acid position corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; and

a valine at an amino acid position corresponding to Chothia canonical amino acid position 104 of a human IgG1 light chain.

114. A method of reducing immunogenicity of a parent antibody by introducing a first amino acid substitution and a second amino acid substitution into the parent antibody to generate a modified antibody, wherein the first amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and the second amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; wherein the modified antibody has a reduction in immunogenicity compared to the parent antibody.

115. The method of claim 114, the method further comprising introducing a third amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; and a fourth amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

116. The method of claim 114 or 115, wherein the method further comprises introducing at least one of the following amino acid substitutions:

- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain;
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain;
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 77 of a human IgG1 heavy chain;
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; and
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

117. The method of any one of claims 114-116, wherein the modified antibody has a melting temperature of 65°C or greater.

118. A method of increasing thermal stability of a parent antibody by introducing a first amino acid substitution and a second amino acid substitution into the parent antibody to generate a modified antibody, wherein the first amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 44 of a human IgG1 heavy chain; and the second amino acid substitution is at an amino acid corresponding to Chothia canonical amino acid position 100 of a human IgG1 light chain; wherein the modified antibody has increase in melting temperature of 2°C or greater compared to the parent antibody as measured by differential scanning calorimetry.

119. The method of claim 118, the method further comprising introducing a third amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 91 of a human IgG1 heavy chain; and a fourth amino acid substitution into the parent antibody at an amino acid corresponding to Chothia canonical amino acid position 3 of a human IgG1 light chain.

120. The method of claim 118 or 119, wherein the method further comprises introducing at least one of the following amino acid substitutions:

- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 49 of a human IgG1 heavy chain;
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 74 of a human IgG1 heavy chain;
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 77 of a human IgG1 heavy chain;
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 78 of a human IgG1 heavy chain; and
- an amino acid substitution at an amino acid corresponding to Chothia canonical amino acid position 92 of a human IgG1 light chain.

121. The method of any one of claims 118-120, wherein the modified antibody has increase in melting temperature of 5°C or greater compared to the parent antibody as measured by differential scanning calorimetry.

122. The method of any one of claims 118-121, wherein the modified antibody has a melting temperature of 65°C or greater.