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(54) **Title:** PLANT-DERIVED ANTIBODIES AND DERIVATIVES THAT REDUCE RISK OF ANTIBODY-DEPENDENT ENHANCEMENT (ADE) OF INFECTION

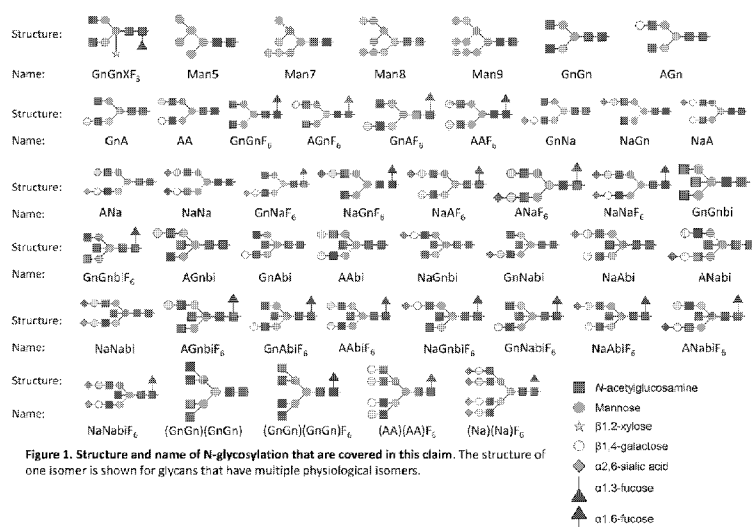


Figure 1. Structure and name of N-glycosylation that are covered in this claim. The structure of one isomer is shown for glycans that have multiple physiological isomers.

(57) **Abstract:** The present invention describes the plant-based production of a therapeutic antibody.

PLANT-DERIVED ANTIBODIES AND DERIVATIVES THAT REDUCE RISK OF ANTIBODY-DEPENDENT ENHANCEMENT (ADE) OF INFECTION

RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional
5 Application No. 61/932,033, filed January 27, 2014, the entire contents of which is hereby
incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under U01 AI075549 awarded by
10 the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The development and implementation of targeted monoclonal antibody (MAb)
therapy has provided new opportunities for controlling a wide range of clinical diseases.
15 Although MAbs produced in mammalian cell culture systems have achieved remarkable
clinical success, their high cost, long manufacturing time, and restricted production capacity
have limited the availability, utility and potential of these drugs. Several of these challenges
might be overcome by using plant expression systems, because they offer highly scalable
production of MAbs at low cost with a low risk of introducing adventitious human or animal
20 pathogens. Functional antibody production requires a eukaryotic host cell that can assemble
four antibody polypeptides into a heterotetramer and perform complex N-linked
glycosylation. Despite this complexity, a MAb was successfully expressed in tobacco plants
only three years after the first plant-made biologic (Hiatt A, Cafferkey R, Bowdish K (1989)
Production of antibodies in transgenic plants. *Nature* 342: 76-78). Since then, a variety of
25 MAbs and their derivatives, such as IgG, IgA, single-chain variable fragments (scFv), and
diabodies have been produced in plants (De Muynck B, Navarre C, Boutry M (2010)
Production of antibodies in plants: status after twenty years. *Plant Biotechnology Journal* 8:
529-563). The largest reported MAb-based molecule produced in plants is a recombinant
immune complex (RIC) (Phoolcharoen W, Bhoo SH, Lai H, Ma J, Arntzen CJ, et al. (2011)
30 Expression of an immunogenic Ebola immune complex in *Nicotiana benthamiana*. *Plant
Biotechnology Journal* 9: 807-816). The ability of plants to express and assemble larger or

more complex MAb-derived molecules such as tetravalent MAbs or bifunctional MAbs has not been described.

N-linked glycosylation of proteins occurs as a series of post-translational modification steps in host cells and depends on the proper folding of the target protein and its transport to the appropriate endomembrane compartment (Roth J (2002) Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions. Chemical reviews 102: 285-303). As such, MAb variants with significant polypeptide structural differences from the native molecule also may have appreciable differences in glycan structures. This potential structural difference may impact the pharmacokinetics, antigen binding, stability, effector functions, immunogenicity, and efficacy of a MAb and its derivatives. Currently, the difference of N-glycosylation between the parent MAb and its structural variants and the precise cause of these differences are not fully understood.

BRIEF SUMMARY OF THE INVENTION

In certain embodiments, the present invention describes the plant-based production of a therapeutic antibody.

In certain embodiments, the present invention provides a plant-produced monoclonal antibody (MAb) specific for a target virus, the MAb comprising a defined and highly-uniform N-glycoform, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells. In certain embodiments, the comparison is to a mammalian cell culture-produced MAb. In certain embodiments, the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGn F₆, AGnF₆, GnAF₆, AAF₆, GnNa, NaGn, NaA, ANa, NaNa, GnNaF₆, NaGnF₆, NaAF₆, ANaF₆, NaNaF₆, GnGnbi, GnGnbiF₆, AGnbi, GnAbi, AAbi, NaGnbi, GnNabi, NaAbi, ANabi, NaNabi, AGnbiF₆, GnAbiF₆, AAbiF₆, NaGnbiF₆, GnNabiF₆, NaAbiF₆, ANabiF₆, NaNabiF₆ (GnGn)(GnGn), (GnGn)(GnGn)F₆, (AA)(AA)F₆, (Na)(Na)F₆ (Figure 1). In certain embodiments, the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, or GnGn F₆. In certain embodiments the N-glycoform is GnGnXF₃, Man5, Man7, Man8, or Man9. As used herein the antigen binding affinity and kinetics is measured by Surface plasmon resonance (SPR) and expressed by parameters of “on rate” k_a , “off rate” k_d , and binding constant K_D .

As used herein the neutralization activity measures the ability of inhibition of an antibody on the infectivity of a specific virus, and expressed by EC50, the concentration of an antibody that induces half of the maximal inhibition. As used herein, the *in vivo* therapeutic activity is defined as the minimal concentrations of an antibody or its derivatives that prevent or clear
 5 infection of a virus in an animal model.

In certain embodiments, the plant-produced MAb is anti-West Nile virus (WNV) MAb E16:

Light chain nucleotide sequence:

ATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGCTACTGGTGTTCATTC
 10 TGATATCGTTATGACACAATCTCCAGATTCTTTGGCTGTTTCTCTTGGAGAGAGG
 GCTACTATCAATTGCAAGGCTTCTCAAGATGTTTCTACTGCTGTTGCTTGGTACCA
 ACAGAAACCTGGACAGCCACCAAACTTCTTATCTCTTGGGCATCTACTAGGCAC
 ACTGGAGTTCCAGATAGATTTTCTGGATCTGGATCTGGAACAGATTTCACTCTTA
 CTATCTCATCTCTTCAAGCTGAGGATGTTGCAGTTTATTACTGTCAGCAACATTAT
 15 ACAACTCCACTTACTTTTCGGACAAGGAACTAAGTTGGAGATCAAAAGAACTGTT
 GCTGCACCATCTGTTTTTCATCTTCCCTCCATCTGATGAGCAGTTGAAATCTGGAAC
 TGCTTCTGTTGTGTGCCTTCTTAATAACTTCTATCCTAGAGAGGCTAAAGTTCAGT
 GGAAGGTGGATAACGCACTTCAATCTGGTAACTCTCAAGAGTCTGTTACAGAGC
 AAGATTCTAAGGACTCAACTTACTCTTTTCATCTACACTTACTTTGTCAAAGCA
 20 GATTACGAGAAACACAAAGTTTACGCATGCGAAGTTACTCATCAAGGACTTTCTT
 CACCAGTTACAAAGTCTTTCAATAGAGGAGAGTGTTAA (SEQ ID NO:1).

Heavy chain nucleotide sequence:

ATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGCTACTGGTGTTCATTC
 TCAAGTTCAATTGGTGCAGTCAGGTGCTGAGGTGAAGAAACCAGGTGCTTCAGTT
 25 AAGGTTTCTTGTAAGGCTTCTGGTTACACATTCACAGATTATTGGATTGAATGGG
 TGAGACAAGCTCCTGGTCAGGGTCTTGAGTGGATGGGAGATATTCTTTGTGGAAC
 TGGAAGAACTAGATACAACGAGAACTTAAGGCTAGAGTTACTATGACTGCTGA
 TACCTCTACATCTACTGCTTACATGGAACCTTAGATCTTTGAGATCAGATGACACT
 GCTGTGTACTATTGTGCTAGGTCAGCTTCTTATGGAGACTACGCTGACTATTGGG
 30 GACAAGGTACTACTGTTACTGTGTCTTCTGCTTCTACCAAGGGACCTTCTGTTTTT
 CCACTTGCTCCTTCTTCTAAGTCTACTTCTGGTGGAACCTGCTGCTTTGGGTTGTTT
 GGTGAAAGATTACTTTCCTGAGCCAGTGACCGTTTCTTGGAACCTCAGGTGCTCTT
 ACATCTGGTGTTCATACTTTCCAGCTGTTCTTCAATCTTCAGGACTTTACTCACT
 TTCTTCTGTTGTTACCGTTCCTTCTTCAAGCTTGGGCACTCAGACCTACATCTGCA

ATGTGAATCACAAACCCAGCAACACCAAGGTTGACAAGAAAGTTGAGCCCAAGT
 CTTGTGACAAGACTCATACGTGTCCACCGTGCCCAGCACCTGAACTTCTTGAGAG
 ACCGTCAGTCTTCTTGTTCCTCCAAAGCCTAAGGATACCTTGATGATCTCCAGG
 ACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGA
 5 AGTTCAACTGGTATGTGGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGA
 GAGAGGAACAGTACAACAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCA
 TCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT
 CCCAGCCCCCATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACC
 ACAAGTGTACACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAG
 10 CTTGACTTGCCTGGTGAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAG
 AGCAATGGGCAACCGGAGAACAACTACAAGACTACACCTCCCGTTCTCGATTCT
 GACGGCTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAA
 CAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTACAATCACTACA
 CACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGA (SEQ ID NO:2).

15 In certain embodiments, the E16 WT MAb may also comprise an alternative heavy chain, which comprises a signal peptide (e.g., MGWSCILFLVATATGVHS SEQ ID NO:41) at its N-terminus that targets the HC into the endomembrane system for secretion (*see, e.g.*, SEQ ID NO:34). Accordingly, an alternative heavy chain nucleotide sequence for E16 WT heavy chain is shown in Figure 17 (SEQ ID NO:33).

20 In certain embodiments, the plant-produced MAb is a single chain variant of E16 (scFv-Fc fusion molecule).

In certain embodiments, the plant-produced MAb is a single chain variant of E16 (scFv- C_H¹⁻³ fusion molecule). The DNA sequence of the variable region of HC (V_H) and LC (V_L) of pHu-E16 was fused together first to generate pHu-E16scFv, and then fused to the
 25 coding sequence of the C_H¹⁻³ of human IgG to form scFv- C_H¹⁻³.

scFv-C_H¹⁻³ sequence:

ATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGCTACTGGTGTTCATTC
 TCAAGTTCAATTGGTGCAGTCAGGTGCTGAGGTGAAGAAACCAGGTGCTTCAGTT
 AAGGTTTCTTGTAAGGCTTCTGGTTACACATTACAGATTATTGGATTGAATGGG
 30 TGAGACAAGCTCCTGGTCAGGGTCTTGAGTGGATGGGAGATATTCTTTGTGGAAC
 TGGAAGAACTAGATACAACGAGAACTTAAGGCTAGAGTTACTATGACTGCTGA
 TACCTCTACATCTACTGCTTACATGGAAGTTAGATCTTTGAGATCAGATGACACT
 GCTGTGTACTATTGTGCTAGGTCAGCTTCTTATGGAGACTACGCTGACTATTGGG
 GACAAGGTACTACTGTTACTGTGTCTTCT GGT TCA ACT TCA GGA GGA GGA

TCAGGTGGTGGTTCAGGAGGTGGAGGATCTTCTGATATCGTTATGACACAATCTC
 CAGATTCTTTGGCTGTTTCTCTTGGAGAGAGGGCTACTATCAATTGCAAGGCTTCT
 CAAGATGTTTCTACTGCTGTTGCTTGGTACCAACAGAAACCTGGACAGCCACCAA
 AACTTCTTATCTCTTGGGCATCTACTAGGCACACTGGAGTTCCAGATAGATTTTCT
 5 GGATCTGGATCTGGAACAGATTTCACCTTACTATCTCATCTCTTCAAGCTGAGG
 ATGTTGCAGTTTATTACTGTCAGCAACATTATACAACCTCCACTTACTTTTCGGACAA
 GGAACATAAGTTGGAGATCAAAGCTAGCACCAAGGGACCTTCTGTTTTTCCACTTG
 CTCCTTCTTCTAAGTCTACTTCTGGTGGAACTGCTGCTTTGGGTTGTTTGGTGAAA
 GATTACTTTCCTGAGCCAGTGACCGTTTCTTGGAACTCAGGTGCTCTTACATCTGG
 10 TGTTCATACTTTCCCAGCTGTTCTTCAATCTTCAGGACTTTACTCACTTTCTTCTGT
 TGTTACCGTTCCTTCT**TCAAGCTT**GGGCACTCAGACCTACATCTGCAATGTGAAT
 CACAAACCCAGCAACACCAAGGTTGACAAGAAAGTTGAGCCCAAGTCTTGTGAC
 AAGACTCATACGTGTCCACCGTGCCAGCACCTGAACTTCTTGGAGGACCGTCAG
 TCTTCTTGTTTCCTCCAAAGCCTAAGGATACCTTGATGATCTCCAGGACTCCTGAA
 15 GTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACT
 GGTATGTGGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGAGAGAGGAA
 CAGTACAACAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCATCAAGATT
 GGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAACCCTCCCAGCCCC
 CATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTA
 20 CACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAGCTTGACTTGC
 CTGGTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGAGCAATGGG
 CAACCGGAGAACAACACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCT
 TCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATG
 TCTTCTCATGCTCCGTGATGCATGAGGCTCTTCACAATCACTACACACAGAAGAG
 25 TCTCTCCTTGCTCTCCGGGTAAAT**TGA** (SEQ ID NO:3).

In certain embodiments, the plant-produced MAb is a single chain variant of E16 (scFv- C_H^{1-3} molecule) consists of a single-chain variable fragment (scFv) of pHu-E16 fused to the heavy chain (HC) constant domains (C_H^{1-3}) of human IgG.

In certain embodiments, the plant-produced MAb is pHu-E16scFv- C_H^{1-3} .

30 In certain embodiments, the N-glycoform selectively binds to Fc receptors or C1q. In certain embodiments, the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGn F₆, AGnF₆, GnAF₆, or AAF₆

In certain embodiments, the MAb is bivalent or tetravalent variant. In certain embodiments, the MAb is Tetra pHu-E16.

In certain embodiments, the MAb comprises a signal peptide (*e.g.*, MGWSCILFLVATATGVHS SEQ ID NO:41) at its N-terminus that targets the HC into the endomembrane system for secretion.

In certain embodiments, the present invention provides an anti-Dengue virus (DV) MAb E60. MAb E60 is a full antibody with mouse LC variable domain (V_L) fused to human LC constant domain (C_L , kappa) and HC variable domain (V_H) to human constant domains (CH^{1-3}) of IgG1. The development of mouse E60 full MAb was described in Oliphant et al, (2006) Journal of Virology, 80: 12149-12159.

In certain embodiments, the plant-produced MAb is DV MAb E60.

10 Light chain nucleotide sequence:

ATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGCTACTGGTGTTCATTC
TGACATCCTGATGACCCAATCTCCATCCTCCATGTCTGTATCTCTGGGAGACTCA
GTCAGCATCACTTGCCATGCAAGTCAGGGCATTAGCGGTAATATAGGGTGGTTGC
AGCAGAAACCAGGGAAATCATTAAAGGGCCTGATCTATCATGGAACCAACTTGG
15 AAGAGGGAGTTCCATCAAGGTTCAAGTGGCAGTGGATCTGGAGCAGATTATTCTCT
CACCATCAGCAGCCTGGAGTCTGAAGATTTTGCAGACTATTACTGTGTACAGTAT
GGTCAGTTTCCTCCGACGTTTCGGTGGAGGCACCAAGCTGGAAATCAAAGCTAGC
AGAACTGTTGCTGCACCATCTGTTTTTCATCTTCCCTCCATCTGATGAGCAGTTGAA
ATCTGGAAGTCTTCTGTTGTGTGCCTTCTTAATAACTTCTATCCTAGAGAGGCTA
20 AAGTTCAGTGGAAAGGTGGATAACGCACTTCAATCTGGTAACTCTCAAGAGTCTGT
TACAGAGCAAGATTCTAAGGACTCAACTTACTCTCTTTCATCTACACTTACTTTGT
CAAAAGCAGATTACGAGAAACACAAAGTTTACGCATGCGAAGTTACTCATCAAG
GACTTTCTTCACCAGTTACAAAGTCTTTCAATAGAGGAGAGTGTTAA (SEQ ID
NO:4).

25 Heavy chain nucleotide sequence:

ATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGCTACTGGTGTTCATTC
TGAGGTCCAGGTGCAACAGTCTGGACCTGAACTGGTGACGCCTGGGGCCTCAGT
GAAGATATCCTGCAAGACTTCTGGATACACTTTCACTGAATATAACCGTCCACTGG
GTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGGCATTAATCCTACC
30 AGTGGTGGTACTAACTACAACCAGAGGTTCAAGGGGCAAGGCCACATTGACTGTA
GACAGGTCTCCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGAT
TCTGCAGTCTATTTTTGTGCAGGAACCCTCTATGGCTACCCTTTTGACTTCTGGGG
CCAAGGCACCACTCTCACAGTCTCCTCAGCTAGCACCAAGGGACCTTCTGTTTTT
CCACTTGCTCCTTCTTCTAAGTCTACTTCTGGTGGAACTGCTGCTTTGGGTTGTTT

GGTGAAAGATTACTTTCCTGAGCCAGTGACCGTTTCTTGGAAGTCAGGTGCTCTT
 ACATCTGGTGTTCATACTTCCAGCTGTTCTTCAATCTTCAGGACTTTACTCACT
 TTCTTCTGTTGTTACCGTTCCTTCTTCAAGCTTGGGCACTCAGACCTACATCTGCA
 ATGTGAATCACAAACCCAGCAACACCAAGGTTGACAAGAAAGTTGAGCCCAAGT
 5 CTTGTGACAAGACTCATACGTGTCCACCGTGCCCAGCACCTGAACTTCTTGGAGG
 ACCGTCAGTCTTCTTGTTCCTCCAAAGCCTAAGGATACCTTGATGATCTCCAGG
 ACTCCTGAAGTCACATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGA
 AGTTCAACTGGTATGTGGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGA
 GAGAGGAACAGTACAACAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCA
 10 TCAAGATTGGTTGAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCT
 CCCAGCCCCCATTGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACC
 ACAAGTGTACACACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAG
 CTTGACTTGCTGGTGAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAG
 AGCAATGGGCAACCGGAGAACAACACTACAAGACTACACCTCCCGTTCTCGATTCT
 15 GACGGCTCCTTCTTCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAA
 CAAGGGAATGTCTTCTCATGCTCCGTGATGCATGAGGCTCTTCACAATCACTACA
 CACAGAAGAGTCTCTCCTTGTCTCCGGGTAAATGA (SEQ ID NO:5).

In certain embodiments, the E60 WT MAb may also comprise an alternative heavy
 chain, which comprises a signal peptide (e.g., MGWSCILFLVATATGVHS SEQ ID NO:41)
 20 at its N-terminus that targets the HC into the endomembrane system for secretion (*see, e.g.*,
 SEQ ID NO:18). Accordingly, in certain embodiments, an alternative heavy chain nucleotide
 sequence for E60 WT is shown in Figure 17 (SEQ ID NO:17).

In certain embodiments, the plant-produced MAb is a single chain variant of DV
 MAb E60 (scFv-Fc fusion molecule).

25 In certain embodiments, the plant-produced MAb is a single chain variant of DV
 MAb E60 (scFv-C_H¹⁻³).

ATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGCTACTGGTGTTCATTC
 TGAGGTCCAGGTGCAACAGTCTGGACCTGAACTGGTGACGCCTGGGGCCTCAGT
 GAAGATATCCTGCAAGACTTCTGGATACACTTTCACTGAATATAACCGTCCACTGG
 30 GTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGGCATTAATCCTACC
 AGTGGTGGTACTAACTACAACCAGAGGTTTCAGGGGCAAGGCCACATTGACTGTA
 GACAGGTCCTCCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGAT
 TCTGCAGTCTATTTTGTGCAGGAACCCTCTATGGCTACCCTTTTGACTTCTGGGG
 CCAAGGCACCACTCTCACAGTCTCCTCAGGTTCAACTTCAGGAGGAGGATCAGGT

GGTGGTTCAGGAGGTGGAGGATCTTCTGGATGGTCTTGTATCATCCTTTTCTTGGT
 TGCAACAGCTACTGGTGTTCATTCTGACATCCTGATGACCCAATCTCCATCCTCCA
 TGTCTGTATCTCTGGGAGACTCAGTCAGCATCACTTGCCATGCAAGTCAGGGCAT
 TAGCGGTAATATAGGGTGGTTGCAGCAGAAACCAGGGAAATCATTTAAGGGCCT
 5 GATCTATCATGGAACCAACTTGGAAGAGGGAGTTCATCAAGGTTCAAGTGGCAG
 TGGATCTGGAGCAGATTATTCTCTCACCATCAGCAGCCTGGAGTCTGAAGATTTT
 GCAGACTATTACTGTGTACAGTATGGTCAGTTTCCTCCGACGTTCCGGTGGAGGCA
 CCAAGCTGGAAATCAAAGCAGCACCAAGGGACCTTCTGTTTTTCCACTTGCTCCT
 TCTTCTAAGTCTACTTCTGGTGGAACTGCTGCTTTGGGTTGTTTGGTGAAAGATTA
 10 CTTTCCTGAGCCAGTGACCGTTTCTTGGAACCTCAGGTGCTCTTACATCTGGTGTTC
 ATACTTTCCAGCTGTTCTTCAATCTTCAGGACTTTACTCACTTTCTTCTGTTGTTA
 CCGTTCCTTCTTCAAGCTTGGGCACTCAGACCTACATCTGCAATGTGAATCACAA
 ACCCAGCAACACCAAGGTTGACAAGAAAGTTGAGCCCAAGTCTTGTGACAAGAC
 TCATACGTGTCCACCGTGCCCAGCACCTGAACTTCTTGGAGGACCGTCAGTCTTC
 15 TTGTTTCCTCCAAAGCCTAAGGATACCTTGATGATCTCCAGGACTCCTGAAGTCA
 CATGTGTAGTTGTGGATGTGAGCCATGAAGATCCTGAGGTGAAGTTCAACTGGTA
 TGTGGATGGTGTGGAAGTGCACAATGCCAAGACAAAGCCGAGAGAGGAACAGT
 ACAACAGCACGTACAGGGTTGTCTCAGTTCTCACTGTTCTCCATCAAGATTGGTT
 GAATGGCAAAGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCAT
 20 TGAGAAGACCATTTCCAAAGCGAAAGGGCAACCCCGTGAACCACAAGTGTACAC
 ACTTCCTCCATCTCGCGATGAACTGACCAAGAACCAGGTCAGCTTGACTTGCCCTG
 GTGAAAGGCTTCTATCCCTCTGACATAGCTGTAGAGTGGGAGAGCAATGGGCAA
 CCGGAGAACAACACTACAAGACTACACCTCCCGTTCTCGATTCTGACGGCTCCTTCT
 TCCTCTACAGCAAGCTCACAGTGGACAAGAGCAGGTGGCAACAAGGGAATGTCT
 25 TCTCATGCTCCGTGATGCATGAGGCTCTTACAATCACTACACACAGAAGAGTCT
 CTCCTTGTCTCCGGGTAAATGA (SEQ ID NO:6).

In certain embodiments, the N-glycoform selectively binds to Fc receptors or C1q.

In certain embodiments, the plant-produced MAb is a bivalent or tetravalent variant.

In certain embodiments, the MAb comprises a signal peptide (*e.g.*,

30 MGWSCILFLVATATGVHS SEQ ID NO:41) at its N-terminus that targets the HC into the endomembrane system for secretion.

In certain embodiments, the plant-produced MAb is specific for a flavivirus, coronavirus, paramyxovirus, lentivirus, or other virus that is prone to ADE.

In certain embodiments, the present invention provides a nucleic acid molecule that encodes DV MAb E60.

In certain embodiments, a plant-produced MAb described herein further comprises a heavy chain (HC) comprising one or more amino acid mutations that affect Fc receptor or/and C1q binding. As described herein these mutations may decrease overall Fc receptor and/or C1q binding and/or increase the selectivity of the binding.

As used herein, L234, L235 and G237 refer to amino acids in the mature heavy chain of IgG1. As described herein, these amino acids may be mutated to affect Fc receptor and/or C1q binding. Additionally, mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes and amino acids that correspond to, or align with, amino acids L234, L235 and G237 may be readily determined by one skilled in the art using known techniques (e.g., BLAST or ALIGN). The numbering of the amino acids may also vary with other alterations to the heavy chain, e.g., the inclusion of a signal peptide. For example, in Figure 17, E16 and E60 HC constructs are shown: L234 is equivalent to L254 in E60 and L255 in E16; L235 is equivalent to L255 in E60 and L256 in E16; and G237 is equivalent G257 in E60 and G258 in E16.

Accordingly, in certain embodiments, the heavy chain of the antibody is a human IgG1 isotype and the one or more mutations are selected from L234AL235A, wherein the leucines at positions 234 and 235 are mutated to alanine, and L234AL235AG237A, wherein the leucines at positions 234 and 235 are mutated to alanine; and glycine at position 237 is mutated to alanine, or mutations that correspond to, or align with, L234AL235A and L234AL235AG237A. In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes. In certain embodiments, the plant-produced MAb is a E16 mutant or an E16 scFv mutant, e.g., an E16 mutant comprising an amino acid sequence shown in Figure 17, such as an E16 heavy chain LALA mutant (SEQ ID NO:38) or an E16 heavy chain LALAGA mutant (SEQ ID NO:40). In certain embodiments, the plant-produced MAb is a E60 mutant or an E60 scFv mutant, e.g., an E60 mutant comprising an amino acid sequence shown in Figure 17, such as an E60 heavy chain LALA mutant (SEQ ID NO:22), an E60 heavy chain LALAGA mutant (SEQ ID NO:24), an E60 scFv LALA mutant (SEQ ID NO:30) or an E60 scFv LALAGA mutant (SEQ ID NO:32).

As described herein, MAbs that only need neutralization activities for their function, but cause ADE, may be mutated to eliminate glycosylation and Fc receptor and/or C1q binding, thereby reducing ADE.

Accordingly, certain embodiments of the invention provide a plant-produced monoclonal antibody (MAb) specific for a target virus, wherein the MAb is aglycosylated and comprises a heavy chain (HC) comprising one or more amino acid mutations that result in an aglycosylated MAb, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb. In certain embodiments, the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

Accordingly, certain embodiments of the invention also provide a plant-produced monoclonal antibody (MAb) specific for a target virus, wherein the MAb is aglycosylated and comprises a heavy chain (HC) comprising one or more amino acid mutations that eliminate Fc receptor or/and C1q binding, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb. In certain embodiments, the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells. In certain embodiments, the MAb comprises two or more mutations.

In certain embodiments, an N-glycosylation site in the MAb is mutated, wherein the mutation results in aglycosylation of the MAb (*i.e.*, the N-glycan is eliminated and not attached to the antibody). In certain embodiments, the N-glycosylation site consensus sequence N-X-S/T is mutated, wherein N is asparagine, X is any amino acid, and S/T is serine or threonine.

As used herein, N297 refers to an amino acid in the mature heavy chain of IgG1. As described herein, this amino acid may be mutated to result in an aglycosylated MAb.

Additionally, a mutation may alternatively be made at equivalent positions in other MAb isotypes and their subtypes and an amino acid that corresponds to, or aligns with, amino acid N297 may be readily determined by one skilled in the art using known techniques (e.g., BLAST or ALIGN). The numbering of the amino acids may also vary with other alterations to the heavy chain, e.g., the inclusion of a signal peptide. For example, in Figure 17, E16 and E60 HC constructs are shown: N297 is equivalent to N317 in E60 and N318 in E16.

Accordingly, in certain embodiments, the N in the consensus sequence is at the position of 297 in human IgG1 or its equivalent position for other MAb isotypes and their subtypes (e.g., a position that corresponds to, or aligns with, N297). In certain embodiments, the HC is a human IgG1 isotype, and the mutation is N297Q (or an N to Q mutation at a

position that corresponds to, or aligns with 297). In certain embodiments, a corresponding mutation may alternatively be made at an equivalent position in other MAb isotypes and their subtypes. In certain embodiments, the plant-produced aglycosylated MAb is a E16 mutant or an E16 scFv mutant, e.g., an E16 mutant comprising an N to Q mutation as shown in SEQ ID NO:36. In certain embodiments, the plant-produced MAb is a E60 mutant or an E60 scFv mutant, e.g., comprising an N to Q mutation as shown in SEQ ID NO:20 or SEQ ID NO:28, respectively.

In certain embodiments, the heavy chain of the aglycosylated MAb is a human IgG1 isotype and the one or more mutations that eliminate Fc receptor or/and C1q binding are selected from L234AL235A and L234AL235AG237A (*see, e.g.*, Figure 17, wherein mutations were made at corresponding positions in various constructs). In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

As described above, certain HC mutations may decrease overall Fc receptor and/or C1q binding and/or increase the selectivity of the binding, thereby reducing ADE. Accordingly, certain embodiments of the invention provide a plant-produced monoclonal antibody (MAb) specific for a target virus, wherein the MAb comprises a heavy chain (HC) comprising one or more amino acid mutations that alter Fc receptor or/and C1q binding, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells. In certain embodiments, the heavy chain comprises two or three amino acid mutations. In certain embodiments, the heavy chain is a human IgG1 isotype and the mutations are selected from L234AL235A and L234AL235AG237A (*see, e.g.*, Figure 17, wherein mutations were made at corresponding positions in various constructs). In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

In certain embodiments, the present invention provides a population of plant-produced monoclonal antibodies (MAb) specific for a target virus, the MAbs comprising a defined and highly-uniform N-glycoform, wherein the N-glycoform is GnGnXF₃, Man₅, Man₇, Man₈, Man₉, GnGn, AGn, GnA, AA, GnGn F₆, AGnF₆, GnAF₆, AAF₆, GnNa, NaGn, NaA, ANa, NaNa, GnNaF₆, NaGnF₆, NaAF₆, ANaF₆, NaNaF₆, GnGnbi, GnGnbiF₆, AGnbi, GnAbi, AAbi, NaGnbi, GnNabi, NaAbi, ANabi, NaNabi, AGnbiF₆, GnAbiF₆, AAbiF₆,

NaGnbiF₆, GnNabiF₆, NaAbiF₆, ANabiF₆, NaNabiF₆ (GnGn)(GnGn), (GnGn)(GnGn)F₆, (AA)(AA)F₆, or (Na)(Na)F₆ as depicted in Figure 1, wherein the MAbs have reduced antibody-dependent enhancement (ADE) as compared to mammalian-produced MAbs, and wherein the MAbs have equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

Certain embodiments of the invention provide a population of plant-produced monoclonal antibodies (MAbs) specific for a target virus, wherein the MAbs are aglycosylated and comprises a heavy chain (HC) comprising one or more amino acid mutations that eliminate Fc receptor or/and C1q binding, wherein the MAbs have reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb. In certain embodiments, the MAbs have equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

Certain embodiments of the invention provide a population of plant-produced monoclonal antibodies (MAbs) specific for a target virus, wherein the MAbs comprise a heavy chain (HC) comprising one or more amino acid mutations that alter Fc receptor or/and C1q binding, wherein the MAbs have reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and wherein the MAbs have equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

In certain embodiments, the present invention provides a method of modulating antibody-dependent enhancement (ADE) of an antibody comprising contacting the antibody with an N-glycan. In certain embodiments, the N-glycan is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, or GnGn F₆. In certain embodiments, the N-glycan is GnGnXF₃, Man5, Man7, Man8, or Man9. In certain embodiments, the methods further comprise mutating the antibody heavy chain (e.g., L234AL235A or L234AL235AG237A, or equivalent mutations at corresponding/alignable positions). In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

In certain embodiments, the present invention provides a method of modulating antibody-dependent enhancement (ADE) of an antibody comprising mutating the amino acid

sequence of the antibody heavy chain. In certain embodiments, the amino acid sequence of the antibody heavy chain is a human IgG1 isotype and comprises mutations L234AL235A or L234AL235AG237A or mutations that correspond to, or align with, these positions. In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

In certain embodiments, the present invention provides a method of producing a MAb having a defined and highly-uniform N-glycoform, wherein the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AGnF₆, GnAF₆, AAF₆, GnNa, NaGn, NaA, ANa, NaNa, GnNaF₆, NaGnF₆, NaAF₆, ANaF₆, NaNaF₆, GnGnbi, GnGnbiF₆, AGnbi, GnAbi, AAbi, NaGnbi, GnNabi, NaAbi, ANabi, NaNabi, AGnbiF₆, GnAbiF₆, AAbiF₆, NaGnbiF₆, GnNabiF₆, NaAbiF₆, ANabiF₆, NaNabiF₆ (GnGn)(GnGn), (GnGn)(GnGn)F₆, (AA)(AA)F₆, (Na)(Na)F₆ as depicted in Figure 1 comprising

- (a) growing a plant comprising a nucleic acid encoding the MAb, and
- (b) isolating the MAb,

wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells. In certain embodiments, the plant is a wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produces N-glycoforms of GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AAF₆, NaNa, NaNaF₆, GnGnbi, or (GnGn)(GnGn) (See Figure 1) as described in reference (Castilho, A. and H. Steinkellner (2012). "Glyco-engineering in plants to produce human-like N-glycan structures." Biotechnology Journal 7(9): 1088-1098).

In certain embodiments, the nucleic acid encodes a MAb with one or more amino acid mutations in its heavy chain (HC) that affect Fc receptor and/or C1q binding. In certain embodiments, the HC is a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations at corresponding/alignable positions. In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

In certain embodiments, the plant is a wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produce N-glycoforms of GnGnXF₃, Man5, Man7,

Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AAF₆, NaNa, NaNaF₆, GnGnbi, or (GnGn)(GnGn) as depicted in Figure 1).

In certain embodiments, the present invention provides another method of producing a MAb having a defined and highly-uniform N-glycoform GnGnXF₃, Man5, Man7, Man8,

5 Man9, GnGn, AGn, GnA, AA, GnGn F₆ comprising

- (a) growing a plant;
- (b) transiently expressing MAb and glycol-enzymes in the plant by co-infiltrating plant with *Agrobacterium* stains containing the genetic constructs of MAb heavy chain (HC), light chain (LC), and glycol-enzymes; and
- 10 (c) isolating the MAb,

wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity kinetics and *in vivo* therapeutic activity kinetics against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

In certain embodiments, the MAb HC construct encodes a HC comprising one or more amino acid mutations that affect Fc receptor and/or C1q binding. In certain embodiments, the HC of a MAb is altered at two or three positions that affect its interaction with C1q or/and Fc receptors. In certain embodiments, the MAb HC construct encodes a human IgG1 HC isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations at corresponding/alignable positions (Hezareh, et al. (2001). "Effector Function Activities of a Panel of Mutants of a Broadly Neutralizing Antibody against Human Immunodeficiency Virus Type 1." Journal of Virology 75(24): 12161-12168). In certain embodiments, the MAb HC construct may encode an alternative MAb isotype/subtype with corresponding mutations.

In certain embodiments, the HC of a MAb is tagged with an endoplasmic reticulum (ER)-retention/retrieval signal.

In certain embodiments, the ER-retention/retrieval signal is a peptide sequence containing the four amino acid H/KDEL (SEQ ID NO:7) or its variant attached to the carboxyl-terminus of the heavy chain of an antibody.

In certain embodiments, the HC of the MAb is tagged with a signal peptide (e.g., MGWSCIILFLVATATGVHS SEQ ID NO:41) at the N-terminus of the heavy chain (HC), which targets the HC into the endomembrane system for secretion.

In certain embodiments, the plant is wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produces N-glycoform of GnGn (See Figure 1) as described in reference (Castilho, A. and H. Steinkellner (2012). "Glyco-engineering in plants to produce human-like N-glycan structures." Biotechnology Journal 7(9): 1088-1098). In

5 certain embodiments, the glyco-enzyme is β 1,4-galactosyltransferase (GalT), α 1,6-fucosyltransferase (FUT8), human β 1,4-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIII), α 1,3-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIV), α 1,6-mannosyl- β 1,6-N-acetylglucosaminyltransferase (GnTV), UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE), N-acetylneuraminic acid phosphate-synthase (NANS),
10 CMP-Neu5Ac transporter (CST), or α 2,6-or α 2,3-sialyltransferase.

In certain embodiments, the genetic construct of one glycol-enzyme is co-infiltrated into a plant with those of MAb LC and HC. In certain embodiments, the genetic constructs of more than one glycol-enzymes are co-infiltrated into a plant with those of MAb LC and HC. In certain embodiments, the plant is a wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produces N-glycoform of GnGn.
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Certain embodiments of the invention provide a method of producing an aglycosylated MAb comprising (a) growing a plant comprising a nucleic acid encoding the MAb, wherein the encoded MAb comprises a mutation that results in aglycosylation of the MAb (e.g. a mutation at an N-glycosylation site in the MAb heavy chain (HC)), and (b)
20 isolating the MAb, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb. In certain embodiments, the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

25 In certain embodiments, a N-glycosylation site consensus sequence N-X-S/T in the MAb HC is mutated, and wherein N is asparagine, X is any amino acid, and S/T is serine or threonine. In certain embodiments, the HC is a human IgG1 isotype, and the mutation is N297Q, or an equivalent mutation that corresponds to, or aligns with, this position. In certain embodiments, corresponding a mutation may alternatively be made at an equivalent
30 position in other MAb isotypes and their subtypes.

In certain embodiments, the encoded MAb further comprises one or more amino acid mutations that eliminate Fc receptor or/and C1q binding. In certain embodiments, the HC is a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with, these

positions. In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

Certain embodiments of the invention provide a method of producing an aglycosylated MAb comprising (a) growing a plant; (b) transiently expressing the MAb in the plant by co-infiltrating the plant with *Agrobacterium* strains containing the genetic constructs of MAb HC and LC, wherein the genetic construct of the MAb HC comprises a mutation that results in aglycosylation of the MAb (e.g, a mutation altering an N-glycosylation site) ; and (c) isolating the MAb, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb. In certain embodiments, the MAb has equivalent antigen binding affinity and kinetics, neutralization activity kinetics and *in vivo* therapeutic activity kinetics against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

In certain embodiments, the genetic construct of the MAb HC comprises a mutation that alters an N-glycosylation site consensus sequence N-X-S/T, and wherein N is asparagine, X is any amino acid, and S/T is serine or threonine. In certain embodiments, the genetic construct of the MAb HC encodes a human IgG1 isotype comprising an N297Q mutation, or an N to Q mutation at a corresponding/alignable position. In certain embodiments, a corresponding mutation may alternatively be made at an equivalent position in other MAb isotypes and their subtypes.

In certain embodiments, the MAb HC genetic construct encodes an HC further comprising one or more mutations that eliminate Fc receptor or/and C1q binding. In certain embodiments, the HC is an IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations at corresponding/alignable positions. In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

In certain embodiments, the plant is a wild-type *Nicotiana benthamiana* or any glyco-engineered *N. benthamiana* plants.

Certain embodiments of the invention provide a method of producing a MAb having altered Fc receptor and/or C1q binding comprising (a) growing a plant comprising a nucleic acid encoding the MAb, wherein the encoded MAb comprises one or more amino acid mutations in its heavy chain that alter Fc receptor and/or C1q binding, (b)isolating the MAb, wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and wherein the MAb has equivalent antigen binding affinity

and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

Certain embodiments of the invention provide a method of producing a MAb having
 5 altered Fc receptor and/or C1q binding comprising (a) growing a plant; (b) transiently expressing MAb in the plant by co-infiltrating the plant with *Agrobacterium* strains containing the genetic constructs of MAb HC and LC, wherein the HC genetic construct encodes an HC comprising one or more mutations that alter Fc receptor and/or C1q binding; and (c) isolating the MAb, wherein the MAb has reduced antibody-dependent enhancement
 10 (ADE) as compared to a mammalian-produced MAb, and wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity kinetics and *in vivo* therapeutic activity kinetics against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

In certain embodiments, the MAb HC is an IgG1 isotype and the one or more
 15 mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations at corresponding/alignable positions. In certain embodiments, corresponding mutations may alternatively be made at equivalent positions in other MAb isotypes and their subtypes.

In certain embodiments, the MAb binds to Fc receptors and/or C1q with increased selectivity. In certain embodiments, the MAb binding to Fc receptors and/or C1q is reduced.

20 In certain embodiments, the method further comprises transiently expressing a glyco-enzyme(s) in the plant by infiltrating the plant with an *Agrobacterium* strain(s) containing the genetic construct of a glycol-enzyme(s). In certain embodiments, the glyco-enzyme is β 1,4-galactosyltransferase (GalT), α 1,6-fucosyltransferase (FUT8), human β 1,4-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIII), α 1,3-mannosyl- β 1,4-N-
 25 acetylglucosaminyltransferase (GnTIV), α 1,6-mannosyl- β 1,6-N-acetylglucosaminyltransferase (GnTV), UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE), N-acetylneuraminic acid phosphate-synthase (NANS), CMP-Neu5Ac transporter (CST), or α 2,6- or α 2,3-sialyltransferase.

In certain embodiments, the genetic construct of one glycol-enzyme is co-infiltrated
 30 into a plant with those of MAb LC and HC. In certain embodiments, the genetic constructs of more than one glycol-enzymes are co-infiltrated into a plant with those of MAb LC and HC.

In certain embodiments, the plant is a wild-type *Nicotiana benthamiana* or a glyco-engineered *N. benthamiana* plant that produce N-glycoforms of GnGnXF₃, Man5, Man7,

Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AAF₆, NaNa, NaNaF₆, GnGnbi, or (GnGn)(GnGn) as depicted in Figure 1. In certain embodiments, the plant is a wild-type *Nicotiana benthamiana* or a glyco-engineered *N. benthamiana* plant that produces N-glycoform of GnGn.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structure and name of N-glycosylation forms that are provided in certain embodiments. The structure of one major isomer is shown for glycans that have multiple physiological isomers.

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Figure 2. Western blot analysis of pHu-E16 variants. **A.** Designs of Hu-E16 variants in this study. **B-D.** Western blot analysis. Hu-E16 variants were extracted from *N. benthamiana* leaves and were separated on SDS-PAGE gels under reducing (**B** and **C**) or non-reducing (**D**) conditions and blotted onto PVDF membranes. The membranes were incubated with a goat anti-human gamma chain antibody or a goat anti-human kappa chain antibody to detect heavy chain (**B** and **Lane 7 of D**) or light chain (**C** and **Lanes 1-6 of D**). Lane 1, pHu-E16 as a reference standard; lane 2, Protein sample extracted from leaves co-infiltrated with pHu-E16 HC and pHu-E16scFv-C_L constructs; lane 3, Sample from leaves co-infiltrated with pHu-E16scFv-C_H¹⁻³ and pHu-E16scFv-C_L; lane 4, Sample from leaves co-infiltrated with pHu-E16scFv-C_H¹⁻³ and pHu-E16 LC; lanes 5 and 7, Sample from leaves infiltrated with pHu-E16scFv-C_H¹⁻³; lane 6, Sample from un-infiltrated leaves. HC: heavy chain, scFv: single-chain variable fragment; C_H¹⁻³: the constant region domains 1 to 3 of HC; LC: light chain; C_L: Constant region of LC; V_L: variable region of LC; V_H: variable region of HC.

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Figure 3. Time course of pHu-E16 expression in *N. benthamiana* plants. Total proteins from leaves infiltrated or co-infiltrated with pHu-E16scFv-C_H¹⁻³, pHu-E16 HC/pHu-E16scFv-C_L, pHu-E16scFv-C_H¹⁻³/pHu-E16scFv-C_L (Tetra pHu-E16), or pHu-E16scFv-C_H¹⁻³/pHu-E16 LC were extracted on days 5 -10 post infiltration and analyzed by ELISA that detects the assembled form of pHu-E16 MAbs variants, Mean ± SD of samples from three independent infiltrations are presented.

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Figure 4. Purification of pHu-E16 variants from *N. benthamiana* leaves. Leaf proteins were extracted on day 9 after agroinfiltration. pHu-E16 variants were purified and analyzed on a 4-20% gradient SDS-PAGE gel under reducing condition and visualized with Coomassie stain. Lane 1, pHu-E16 as a reference standard; lane 2, pHu-E16 HC/pHu-

E16scFv- C_L ; lane 3, Tetra pHu-E16; lane 4, pHu-E16scFv- C_H^{1-3} /pHu-E16 LC; lanes 5, pHu-E16scFv- C_H^{1-3} . One representative of several independent experiments is shown.

Figure 5. FcγR and C1q binding and antibody-dependent enhancement of pHu-E16 variants.

- A.** SPR analysis of FcγR and C1q binding. FcγRs including CD16A (CD16V¹⁵⁸, CD16F¹⁵⁸), CD32A (CD32aR¹³¹ and CD32aH¹³¹ fused to an aglycosylated Fc region of IgG2 (CD32aR¹³¹-G2agl and CD32aH¹³¹-G2agl)), CD32B (CD32b fused to an aglycosylated Fc region of IgG2 (CD32b-G2agl)) and CD64, and C1q were injected over the surfaces with Hu-E16 variants captured on immobilized WNV E protein. Buffer injection was subtracted as blank and responses were normalized to same level of captured antibodies.
- B.** Hu-E16 variants antibody-dependent enhancement of WNV infection. Serial dilutions of Hu-E16 variants were mixed with WNV RVP and added to CD32A+ K562 cells. Forty-eight hours later, cells were analyzed by flow cytometry for GFP expression.

Figure 6. Antigen binding of pHu-E16 variants to DIII of WNV E.

- A.** ELISA analysis. Serial dilutions of pHu-E16 variants were incubated on plates coated with WNV DIII and detected with a HRP-conjugated anti-human gamma antibody. Dilutions of pHu-E16 were used in parallel as reference standards. A commercial generic human IgG (Southern Biotech) was used as a negative control. One set of representative O.D. 450 nm readings from several independent experiments is presented.
- B.** Binding of pHu-E16scFv- C_H^{1-3} to domain III of WNV E displayed on the cell surface of yeast. Yeast cells displaying domain III of WNV E protein were stained with pHu-E16scFv- C_H^{1-3} , mHu-E16 (positive control), or a plant-produced humanized MAb against Ebola virus GP1 protein (negative control). Yeast cells were then processed by flow cytometry. Representative data from three independent experiments are shown.

Figure 7. Neutralization of WNV by pHu-E16 variants. WNV (strain New York 1999) was incubated with serial dilutions of A) mHu-E16 (positive control 1), or B) pHu-E16 (positive control 2), C) pHu-E16scFv- C_H^{1-3} , or D) Tetra pHu-E16 and used to infect Vero cells. Cells were then fixed, permeabilized, analyzed by focus reduction assay and quantitated by Biospot analysis. Mean ± SEM is shown from one of several independent experiments. EC50 values are listed below the graphs.

Figure 8. pHu-E16 variants mediated protection in mice. **A.** Five week-old C57BL/6 mice were passively transferred saline, 100 ng of mHu-E16, or serial 10-fold increases in dose (ranging from 1 to 100 ng, $n=10$ mice per dose) of pHu-E16scFv- C_H^{1-3} via an intraperitoneal route on the same day as subcutaneous infection with 10^2 PFU of WNV. Survival data from at least two independent experiments were analyzed by log-rank test. **B.**

Wild type C57BL/6 mice were infected with 10^2 PFU of WNV and then given a single dose of the indicated doses of pHu-E16 scFv-CH¹⁻³, Tetra pHu-E16 or mHu-E16 via an intraperitoneal route at day +4 after infection. Survival data from at least two independent experiments ($n = 10$ mice per dose) were analyzed by the log-rank test.

Figure 9. Western blot analysis of pE60. pE60 was extracted from *N. benthamiana* leaves and were separated on SDS-PAGE gels under reducing (**A and B lanes 5-6**) or non-reducing (**B lanes 2-4**) conditions and blotted onto PVDF membranes. The membranes were incubated with a goat anti-human gamma chain antibody or a goat anti-human kappa chain antibody to detect heavy chain (**A**) or light chain (**B**). Lane 1, Molecular weight marker; lanes 2 and 5, Mammalian cell-produced E60 as a reference standard; lanes 3 and 6, Protein sample extracted from leaves infiltrated with buffer as a negative control; lanes 4 and 7, Sample from leaves co-infiltrated with E60 LC and E60 HC. HC: heavy chain, LC: light chain, (HL)₂: assembled tetrameric form of E60 antibody.

Figure 10. Purification of E60 from *N. benthamiana* leaves. Leaf proteins were extracted from wild-type (Lanes 3 and 6) or GnGn (Lanes 2 and 7) *N. benthamiana* plants on day 9 after agroinfiltration. pE60 was purified and analyzed on a 4-20% gradient SDS-PAGE gel under reducing (Lanes 1-3) or non-reducing (Lanes 5-7) condition and visualized with Coomassie stain. Lanes 1 and 6, pE60 purified from WT plants; Lanes 2 and 7, pE60 purified from GnGn plants; Lanes 3 and 5, pHu-E16 as a reference standard. One representative of several independent experiments is shown.

Figure 11. Binding of plant-derived E60 to domain II of WNV E displayed on the cell surface of yeast. Negative control yeast and yeast cells displaying domain I-II of DENV E protein were stained with E60 from WT *N. benthamiana* plants (WTpE60), GnGn *N. benthamiana* plants (Δ XFpE60), Mammalian cells (mE60, positive control), or pGP1 (negative control). Yeast cells were then processed by flow cytometry. Representative data from at least three independent experiments are shown.

Figure 12. Neutralization of DENV by plant-produced E60. DENV was incubated with serial dilutions of WTpE60, Δ XFpE60, and mE60 (positive control) and used to infect human U937 cells. Cells were then fixed, permeabilized, analyzed by focus reduction assay and quantitated by Biospot analysis. Mean $\pm$ SEM is shown from at least two independent experiments.

Figure 13. N-linked glycan analysis of E16 antibody produced in glycoengineered *N. benthamiana*. Glycosylated peptides containing the Asn297 residue of the CH region

were obtained from E16 purified from Δ XF Plants (A) or mammalian cells E16 (B) by tryptic digestion, and analyzed by LC-ESI-MS. Due to an incomplete tryptic digestion two glycopeptides were generated that differ by 482 Da. Glycopeptide (GP) 1 is indicated with an asterisk, GP 2 is highlighted.

Figure 14. Neutralization of WNV by pHu-E16 and pHu-E16scFv-CH¹⁻³ produced in glycoengineered *N. benthamiana*. WNV RVPs were pre-incubated with serial dilutions of Δ XF plants-derived pHu-E16 (Δ XFpE16, A), pHu-E16scFv-CH¹⁻³ (Δ XFpE16scFv-CH, B), or WT plant-derived pHu-E16 (WTPe16, C) in the presence of medium (w/o C1q, black line) or 50 μ g/ml of human C1q (with C1q, red line), and used to infect permissive Raji-DC-SIGN-R cells. After 40 hours of incubation, cells were fixed and analyzed by flow cytometry for GFP expression. Data are representative of at least two independent experiments.

Figure 15. Δ XFpE16 and Δ XFpE16scFv-CH mediated protection in mice. A. Five week-old C57BL/6 mice were passively transferred saline, or serial increases in dose (ranging from 1 to 1000 ng, n = 10 mice per dose) of Δ XFpE16 or Δ XFpE16scFv-CH via an intraperitoneal route on the same day as subcutaneous infection with 10² PFU of WNV. Survival data from at least two independent experiments were analyzed by log-rank test. mE16 (100 ng) was used in parallel as a positive control. B. Wild type C57BL/6 mice were infected with 10² PFU of WNV and then given a single dose of Δ XFpE16 (50 μ g), Δ XFpE16scFv-CH (500 μ g) or mHu-E16 (50 μ g or 500 μ g) via an intraperitoneal route at day +4 after infection. Survival data from at least two independent experiments (n = 10 mice per dose) were analyzed by the log-rank test.

Figure 16. Relative abundance in percentage of major glyco-structures detected on HuE16 variants.

Figure 17. Nucleic acid and amino acid sequences for various E60 and E16 constructs. Each nucleic acid construct also includes a sequence that encodes a signal peptide (MGWSCILFLVATATGVHS SEQ ID NO:41) at the N-terminus of the heavy chain (HC), which targets the HC into the endomembrane system for secretion.

DETAILED DESCRIPTION OF THE INVENTION

One of the major impediments towards developing vaccines and antibody-based therapeutics for many viruses is the risk of antibody-dependent enhancement (ADE), which may render vaccinated or anti-viral antibody treated subjects more susceptible to infection.

The ability of obtaining MAb variants with specific and selective interaction with Fc receptors or C1q is crucial for reducing ADE. N-glycosylation of a MAb plays an important role in its interaction with the Fc receptors and C1q. Therefore, the ability of obtaining highly uniform N-glycans for MAb drug is crucial for identifying MAb variants with reduced or eliminated ADE. Previous studies on this subject have been limited by the difficulty in obtaining antibodies with a homogenous glycoform due to the heterogeneous N-glycan nature of mammalian cells. Previous attempts to obtain various defined glycoforms for selecting the right glycoforms to reduce ADE were unsuccessful. This is due to the fact that current approaches to alter the glycoform of IgGs by glycosylation inhibitors or treatment with glycosidases are inadequate in producing specifically defined glycoforms. Other attempts were made to use aglycosylated MAbs (MAbs have no N-glycosylation) for reducing ADE. While aglycosylated MAbs cannot bind FcγRs or cause ADE, they may become less stable and lose the necessary effector function (CDC or ADCC) required for their full therapeutic effect. Accordingly, aglycosylated MAbs with reduced ADE may be useful where only neutralization activities are needed for their function.

The present study indicated that it is possible to use plants to produce monoclonal antibody (MAb) drugs to reduce or even eliminate their ability to induce ADE. In certain embodiments, the present plant system is superior in producing MAbs with highly uniform N-glycans. This reduces the risk of ADE and enhances the safety of many MAb drugs for viral infections. Because in certain embodiments the present plant-produced MAbs are still glycosylated, they are stable and retain selective ADCC and CDC activity for full therapeutic function and potency, while losing or greatly reducing ADE.

Plants are uniquely able to produce MAb or MAb derivatives with highly homogeneous or uniform N-glycosylation patterns. For example, > 90% of pE16 MAb produced in wild-type (WT) *N. benthamiana* plants display the N-glycosylation form of GnGnXF3 (Figure 16). If an endoplasmic reticulum (ER)-retention/retrieval signal such as a peptide sequence containing the four amino acid H/KDEL (SEQ ID NO:7) or its variant is attached to the carboxyl-terminus of the heavy chain of an antibody, predominantly ER-type oligomannosidic (Man7, Man8 and Man9, Figure 1) N-linked glycans will be produced for MAbs by WT and other plant lines. In addition, when fused to part of heavy chain, single-chain variants of an MAb such as scFv-Fc and scFv-CH¹⁻³ molecules also displayed Man8 and Man9-types of oligomannosidic structures when expressed in plants. Several plant lines have been developed by genetically suppressing or eliminating enzymes for the biosynthesis of plant-specific glycans and by introducing glycoenzymes from mammalian cells (Lai, H.,

M. Engle, et al. (2010). "Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice." *Proceedings of the National Academy of Sciences of the United States of America* 107(6): 2419-2424). For example, a *N. benthamiana* plant line (Δ XF) was generated by RNA interference (RNAi) technology with down-regulated expression of the endogenous β 1,2-xylosyltransferase and α 1,3-fucosyltransferase genes. *N*-glycan analysis showed that Δ XF line-produced endogenous glycoproteins exhibited a profile with complex-type *N*-glycans virtually lacking the plant-specific xylose and a significant reduction of α 1,3-fucose for endogenous proteins (Loos, A., B. Van Droogenbroeck, et al. (2011). "Expression of antibody fragments with a controlled N-glycosylation pattern and induction of endoplasmic reticulum-derived vesicles in seeds of *Arabidopsis*." *Plant Physiol* 155(4): 2036-48). Moreover, MAbs produced in this plant line have a homogenous mammalian N-glycoform with terminal N-acetylglucosamine (Gn) residues (i.e., GnGn structures), lacking unwanted β 1,2-xylose and core α 1,3-fucose residues (Figure 13) (Loos, A., B. Van Droogenbroeck, et al. (2011). "Expression of antibody fragments with a controlled N-glycosylation pattern and induction of endoplasmic reticulum-derived vesicles in seeds of *Arabidopsis*." *Plant Physiol* 155(4): 2036-48). Similarly, the plant lines with down-regulated or knocked-out endogenous β 1,2-xylosyltransferase and α 1,3-fucosyltransferase genes have been generated for the aquatic plant *Lemna minor*, the moss *Physcomitrella patens*, *Medicago sativa* and rice cells. Other glycol-engineered plant lines include *N. benthamiana* lines that perform β -1,4-galactosylation (produces AA N-glycans, created by expressing human β 1,4-galactosyltransferase (GalT) fused to the amino-terminal cytoplasmic-transmembrane-stem (CTS) region of rat α 2,6-sialyltransferase (ST)), and α 1,6-fucosylation (expressing human α 1,6-fucosyltransferase (FUT8)). Plant lines that produce bisected, tri- and tetra-antennary N-glycans (for example, GnGnbi, and (GnGn)(GnGn), Figure 1) (by expressing human β 1,4-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIII), α 1,3-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIV) and α 1,6-mannosyl- β 1,6-N-acetylglucosaminyltransferase (GnTV)) and terminal sialylation (by expressing UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE), N-acetylneuraminic acid phosphate-synthase (NANS), CMP-Neu5Ac transporter (CST), GalT, and α 2,6-or α 2,3-sialyltransferase (ST)) are also developed.

WT plants and each of these plant lines produces a defined glycoform for MAbs as shown in Figure 1 with GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGn F₆ as the most promising glycoforms. In addition to these stable plant lines, MAbs with particular glycoforms can also be produced by transiently co-expressing the genetic

construct of glycol-enzymes individually or in combinations (including GalT, FUT8, GnTIII, GnTIV, GnTV, GNE, MANS, CST and ST) with the MAb HC and LC constructs in either WT or Δ XF plant lines through plant transient expression systems. Both of these approaches allow the selection of particular glycoforms that reduce or eliminate ADE, while maintain the therapeutic potency of the MAb. In addition to N-glycosylation, amino acid replacement in the HC of a MAb, e.g. L234AL235A and L234AL235AG237A (Hezareh, et al. (2001).

"Effector Function Activities of a Panel of Mutants of a Broadly Neutralizing Antibody against Human Immunodeficiency Virus Type 1." *Journal of Virology* 75(24): 12161-12168)

has also been used to alter the antibody interaction with Fc receptors and C1q. Such

glycoforms and glycoforms + HC amino acid replacement combinations have been identified by the present studies for an anti-West Nile virus antibody and an anti-Dengue virus antibody. This approach is applicable to many other antibodies or antibody-derivative-based drugs that treat viruses that are prone to ADE.

Dengue virus (DV)

Dengue virus (DV) is a member of the Flavivirus genus of the Flaviviridae family. DV is the most common mosquito-transmitted viral disease in the world, with ~50 to 100 million infections per year. DV infection causes a spectrum of disease ranging from Dengue fever (DF) to the life-threatening Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS). DV is a category A NIAID priority pathogen, a potential bioterrorism agent, and imposes a substantial economic and social liability, as it remains a leading cause of hospitalization and death of children in at least eight Southeast Asian countries. One of the major impediments towards developing vaccines and antibody-based therapeutics for DV and other viruses is the risk of ADE, which may render vaccinated or anti-DV antibody treated subjects more susceptible to infection. ADE occurs because pre-existing sub-neutralizing concentrations of antibodies (including therapeutic monoclonal antibodies (MAbs)) and the infecting DV form complexes that bind to Fc- γ receptor (Fc γ R)-bearing cells, resulting in increased virus uptake and replication. The binding of human IgG to Fc γ R is highly sensitive to the presence and the nature of the N-linked glycosylation at position N297 in its CH2 domain. Previous studies on this subject have been limited by the difficulty in obtaining antibodies with a homogenous glycoform due to the heterogeneous N-glycan nature of mammalian cells and incompetent chemical modification method. While aglycosylated MAbs cannot bind Fc γ Rs or cause ADE, they may become less stable, have shorter half-life in circulation (altered pharmacokinetics) and lose the necessary effector function required for

their full therapeutic effect. Therefore, aglycosylated MAbs are only desirable when they function solely through neutralization. Recent progress in plant glycoengineering has allowed the generation of transgenic plant lines that produce MAbs with defined and uniform mammalian N-linked glycans. This progress has allowed the study of how Fc glycosylation of MAbs affects ADE.

West Nile Virus (WNV)

West Nile virus (WNV) is a member of the Flavivirus genus of the Flaviviridae family, which also includes the Japanese encephalitis virus (JE), Tick-borne encephalitis virus (TBE), St. Louis Encephalitis virus (SLEV), Murray Valley encephalitis virus, dengue virus (including the four serotypes of: DEN-1, DEN-2, DEN-3, and DEN-4), and the family prototype, yellow fever virus (YF). Flavivirus infections are a global public health with about half of the flaviviruses causing human diseases.

WNV is a neurotropic, enveloped virus with a single-stranded, positive polarity, 11 kilobase RNA genome. Until 1999, WNV was found in the Eastern Hemisphere, with wide distribution in Africa, Asia, the Middle East, and Europe. In 1999, WNV entered the Western Hemisphere as a point introduction in New York City. Greater than 29,000 human cases have been diagnosed with severe WNV infection in the continental United States during the last decade, and many more have been infected and remain undiagnosed. Advanced age is by far the greatest risk factor for severe neurological disease, long-term morbidity, and death, although a genetic basis of susceptibility has also been recently identified.

Historically, there has been a lack of effective and specific antiviral treatment for infection by WNV or other flaviviruses. While several small molecules compounds have been recently described with antiviral activity against WNV *in vitro*, only few have demonstrated efficacy *in vivo*. Interferon (IFN), which is used as part of combination therapy against the distantly related hepatitis C virus, potently inhibits flaviviruses including WNV when used as prophylaxis. However, its effect is markedly attenuated once viral replication has commenced as flavivirus non-structural proteins antagonize IFN signaling pathways. Current treatment for WNV infection is supportive and no vaccine or therapeutic agent has been approved for human use. New threats of WNV globally and lack of available treatments warrant studies to develop effective therapeutics and production technologies that can rapidly transfer the candidates into the clinical care settings in a cost-conscious manner.

Recently, a plant-derived humanized murine MAb was developed with promising therapeutic potential. This MAb (E16) binds to a highly conserved epitope on the envelope

protein of WNV in all North American isolates, blocks viral fusion, and shows promising post-exposure therapeutic activity. Nonetheless, detailed studies show that while the E16 is therapeutically effective, peripheral delivery of this antibody has a limited window of efficacy in rodents. For example, administration of a single dose of hu-E16 through an intravenous or intraperitoneal route at day 5 postinfection or earlier improves survival rates. However, delivery of E16 directly into the brain at day 6 after infection can protect hamsters against lethal WNV infection.

Even though antibodies have been identified as potential prophylactic and or therapeutic medicaments for WNV or other infectious diseases, their ultimate application as beneficial therapeutics is limited lack of efficacy due to the short therapeutic window and the risk of antibody-dependent enhancement (ADE). Thus, there remains a need for effective, safe vaccines. In addition, there is a need to alleviate the high production costs and scalability associated with the mammalian cell culture production system. Moreover, if biological drugs are too costly to produce for resource poor health care systems and cannot be easily made into generics, their therapeutic potential may never be realized. As such, the development of production platforms that are cost-effective, scalable, and safe for biological therapeutics is urgently needed.

Despite annual WNV outbreaks in North America there is a lack of effective and specific antiviral treatment. The high production costs and limited scalability associated with mammalian cell culture production may restrict the use of therapeutic antibodies against WNV and other flaviviruses in resource-poor settings in the future. Here, the feasibility of producing in plants a candidate MAb therapeutic against WNV infection was investigated. The inventors have shown that plant-derived MAb therapeutics have similar potency as their mammalian-cell counterparts, and production of biological therapeutics in plants provides a platform that can address the cost and scalability issues associated with the mammalian cell culture production system. Further, these plant-derived therapeutics circumvent the issue of ADE.

In this invention, the inventors generated several monoclonal antibody (MAb) therapeutics in plants and in mammalian culture cells that can effectively treat West Nile virus (WNV) infection, do not induce ADE, and can also be rapidly scaled-up for commercial production.

The plant-derived MAbs retain the biological and therapeutic activity of the parental MAb in high-affinity binding and potent neutralizing activity *in vitro* against the target virus, and in protecting mice against viral-induced mortality.

Most of the current antibodies and their fragments are developed in either mammalian cell or bacterial cultures. Generation of these molecules in current systems has been hindered by difficulties especially in obtaining properly folded full-length molecules in sufficient quantities that retain conformationally-sensitive epitopes. The present invention showed that
5 in addition to mammalian cells, plants can also be readily used to produce a MAb successfully without any of these production issues. Since plants can rapidly express, accumulate and assemble MAb, and this can be expanded for commercial production without high-capital investments.

Transgenic plants are suitable for MAb production as they can be rapidly expanded in
10 commercial production without the high-capital investment associated with traditional MAb bioreactor facilities. pHu-E16 was expressed rapidly in *N. benthamiana* leaves within 4 to 8 days of infiltration and efficiently assembled into a native IgG form. Without any genetic optimization, pHu-E16 accumulated at an average of 0.8 mg/g of fresh leaf weight, greater than the highest expression level for MAbs in plants ever reported (Giritch A, et al. (2006)
15 Rapid high-yield expression of full-size IgG antibodies in plants coinfecting with noncompeting viral vectors. *Proc Natl Acad Sci U S A.* 103: 14701-14706). The rapid high-level production and assembly of pHu-E16 convincingly demonstrate the viability of this system for the more large-scale cost effective production of MAbs.

It is well-known that downstream processing is an important component of a
20 pharmaceutical protein production technology. In the present invention, there is described a simple three-step extraction and purification scheme that can be used to purify plant-generated MAb efficiently and in a manner that is scalable for mass production and conforms to cGMP regulations, thereby providing a method for the production of a pharmaceutically acceptable preparation of MAb. The rapid high-level accumulation of MAb in plants and the
25 availability of a scalable and cGMP compliant processing scheme provides advantages over the mammalian cell culture for future low-cost commercial production of MAb.

The N-linked glycosylation of proteins in plants is generally similar to that in mammalian cells. However, plants have unique plant-specific β -1,2-xylose and core α -1,3-fucose residues on complex N-linked glycans and lack terminal β -1,4-Gal and N
30 acetylneuraminic acid (Neu5Ac) residues (Gomord V, et al. (2004) Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge. *Plant Biotechnol J.* 2: 83-100). The impact of such differences on the activity of MAb therapeutics *in vivo* has not been evaluated, although glycan variations in the Fc region of IgG modulate the binding and activation of C1q (Raju TS (2008) Terminal sugars of Fc glycans influence antibody

effector functions of IgGs. *Curr Opin Immunol.* 20: 471-478; Wang F, et al. (2006) Structural and functional characterization of glycosylation in an immunoglobulin G1 to *Cryptococcus neoformans* glucuronoxylomannan. *Mol Immunol.* 43: 987-998). Since pHu-E16 HC has an ER-retention KDEL (SEQ ID NO:8) sequence, it is likely retained in the ER resulting in a predominately high mannose form of glycosylation (Ko K, et al. (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proc Natl Acad Sci U S A.* 100: 8013-8018), which contributes to the reduced affinity to C1q (Qun Z, et al. (2008) Development of a simple and rapid method for producing non-fucosylated oligomannose containing antibodies with increased effector function. *Biotechnol Bioeng.* 99: 652-665).

The difference between plant and mammalian glycosylation patterns raises concerns for the immunogenicity of plant-derived MAb therapeutics. The possibility of inducing plant-glycan specific antibodies could reduce therapeutic efficacy by accelerating clearance from plasma, or cause potential adverse effects through immune complex formation. Immunization studies with plant glycoproteins in different animal models have yielded inconsistent results: rats and rabbits develop antibodies to plant specific xylose and α -1,3-fucose, yet mice generate no antibody response against these glycans (Chargelegue D, et al. (2000) A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. *Transgenic Res.* 9: 187-194; Jin C, et al. (2008) A plant derived human monoclonal antibody induces an anticarbohydrate immune response in rabbits. *Glycobiology*). Moreover, no adverse effects were observed in patients with topical application of plant-produced MAbs with plant unique carbohydrates (Zeitlin L, et al. (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat Biotech.* 16: 1361-1364; Ma JK, et al. (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nat Med.* 4: 601-606). To date, the immunogenicity of systemic administered plant-produced MAbs has not been evaluated in humans.

To avoid problems associated with plant-specific glycans, "humanized" *N. benthamiana*, *Arabidopsis thaliana* and *Lemna minor* plant lines have been generated by genetic knockout or RNA interference (RNAi) strategies (Schahs M, et al. (2007) Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. *Plant Biotechnol J.* 5: 657-663; Strasser R, et al. (2008) Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N glycan structure. *Plant Biotechnol J.* 6: 392-402; Cox KM, et al. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat Biotechnol.* 24:

1591-1597). In these plants, enzymes for the biosynthesis of plant specific glycans are inactivated, resulting in structurally equivalent MAbs as those derived in mammalian cells. Moreover, the glycan uniformity of MAbs produced by these optimized plant lines is better than those from mammalian cell cultures. Indeed, an anti-human CD30 MAb produced from
5 these genetically modified plants had only a single predominant N-glycan species and showed improved antibody-dependent cell-mediated cytotoxicity (ADCC) compared to the same MAb produced in mammalian cells (Cox KM, et al. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat Biotechnol.* 24: 1591-1597). This improvement is most likely due to the removal of fucose, which results in
10 improved FcγR binding of MAbs (Shields RL, et al. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcγγ RIII and antibody-dependent cellular toxicity. *J Biol Chem.* 277: 26733-26740). The therapeutic utility of pHu-E16 can be improved by expression in such “humanized” *N. benthamiana* lines.

In brief, the Example provided below demonstrates that plant-derived MAbs can
15 function effectively as post-exposure therapy against a potentially lethal infectious disease. Plants are an efficient platform to produce Hu-E16 with high-yield, speed, enhanced scalability, and cost-effectiveness, satisfying all major metrics for a successful therapeutic candidate. This technology can be readily applied in the future to antiviral antibodies against other emerging infectious disease threats, and may be most useful in resource poor settings
20 such as the developing world.

E16 is a monoclonal antibody that strongly neutralizes WNV and a humanized version of this antibody has been described in the art that retains its neutralizing activity and avidity (Oliphant T, et al. (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nat Med.* 11: 522-530).

25 The generalized structure of antibodies or immunoglobulin is well known to those of skill in the art, these molecules are heterotetrameric glycoproteins, typically of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is covalently linked to a heavy chain by one disulfide bond to form a heterodimer, and the heterotrimeric molecule is formed through a covalent disulfide linkage between the
30 two identical heavy chains of the heterodimers. Although the light and heavy chains are linked together by one disulfide bond, the number of disulfide linkages between the two heavy chains varies by immunoglobulin isotype. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at the amino-terminus a variable domain (VH), followed by three or four constant domains (CH1, CH2, CH3, and

CH4), as well as a hinge region between CH1 and CH2. Each light chain has two domains, an amino-terminal variable domain (VL) and a carboxy-terminal constant domain (CL). The VL domain associates non-covalently with the VH domain, whereas the CL domain is commonly covalently linked to the CH1 domain via a disulfide bond. Particular amino acid residues are
5 believed to form an interface between the light and heavy chain variable domains (Chothia et al., 1985, J. Mol. Biol. 186:651-663.)

Certain domains within the variable domains differ extensively between different antibodies *i.e.*, are "hypervariable." These hypervariable domains contain residues that are directly involved in the binding and specificity of each particular antibody for its specific
10 antigenic determinant. Hypervariability, both in the light chain and the heavy chain variable domains, is concentrated in three segments known as complementarity determining regions (CDRs) or hypervariable loops (HVLs). CDRs are defined by sequence comparison in Kabat et al., 1991, In: Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., whereas HVLs are structurally defined
15 according to the three-dimensional structure of the variable domain, as described by Chothia and Lesk, 1987, J. Mol. Biol. 196: 901-917. Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred. As defined by Kabat, CDR-L1 is positioned at about residues 24-34, CDR-L2, at about residues 50-56, and CDR-L3, at about residues 89-97 in the light chain variable domain; CDR-H1 is positioned at about
20 residues 31-35, CDR-H2 at about residues 50-65, and CDR-H3 at about residues 95-102 in the heavy chain variable domain. The CDR1, CDR2, CDR3 of the heavy and light chains therefore define the unique and functional properties specific for a given antibody.

The three CDRs within each of the heavy and light chains are separated by framework regions (FR), which contain sequences that tend to be less variable. From the amino terminus
25 to the carboxy terminus of the heavy and light chain variable domains, the FRs and CDRs are arranged in the order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. The largely β -sheet configuration of the FRs brings the CDRs within each of the chains into close proximity to each other as well as to the CDRs from the other chain. The resulting conformation contributes to the antigen binding site (see Kabat et al., 1991, NIH Publ. No. 91-3242, Vol. I,
30 pages 647-669), although not all CDR residues are necessarily directly involved in antigen binding.

The terms, "antibody", is used herein in the broadest sense and specifically encompass monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments such as variable

domains and other portions of antibodies that exhibit a desired biological activity, *e.g.*, WNV binding and or neutralizing.

The term "monoclonal antibody" (MAb) refers to an antibody of a population of substantially homogeneous antibodies; that is, the individual antibodies in that population are identical except for naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic determinant, an "epitope". Therefore, the modifier "monoclonal" is indicative of a substantially homogeneous population of antibodies directed to the identical epitope and is not to be construed as requiring production of the antibody by any particular method. It should be understood that monoclonal antibodies can be made by any technique or methodology known in the art; including *e.g.*, the hybridoma method (Kohler et al., 1975, Nature 256:495), or recombinant DNA methods known in the art (see, *e.g.*, U.S. Pat. No. 4,816,567), or methods of isolation of monoclonal recombinantly produced using phage antibody libraries, using techniques described in Clackson et al., 1991, Nature 352: 624-628, and Marks et al., 1991, J. Mol. Biol. 222: 581-597.

Chimeric antibodies consist of the heavy and light chain variable regions of an antibody from one species (*e.g.*, a non-human mammal such as a mouse) and the heavy and light chain constant regions of another species (*e.g.*, human) antibody and can be obtained by linking the DNA sequences encoding the variable regions of the antibody from the first species (*e.g.*, mouse) to the DNA sequences for the constant regions of the antibody from the second (*e.g.*, human) species and transforming a host with an expression vector containing the linked sequences to allow it to produce a chimeric antibody. Alternatively, the chimeric antibody also could be one in which one or more regions or domains of the heavy and/or light chain is identical with, homologous to, or a variant of the corresponding sequence in a monoclonal antibody from another immunoglobulin class or isotype, or from a consensus sequence. Chimeric antibodies can include fragments of such antibodies, provided that the antibody fragment exhibits the desired biological activity of its parent antibody, for example binding to the same epitope (see, *e.g.*, U.S. Pat. No. 4,816,567; and Morrison et al., 1984, Proc. Natl. Acad. Sci. USA 81: 6851-6855).

The methods of the invention can also be used to prepare antibody fragments. The term "antibody fragment" refers to a portion of a full length antibody, in which a variable region or a functional capability is retained, for example, specific West Nile Virus epitope binding. Examples of antibody fragments include, but are not limited to, a Fab, Fab', F(ab')₂, Fd, Fv, scFv and scFv-Fc fragment, a diabody, a linear antibody, a single-chain antibody, a

minibody, a diabody formed from antibody fragments, and multispecific antibodies formed from antibody fragments.

Full length antibodies can be treated with enzymes such as papain or pepsin to generate useful antibody fragments. Papain digestion is used to produce two identical antigen-binding antibody fragments called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment. The Fab fragment also contains the constant domain of the light chain and the CH1 domain of the heavy chain. Pepsin treatment yields a F(ab')₂ fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

Fab' fragments differ from Fab fragments by the presence of additional residues including one or more cysteines from the antibody hinge region at the C-terminus of the CH1 domain. F(ab')₂ antibody fragments are pairs of Fab' fragments linked by cysteine residues in the hinge region. Other chemical couplings of antibody fragments are also known.

"Fv" fragment contains a complete antigen-recognition and binding site consisting of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. In this configuration, the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody.

A "single-chain Fv" or "scFv" antibody fragment is a single chain Fv variant comprising the VH and VL domains of an antibody where the domains are present in a single polypeptide chain. The single chain Fv is capable of recognizing and binding antigen. The scFv polypeptide may optionally also contain a polypeptide linker positioned between the VH and VL domains in order to facilitate formation of a desired three-dimensional structure for antigen binding by the scFv (see, *e.g.*, Pluckthun, 1994, In *The Pharmacology of monoclonal Antibodies*, Vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315).

Other recognized antibody fragments include those that comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) to form a pair of antigen binding regions. These "linear antibodies" can be bispecific or monospecific as described in, for example, Zapata et al. 1995, *Protein Eng.* 8(10):1057-1062.

Any of the above antibody fragments or variants can be produced by the methods described herein and isolated for use as therapeutic and or prophylactic medicaments. As such, the antibody compositions of the invention may advantageously be prepared as pharmaceutical formulations in suitable pharmaceutical excipients or delivery vehicles. The antibody formulations will be prepared by at least one purification step in which the

recombinant cellular material is removed. The methods described below are scaleable for the production of large quantities of the huE16 antibody for therapeutic and or prophylactic uses against WNV infection.

5

EXAMPLE

Generation and analysis of novel plant-derived antibody-based therapeutic molecules against West Nile virus and Dengue virus

Previously, a plant-derived monoclonal antibody (MAb) (pHu-E16) was engineered that efficiently treated West Nile virus (WNV) infection in mice. In this study, a plant-
 10 derived MAb against dengue virus (DENV, pE60) was engineered. Several pHu-E16 and pE60 variants were also developed to improve its efficacy. These variants included a single-chain variable fragment (scFv) of pHu-E16 fused to the heavy chain (HC) constant domains (C_H^{1-3}) of human IgG (pHu-E16scFv- C_H^{1-3}) and a tetravalent molecule (Tetra pHu-E16) assembled from pHu-E16scFv- C_H^{1-3} with a second pHu-E16scFv fused to the light chain
 15 (LC) constant region. pE60, pHu-E16scFv- C_H^{1-3} and Tetra pHu-E16 were efficiently expressed and assembled in Wild-type (WT) and glycoengineered plants. To assess the impact of differences in N-linked glycosylation on pHu-E16 variant assembly and function, additional pHu-E16 variants were expressed with various combinations of HC and LC components. The study revealed that proper pairing of HC and LC was essential for the
 20 complete N-glycan processing of antibodies in both plant and animal cells. Associated with their distinct N-glycoforms, pHu-E16, pHu-E16scFv- C_H^{1-3} and Tetra pHu-E16 exhibited differential binding to C1q and specific Fc receptors (FcγR). Notably, none of the plant-derived Hu-E16 variants showed antibody-dependent enhancement (ADE) activity in CD32A⁺ cells, suggesting the potential of plant-produced antibodies to minimize the adverse
 25 effect of ADE. Importantly, all plant-derived MAb variants exhibited at least equivalent *in vitro* neutralization and *in vivo* protection in mice compared to mammalian cell-produced Hu-E16 or E60. This study demonstrates the capacity of plants to express and assemble a large, complex and functional IgG-like tetravalent mAb variant and also provides insight into the relationship between MAb N-glycosylation, FcγR and C1q binding, and ADE. These new
 30 insights may allow the development of safer and cost effective MAb-based therapeutics for flaviviruses.

West Nile virus (WNV) is a neurotropic virus that infects the central nervous system (CNS) of human and animals. Historically, WNV was an Old World disease found mostly in the Eastern Europe, Africa, and the Middle East. However, in 1999, WNV entered the

Western hemisphere and subsequently spread across the United States (US), Canada, the Caribbean region and Latin America (Petersen, et al. (2013). "West Nile virus: review of the literature." *Jama* **310**(3): 308-315). In the US, the frequency and severity of WNV outbreaks have increased significantly in recent years with 2012 as the deadliest (286 fatalities) on record. Elderly and immunocompromised humans are the most vulnerable for developing severe neurological disease, long-term morbidity, and death (Bode, A. V., J. J. Sejvar, et al. (2006). "West Nile virus disease: a descriptive study of 228 patients hospitalized in a 4-county region of Colorado in 2003." *Clinical Infectious Diseases* **42**(9): 1234-40), although genetic factors also are associated with an increased risk of disease. Currently, there is no vaccine or therapeutic approved for human use. The global threat of WNV epidemics and the lack of treatment warrant the development of antiviral therapeutics and production platforms that can expeditiously bring products to market at low cost. A plant-derived, humanized murine MAb (pHu-E16) that binds to an epitope on domain III (DIII) of WNV envelope (E) protein was previously reported, as a post-exposure therapeutic candidate for WNV (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 2419-2424). It was demonstrated that pHu-E16 was produced at high levels and assembled efficiently in both *Nicotiana benthamiana* and lettuce plants (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 2419-2424; Lai H, He J, Engle M, Diamond MS, Chen Q (2012) Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. *Plant Biotechnology Journal* **10**: 95-104). pHu-E16 also retained antigen binding specificity and affinity, and neutralized WNV infection equivalently relative to the mammalian cell-produced Hu-E16 (mHu-E16). Furthermore, pHu-E16 also protected mice from WNV infection and mortality equivalently compared to mHu-E16 in both pre- and post-exposure treatment models. Because WNV is a neurotropic virus, peripheral delivery of pHu-E16, however, likely will have a limited window of efficacy due to its inability to efficiently cross the blood brain barrier (BBB) and accumulate in the brain at concentrations sufficient for neutralization. Thus, it would be desirable to develop pHu-E16 variants, such as bifunctional MAbs, that can cross the BBB while retaining targeted therapeutic activity.

To test the ability of plants in producing such complex MAb variants, several pHu-E16 derivatives were expressed including a pHu-E16scFv fused to the heavy chain (HC)

constant domains (C_H^{1-3}) of human IgG (pHu-E16scFv- C_H^{1-3}) and a large tetravalent molecule (Tetra pHu-E16) assembled from pHu-E16scFv- C_H^{1-3} with another pHu-E16scFv fused to the light chain (LC) constant region (C_L) (pHu-E16scFv- C_L). It was demonstrated that plants can express and assemble these pHu-E16 variants efficiently. These pHu-E16 variants also showed at least equivalent protection as the parent pHu-E16 or mHu-E16 against a lethal WNV challenge in a mouse model. The results also revealed differences in N-linked glycosylation pattern between different MAb variants, and demonstrated that the proper pairing of HC and LC was essential for the complete N-glycan processing of antibodies in both plant and animal cells. It was also found that pHu-E16, pHu-E16scFv- C_H^{1-3} and Tetra pHu-E16 exhibited differential binding to specific Fc receptors (FcγRs) and C1q, the complement opsonin that activates the classical complement pathway C3 convertase. Furthermore, in K562 cells, none of the plant-derived Hu-E16 variants showed significant antibody-dependent enhancement (ADE) activity, a phenomenon that is a major impediment for developing MAb-based therapeutics against flaviviruses, such as the related Dengue virus. Overall, this study demonstrates the capacity of a plant to express and assemble efficiently a large and complex IgG-like tetravalent MAb biologic, which offers a step forward toward the development of bifunctional MAbs in plants. This study also contributes to the understanding of the basic biological process of antibody glycosylation, and the relationship between MAb N-linked glycosylation, FcγR and C1q binding, and ADE.

RESULTS

Expression and assembly of pHu-E16 scFv- C_H^{1-3} in *N. benthamiana*

An important prerequisite for the generation of bifunctional Hu-E16 MAb is the correct assembly of its functional component: pHu-E16scFv- C_H^{1-3} . The DNA sequence of the variable region of HC (V_H) and LC (V_L) of pHu-E16 (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424) was fused together first to generate pHu-E16scFv, and then fused to the coding sequence of the C_H^{1-3} of human IgG (**Fig 2A**). The resulting coding sequence of pHu-E16scFv- C_H^{1-3} was cloned into a MagnICON-based plant expression vector (Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, et al. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfecting with noncompeting viral vectors. Proceedings of the National Academy of Sciences of the United States of America 103: 14701-14706), and transformed into *Agrobacterium tumefaciens*. *A. tumefaciens* strains containing the pHu-E16scFv- C_H^{1-3} construct were co-delivered into *N. benthamiana* leaves

along with the promoter module and an integrase construct through agroinfiltration (Leuzinger K, Dent M, Hurtado J, Stahnke J, Lai H, et al. (2013) Efficient Agroinfiltration of Plants for High-level Transient Expression of Recombinant Proteins. *Journal of Visualized Experiments*: doi:10.3791/50521; Chen Q, Lai H, Hurtado J, Stahnke J, Leuzinger K, et al. (2013) Agroinfiltration as an Effective and Scalable Strategy of Gene Delivery for Production of Pharmaceutical Proteins. *Adv Tech Biol Med* 1: 9). Western blot analysis after reducing or non-reducing gel electrophoresis confirmed that pHu-E16scFv-CH¹⁻³ was produced in leaves with the expected molecular weight (**Fig 2B, Lane 5**), and that it assembled into a dimer (**Fig 2D, Lane 7**). ELISA results also indicated that pHu-E16scFv-CH¹⁻³ reached the highest level of accumulation 9 days post infiltration (dpi) with *A. tumefaciens*, with an average accumulation of 0.8 mg/g leaf fresh weight (LFW) (**Fig 3**). This level is similar that of the parent pHu-E16 MAb was reported previously, which is among the highest expression levels for MAbs in plants ever reported (De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. *Plant Biotechnology Journal* 8: 529-563; Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2419-2424; Bendandi M, Marillonnet S, Kandzia R, Thieme F, Nickstadt A, et al. (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. *Annals of Oncology* 21: 2420-2427). Similar expression pattern and level was observed when pHu-E16scFv-CH¹⁻³ was expressed in a glycoengineered Δ XF N. benthamiana line which decorates proteins with mammalian type GnGn glycans (He, et al. (2014). "Generation and Analysis of Novel Plant-Derived Antibody-Based Therapeutic Molecules against West Nile Virus." *PLoS ONE* 9(3): e93541 DOI:10.1371/journal.pone.0093541; Lai, et al. (2014). "Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants." *Plant Biotechnology Journal* 12(8): 1098-1107.

E60 mAb expression and assembly in N. benthamiana

Gel electrophoresis (SDS-PAGE) and western blot were used to determine the ability of WT and Δ XF N. benthamiana plants in expressing and assembling E60 mAb in their cells. Δ XF is a N. benthamiana N-glycosylation mutant that produces glycoproteins with the mammalian-type GnGn glycan structure due to the inactivation of enzymes for plant-specific xylose and core fucose residues (Strasser et al. 2008). Western blot analysis of reduced

samples indicated that E60 was expressed in WT plants with the expected molecular weight of HC (50 kDa) and LC (25 kDa) (**Fig. 9**). Results from non-reduced samples confirmed that HC and LC had assembled into the expected tetrameric full antibody (**Fig. 9**). Similar to the positive control mAb, the assembled E60 was detected as a band larger than the calculated size (150 kDa), suggesting its possible glycosylation. The expression and assembly of E60 in Δ XF plants have also been demonstrated with equivalent results (data not shown). The temporal expression pattern of E60 in plants was monitored by ELISA, indicating that the expression pattern of E60 is similar to what was observed for several mAb variants against WNV in *N. benthamiana* (He, et al. (2014). "Generation and Analysis of Novel Plant-Derived Antibody-Based Therapeutic Molecules against West Nile Virus." *PLoS ONE* 9(3): e93541 DOI:93510.91371/journal.pone.0093541; Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2419-2424; Lai, et al. (2014). "Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants." *Plant Biotechnology Journal* 12(8): 1098-1107).

Plant-derived pHu-E16 exhibited a highly homogeneous glycosylation pattern

pHu-E16 was expressed in both WT and Δ XF *N. benthamiana* plant line. N-glycan profile analysis revealed that 90% of pHu-E16 produced in WT plants exhibited the expected GnGnXF glycoform (**Figure 16**). pHu-E16 produced in Δ XF *N. benthamiana* plant line exhibited the expected mammalian-type complex N-glycan GnGn with a high degree (~ 95%) of uniformity (**Fig 13A**). Furthermore, no plant-specific glycan residues (i.e. β 1,2-xylose and core α 1,3-fucose) were detected in either Δ XF-expressed pHu-E16. Homogeneous glycans in plant-produced pHu-E16 should enhance the identification of glycoforms that reduced the risk of ADE while retaining the full therapeutic activity. In contrast, E16 produced in mammalian cells (mE16) displayed a mixture of several glycoforms (**Fig 13B and Fig 16**).

pHu-E16 scFv- C_H^{1-3} exhibited altered N-linked glycosylation pattern

A typical feature of IgG antibodies is a conserved N-glycosylation site at position Asn²⁹⁷ in the C_H^2 domain and it is well documented that the pattern and extent of N-linked glycosylation affects the stability and function of an antibody (Houde D, Peng Y, Berkowitz SA, Engen JR (2010) Post-translational modifications differentially affect IgG1 conformation and receptor binding. *Mol Cell Proteomics* 9: 1716-1728). Accordingly, the N-glycoforms of pHu-E16scFv- C_H^{1-3} were next examined by liquid-chromatography-electrospray ionization-

mass spectrometry (LC-ESI-MS) to assess whether the extent N-linked glycosylation was similar to the parent pHu-E16. pHu-E16 exhibited the expected complex-type N-glycans (GnGnXF, **Fig 16**), typical for plant-produced IgGs (Loos A, Steinkellner H (2012) IgG-Fc glycoengineering in non-mammalian expression hosts. Arch Biochem Biophys 526: 167-173). However, pHu-E16scFv-C_H¹⁻³ displayed oligomannosidic structures, predominantly Man8 and Man9 (**Fig 16**). The predominantly endoplasmic reticulum (ER)-type N-glycans of pHu-E16scFv-C_H¹⁻³ were unexpected, as this molecule was targeted for secretion and not tagged with an ER-retention signal KDEL (SEQ ID NO:8). To investigate if this is a phenomenon specific to plant cells, the N-glycan profiles of equivalent Hu-E16 variants produced in mammalian cells (mHu-E16 and mHu-E16scFv-C_H¹⁻³) were also compared. A substantial amount of oligomannosidic glycans (oligoman, mostly M5 and M7) in mHu-E16scFv-C_H¹⁻³ were observed, in addition to the predominant complex-type glycans (GnGnF⁶ and AGnF⁶), whereas no such glycans were observed in mHu-E16 (**Fig 16**). This result suggests that the unusual N-linked glycosylation pattern of scFv-C_H¹⁻³ is not unique to plants but also is shared by mammalian cells, even though the percentage and the particular species of oligomannosidic glycans differ between cells from the two species.

Expression and N-linked glycosylation of a tetravalent pHu-E16 and other pHu-E16 variants

The success of pHu-E16 scFv-C_H¹⁻³ expression and assembly in plants indicates the feasibility of developing an IgG-like tetravalent MAb variant. Such novel form of anti-WNV therapeutics either can provide four WNV antigen-binding sites or function as a bifunctional MABs that offer divalent binding to two different antigens. For example, a bifunctional MABs could be engineered to bind a BBB receptor as its second antigen to enhance its transport into the brain, extending the window of treatment for WNV infection. Thus, to understand the basis of the difference in N-linked glycans between pHu-E16scFv-C_H¹⁻³ and its parent pHu-E16 and develop a potentially more efficacious WNV therapeutic, three additional pHu-E16 variants with various combinations of HC and LC components were engineered and expressed. First, pHu-E16scFv was fused to the plant-codon optimized C_L (pHu-E16scFv-C_L). Three pHu-E16 variants were then produced in *N. benthamiana* plants by the co-expression of the following Hu-E16 construct combinations: scFv-C_H¹⁻³/LC, HC/scFv-C_L, and scFv-C_H¹⁻³/scFv-C_L (**Fig 2A**). Western blot analysis after reducing gel electrophoresis confirmed that each component of the variants was produced in plants with the expected molecular weight (**Fig 2B and C, Lanes 2 - 4**). These protein bands are specific to the infiltrated molecular constructs, as they were not detected in samples from negative control plant leaves (**Fig 2B**

and C, Lane 6). Moreover, western blotting after non-reducing gel electrophoresis reveals that the two polypeptides in each combination assembled into an IgG-like heterotetramer (**Fig 2D, Lanes 2 – 4 and Fig 2A**). The successful expression and assembly of pHu-E16scFv-C_H¹⁻³ with pHu-E16scFv-C_L to form a tetravalent molecule (Tetra pHu-E16, **Fig 2B-D, Lane 3, and Fig 1A**) in plants is noteworthy, as the assembly of such large and complex MAb variants has not been reported previously. Yield analysis by ELISA established that Tetra pHu-E16 and other variants reached the highest level of production at 9 dpi. Nonetheless, the expression level varied between the three pHu-E16 variants, with HC/scFv-C_L's comparable to that of the parent pHu-E16 and pHu-E16scFv-C_H¹⁻³ (0.69 mg/g LFW), scFv-CH¹⁻³/LC's at 0.43 mg/g LFW, and Tetra pHu-E16 at 0.24 mg/g LFW (**Fig 3**).

In addition to developing a tetravalent pHu-E16 as a possible novel WNV therapeutic molecule, the expression of the three pHu-E16 variants also allowed the cause of the unexpected N-linked glycosylation of pHu-E16scFv-C_H¹⁻³ to be studied, specifically to address whether LC or the proper pairing of HC and LC affected N-linked glycosylation of MAb and its variants. Co-expression of LC of pHu-E16 significantly increased the percentage of complex-type glycans in pHu-E16scFv-C_H¹⁻³ (**Fig 16**). In Tetra pHu-E16, the co-expression and pairing of scFv-C_H¹⁻³/scFv-C_L also increased the amount of complex-type N-glycans (**Fig 16**), albeit not to the same level as in the scFv-C_H¹⁻³/LC combination. In contrast, pairing of the HC of pHu-E16 with scFv-C_L yielded a predominantly complex-type N-linked glycosylation pattern, similar to that of the parent pHu-E16 (**Fig 16**). These results indicated the importance of LC and its pairing with HC for the complete processing of N-linked glycans on antibodies.

Purification of pHu-E16 and pE60 variants from *N. benthamiana* leaves

For Tetra pHu-E16 and other variants and pE60 to be feasible therapeutic candidates for WNV and DENV, respectively, an efficient extraction and purification process must be developed and validated. A scalable extraction and purification process previously developed for pHu-E16 was applied to the purification of these variants (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2419-2424). Analysis of processing samples with gel electrophoresis indicate that in spite of their structural complexity, Tetra pHu-E16, pHu-E16scFv-C_H¹⁻³, pE60 and other variants were readily extracted from plant tissue and enriched to >90% purity by a combination of ammonium sulfate precipitation and protein A

chromatography (**Fig 4 and Fig 10**). These purified pHu-E16 variants were used for the further functional characterization.

pHu-E16 variants show differential binding to human Fcγ receptors and C1q and reduced antibody-dependent enhancement activity in K562 cells

5 N-linked glycosylation in the Fc region of an antibody affects binding to FcγRs and C1q (Houde D, Peng Y, Berkowitz SA, Engen JR (2010) Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics 9: 1716-1728). Accordingly, the binding of pHu-E16scFv-CH¹⁻³ and Tetra pHu-E16 to C1q and different human FcγRs, including CD16A-V¹⁵⁸, CD16A-F¹⁵⁸, CD32A-R¹³¹, CD32A-H¹³¹,
10 CD32B, and CD64, was investigated by surface plasmon resonance (SPR) analysis using pHu-E16 and mHu-E16 as references. Compared to mHu-E16, pHu-E16 showed reduced binding to all FcγRs and C1q (**Fig 5A**). In comparison, pHu-E16scFv-CH¹⁻³ showed slightly enhanced binding to CD64 and the low-affinity (F¹⁵⁸) isoform of CD16A, maintained similar binding to the high-affinity (V¹⁵⁸) CD16A isoform, and had reduced binding to CD32A and B
15 (**Fig 5A**). Tetra pHu-E16 showed a similar binding profile of FcγRs and C1q as pHu-E16scFv-CH¹⁻³, except with reduced binding to CD64 and the high affinity isoform (V¹⁵⁸) of CD16A (**Fig 5A**).

The interaction between Fc moieties and FcγRs or C1q also regulates antibody-mediated enhancement of viral infections (ADE), a phenomenon that is relevant to disease
20 pathogenesis of some flaviviruses (Morens DM (1994) Antibody-dependent of enhancement of infection and the pathogenesis of viral disease. Clin Inf Dis 19: 500-512; Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM (2010) Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. Lancet Infect Dis 10: 712-722; Mehlhop E, Ansarah-Sobrinho C, Johnson S, Engle M, Fremont DH, et al. (2007) Complement protein C1q inhibits antibody-dependent
25 enhancement of flavivirus infection in an IgG subclass-specific manner. Cell Host Microbe 2: 417-426). As such, it was investigated if plant-produced Hu-E16 variants would have a unique ADE profile *in vitro* compared to that of mHu-E16. Notably, all pHu-E16 variants lost their ADE activity in human K562 cells, which predominantly express CD32A, indicating the
30 potential of plants to produce antibodies or antibody-like molecules that minimize ADE (**Fig 5B**).

Antigen binding and neutralization activity of pHu-E16 variants and pE60

The binding kinetics and function of pHu-E16 variants was also assessed in several assays. The binding of pHu-E16 variants to WNV E DIII was determined by ELISA in which

DIII was coated on the plate (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424). E16scFv-C_H¹⁻³, Tetra pHu-E16 and other pHu-E16 variants bound in a similar manner to DIII as pHu-E16 (**Fig 6A**). Recognition of pHu-E16 variants for DIII was corroborated in a binding assay with yeast that displayed DIII on their surface. Flow cytometric analysis showed that the percentage of positive yeast and the mean fluorescence intensity of binding by saturating concentrations of pHu-E16scFv-C_H¹⁻³ and mHu-E16 were similar (**Fig 6B**). Similar results were obtained for Tetra pHu-E16 and other variants (data not shown). For the DENV therapeutic candidate of pE60, flow cytometric analysis demonstrated that plant-derived pE60 exhibited similar antigen (DENV DI-II) binding specificity as its mammalian-produced counterpart (**Fig 11**). The binding of these variants to WNV E DIII was quantitated by a SPR assay; as expected, E16scFv-C_H¹⁻³ and Tetra pHu-E16 had similar binding affinity and kinetics for WNV E protein and DIII compared to its parent mHu-E16 with K_D = 10 to 25 nM. These results confirm that E16scFv-C_H¹⁻³ and Tetra pHu-E16 retain antigen binding activity and specificity compared to pHu-E16 and mHu-E16.

To evaluate the neutralization potential of pHu-E16 variants, a focus reduction neutralization assay that measures antibody inhibition of WNV or DENV infection was used (Lai H, He J, Engle M, Diamond MS, Chen Q (2012) Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. Plant Biotechnology Journal 10: 95-104; Fuchs A, Pinto AK, Schwaebler WJ, Diamond MS (2011) The lectin pathway of complement activation contributes to protection from West Nile virus infection. Virology 412: 101-109). pHu-E16scFv-C_H¹⁻³ and Tetra pHu-E16 neutralized WNV infection comparably to mHu-E16 and pHu-E16 (**Fig 7**, EC₅₀: mHu-E16 = 3.6 ng/ml, pHu-E16 = 3.7 ng/ml, pHu-E16scFv-C_H¹⁻³ = 5.2 ng/ml, Tetra pHu-E16 = 3.0 ng/ml, respectively). Thus, E16scFv-C_H¹⁻³ and Tetra pHu-E16 retained potent neutralizing activity against infectious WNV. Furthermore, along with pHu-E16 produced in ΔXF N. benthamiana plants, E16scFv-C_H¹⁻³ produced in ΔXF plants also retained its WNV neutralizing activity (**Fig 14**). For anti-DENV MAb E60, a potent neutralizing activity was observed for both WT and ΔXF N. benthamiana plant-produced E60 (**Fig 12**)

pHu-E16 scFv-C_H¹⁻³ and Tetra pHu-E16 have potent prophylactic and therapeutic potential

Prophylaxis and therapeutic studies were performed to evaluate the activity of pHu-E16 variants *in vivo*. Pre-treatment studies were performed in 5 week-old wild type

C57BL/6 mice ($n \geq 10$, per group) to compare the concentrations of pHu-E16scFv- C_H^{1-3} and mHu-E16 that prevent WNV infection. Mice were inoculated with 10^2 PFU of WNV (New York 2000 strain), which causes a baseline mortality of 80 to 90% in this model (Engle M, Diamond MS (2003) Antibody prophylaxis and therapy against West Nile Virus infection in wild type and immunodeficient mice. J Virol 77: 12941-12949). Increasing amounts (1 to 100 ng) of pHu-E16scFv- C_H^{1-3} or 100 ng of mHu-E16 were administered as a single dose on the day of infection. Results showed that 80% of mice were protected from lethal infection when 100 ng of pHu-E16scFv- C_H^{1-3} was administered ($P < 0.05$), whereas the same dosage of mHu-E16 protected 60% of mice (**Fig 8A**). A single injection of as low as 10 ng of pHu-E16scFv- C_H^{1-3} also prevented mortality (**Fig 8A**).

Post-exposure therapeutic treatments were performed by passively administering a single dose (50 or 500 μ g) of pHu-E16scFv- C_H^{1-3} or Tetra pHu-E16 after subcutaneous inoculation of 10^2 PFU of WNV. Since WNV spreads to the brain in mice by day 4 after infection, the efficacy of pHu-E16 variants was investigated at this time point (**Fig 8B**).

Notably, 80% of mice were protected from lethal infection when 500 μ g of pHu-E16scFv- C_H^{1-3} was given 4 days after WNV inoculation, similar to that observed with mHu-E16 performed in parallel (**Fig 8B**, $P > 0.9$). A single administration of 50 μ g of Tetra pHu-E16 protected up to 90% of mice from lethal infection, compared with an 80% survival rate achieved by mHu-E16 (**Fig 8B**).

Similarly, the prophylactic and therapeutic efficacy of glycoengineered plant (Δ XF)-derived pHu-E16 scFv- C_H^{1-3} and pHu-E16 was also demonstrated in lethal WNV challenge studies in mice (**Fig 15**). Overall, pHu-E16, pHu-E16 scFv- C_H^{1-3} and Tetra pHu-E16 had at least equivalent prophylactic and therapeutic efficacy in mice compared to mHu-E16 regardless of whether they were produced in WT or glycoengineered plant lines.

DISCUSSION

MAb expression was first attempted in stable transgenic plants (Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342: 76-78). It suffered from low yield, long lead time to generate and select transgenic plants, and unstable seed banks largely due to the randomness of transgene integration in the plant genome (position effect), the lack of control in transgene copy number, and the shortage of strong promoters to drive transgene expression (De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. Plant Biotechnology Journal 8: 529-563). This greatly undermined the cost-saving potential of the plant expression systems. The development of transient expression systems based on deconstructed virus vectors has

increased the speed and yield of MAb production in plants (Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, et al. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfecting with noncompeting viral vectors. *Proceedings of the National Academy of Sciences of the United States of America* 103: 14701-14706; Chen Q, He J, Phoolcharoen W, Mason HS (2011) Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Human Vaccines* 7: 331-338; Lico C, Chen Q, Santi L (2008) Viral vectors for production of recombinant proteins in plants. *Journal of cellular physiology* 216: 366-377; Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H (2009) A DNA replicon system for rapid high-level production of virus-like particles in plants. *Biotechnology and Bioengineering* 103: 706-714). For example, with the MagnICON and geminiviral vectors, MAbs can be obtained within 10 days of vector infiltration into *N. benthamiana* and lettuce plants with yields up to 1mg MAb/g LFW (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2419-2424; Lai H, He J, Engle M, Diamond MS, Chen Q (2012) Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. *Plant Biotechnology Journal* 10: 95-104; Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, et al. (2010) High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. *Biotechnology and bioengineering* 106: 9-17; He J, Lai H, Brock C, Chen Q (2012) A Novel System for Rapid and Cost-Effective Production of Detection and Diagnostic Reagents of West Nile Virus in Plants. *Journal of Biomedicine and Biotechnology* 2012: 1-10; Zeitlin L, Pettitt J, Scully C, Bohorova N, Kim D, et al. (2011) Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. *Proceedings of the National Academy of Sciences* 108: 20690-20694). While MAb derivatives, such as scFv, diabodies, and RIC have been produced in plants (De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. *Plant Biotechnology Journal* 8: 529-563; Phoolcharoen W, Bhoo SH, Lai H, Ma J, Arntzen CJ, et al. (2011) Expression of an immunogenic Ebola immune complex in *Nicotiana benthamiana*. *Plant Biotechnology Journal* 9: 807-816; Phoolcharoen W, Dye JM, Kilbourne J, Piensook K, Pratt WD, et al. (2011) A nonreplicating subunit vaccine protects mice against lethal Ebola virus challenge. *Proceedings of the National Academy of Sciences* 108: 20695-20700), the ability of plants to express and assemble larger or more complex MAb-derived molecules such as IgG-like tetravalent MAbs or bifunctional MAbs has not been reported.

Here, the feasibility of plant cells in producing such an IgG-like tetravalent MAb against WNV based on MAb Hu-E16 was investigated. The results showed that two Hu-E16scFvs each genetically fused to the C_H¹⁻³ and C_L fragment of IgG can be produced efficiently in both WT and glycoengineered *N. benthamiana* leaves. The Hu-E16scFv-C_H¹⁻³ and Hu-E16scFv-C_L efficiently assembled into an IgG-like molecule. The study also demonstrated that the tetravalent pHu-E16 can be recovered efficiently to high (>90%) purity, and retain antigen-binding and neutralization activity *in vitro*. Most importantly, a single dose of Tetra-pHu-E16 protected mice at least equivalently as mHu-E16 against a lethal WNV infection several days after exposure. To our knowledge, this is the first example in a plant of expression and assembly of a large and complex IgG-like tetravalent functional molecule. Tetra pHu-E16 was expressed rapidly in *N. benthamiana* leaves and reached its highest level of accumulation 9 days after leaf infiltration. While its expression level is slightly lower than that of pHu-E16, this level is within the range feasible for commercial production (Chen Q (2013) Virus-like Particle Vaccines for Norovirus Gastroenteritis. In: Giese M, editor. Molecular Vaccines. Vienna: Springer, DOI:10.1007/978-3-7091-1419-3_8. pp. 153-181), and likely can be increased by further genetic optimization (De Muynck B, Navarre C, Boutry M (2010) Production of antibodies in plants: status after twenty years. Plant Biotechnology Journal 8: 529-563). The rapid accumulation and assembly of Tetra pHu-16 and the availability of a scalable and current Good Manufacturing Practice (cGMP) compliant purification scheme suggest the viability of such molecules as a platform for novel therapeutic candidate.

Another goal of this research is to further increase the efficacy of Hu-E16 MAb as a proof-of-principle for antibody treatment of viral infections in the CNS. Even though mHu-E16, pHu-E16, pHu-E16scFv-C_H¹⁻³, and Tetra pHu-E16 showed excellent efficacy, their window of clinical treatment will be limited via peripheral delivery systems (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424; Oliphant T, Engle M, Nybakken G, Doane C, Johnson S, et al. (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nature Medicine 11: 522-530). These current MAb-based therapeutic candidates cannot efficiently cross the BBB and accumulate to high levels in the brain unless there is widespread inflammation and breakdown of the BBB. Thus, it would be desirable to develop pHu-E16 variants that can cross the BBB more efficiently. One possible strategy is to develop IgG-like bifunctional MAbs that with a similar structure

as the Tetra pHu-E16, but assembled from two scFvs with distinct affinity and binding to two disparate antigens. For example, one of the two scFvs could bind to a specific receptor (e.g., insulin receptor on BBB to facilitate its transport into the brain, and the other scFv would retain its binding and neutralization against WNV in the brain. The successful production and assembly of Tetra pHu-E16 and the demonstration of its therapeutic activity here bring us closer to developing such Hu-E16 bifunctional MABs to prolong the window of its efficacy.

The N-linked glycosylation of MABs affects its stability, FcγR and C1q binding, pharmacokinetics, effector function and efficacy (Houde D, Peng Y, Berkowitz SA, Engen JR (2010) Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics 9: 1716-1728). N-linked glycosylation occurs as a series of post-translational modification steps initiated in the ER and processed through different compartments of the Golgi complex of the host cell. Altering the structure of a protein or its folding may affect glycan modification and the corresponding cellular localization (Loos A, Van Droogenbroeck B, Hillmer S, Grass J, Pabst M, et al. (2011) Expression of antibody fragments with a controlled N-glycosylation pattern and induction of endoplasmic reticulum-derived vesicles in seeds of Arabidopsis. Plant Physiol 155: 2036-2048; Feige MJ, Groscurth S, Marcinowski M, Shimizu Y, Kessler H, et al. (2009) An unfolded CH1 domain controls the assembly and secretion of IgG antibodies. Mol Cell 34: 569-579). However, the impact of structural variations in the native IgG on N-linked glycosylation has not been thoroughly studied. Here, the difference in the N-linked glycans of pHu-E16 IgG and its pHu-E16scFv-CH¹⁻³ variant was first examined. The results revealed that their N-linked glycan profiles were different from one another. pHu-E16 exhibited the expected complex-type N-linked glycans (Loos A, Steinkellner H (2012) IgG-Fc glycoengineering in non-mammalian expression hosts. Arch Biochem Biophys 526: 167-173), confirming it was fully processed through the endomembrane system and secreted into the apoplast as designed. In contrast, pHu-E16scFv-CH¹⁻³ predominantly displayed Man8 and Man9-types of oligomannosidic structures, which indicates incomplete glycan processing and suggests its aberrant localization in ER or ER-derived vesicles. A similar observation in glycoforms and aberrant subcellular deposition was reported for Arabidopsis (*Arabidopsis thaliana*) seed-derived 2G12scFV-Fc, a MAb variant against Human immunodeficiency virus (HIV), and to a lesser extent, for HA78scFv-Fc, an anti-hepatitis A virus MAb also produced in Arabidopsis seed (Loos A, Van Droogenbroeck B, Hillmer S, Grass J, Pabst M, et al. (2011) Expression of antibody fragments with a controlled N-glycosylation pattern and induction of endoplasmic reticulum-derived vesicles in seeds of Arabidopsis. Plant Physiol

155: 2036-2048). However, in the same study, leaf-derived HA78scFv-Fc displayed the expected complex-type N-glycans, whereas leaf-produced 2G12scFv-Fc exhibited the ER-typical oligomannosidic glycans and was functionally inactive (Loos A, Van Droogenbroeck B, Hillmer S, Grass J, Pabst M, et al. (2011) Expression of antibody fragments with a controlled N-glycosylation pattern and induction of endoplasmic reticulum-derived vesicles in seeds of Arabidopsis. *Plant Physiol* 155: 2036-2048). These observations suggest that beside the polypeptide component of scFv-Fcs, the type of plant tissue (e.g., seeds vs. leaves) and plant species may also impact the N-linked glycosylation status of scFv-Fc variants. It should be noted that the structure of 2G12scFv-Fc and HA78scFv-Fc is different from that of pHu-E16scFv- C_H^{1-3} , as their scFvs were fused to the Fc fragment (hinge- C_H^{2-3}) of IgG, thus, lacking the HC constant region 1 domain (C_H^1). To investigate if this were a phenomenon unique to plant cells, the N-linked glycan profiles of the equivalent Hu-E16 counterpart variants produced in mammalian cells were compared. The results indicated that in addition to the predominant complex-type glycans (GnGnF⁶ and AGnF⁶), mHu-E16scFv- C_H^{1-3} also had a substantial amount of oligomannosidic glycans, mostly M5 and M7, whereas no such glycans were observed in the native IgG version of mHu-E16. This result suggests that at least for Hu-E16, the unusual N-linked glycosylation of scFv- C_H^{1-3} is not unique to plants but shared by mammalian cells, even though the percentage and the particular species of oligomannosidic glycans varies between the two cell types.

To provide further insight for this N-glycosylation difference, three additional pHu-E16 variants were expressed with various combinations of HC and LC components. Examining whether LC or the proper pairing of HC and LC regulated the proper N-linked glycosylation of MAbs and its variants was of particular interest. Co-expression of LC with pHu-E16scFv- C_H^{1-3} significantly increased its level of complex N-linked glycans percentage. In Tetra pHu-E16, the co-expression and pairing of scFv- C_L with scFv- C_H^{1-3} also increased the amount of complex-type N-linked glycans. Furthermore, the pairing of the HC of pHu-E16 with scFv- C_L resulted in a predominantly complex-type N-linked glycosylation pattern, similar to seen with the parent pHu-E16. These results support the hypothesis that C_L domain of an antibody is essential for its secretion in the cell (Feige, M. J., S. Groscurth, et al. (2009). "An unfolded CH1 domain controls the assembly and secretion of IgG antibodies." *Mol Cell* 34(5): 569-79). It was suggested previously that HC dimers are retained in the ER by the chaperone BiP and are released only upon binding with C_L to ensure its proper folding. The results corroborate this and highlight the importance of LC pairing with HC for the proper processing of antibody N-linked glycosylation. It was speculated that in the absence of LC

pHu-E16scFv-C_H¹⁻³ was retained in the ER by BiP, preventing its transport within the endomembrane system and resulting in the observed ER-typical oligomannosidic glycoform. In contrast, co-expression of LC or pHu-E16scFv-C_L enabled the C_L domain to release pHu-E16scFv-C_H¹⁻³ from BiP and to transport it to the Golgi compartments for further assembly,
 5 glycan processing and secretion. While the N-linked glycosylation of the scFv-C_H¹⁻³ and scFv-Fc variant may be regulated by many factors, the results suggest that the lack of C_L and its pairing with the C_H¹ contributes to the aberrant N-linked glycosylation and intracellular localization of pHu-E16scFv-C_H¹⁻³ and other previously reported scFv-Fcs, even though the degree of aberration may vary depending on the specific scFv-Fc/scFv-C_H¹⁻³ or the cell type
 10 in which it was produced.

In this study, wild-type (WT) *N. benthamiana* plants were first used for expressing the pHu-E16 variants. It was also demonstrated that pHu-E16 produced in WT plants have a highly homogeneous (90%) population of the expected glycoform, GnGnXF, in contrast of a more heterogeneous mixture of Hu-E16 produced in mammalian cells. While N-linked
 15 glycosylation of proteins in plants is similar to that of mammalian cells, WT plants add plant-specific β-1,2-xylose and core α-1,3-fucose residues to complex N-linked glycans and lack terminal β1,4-Gal and N-acetylneuraminic acid (Neu5Ac) residues (Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, et al. (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnology Journal* 8: 564-587; Bosch D, Castilho A, Loos A, Schots A, Steinkellner H (2013) N-Glycosylation of Plant-produced Recombinant Proteins. *Curr Pharm Des* 19: 5503-5512).
 20 These differences in protein glycosylation between WT plant and mammalian cells were one of the reservations of plant-expression platforms for human therapies, because they could produce improper glycoforms that alter efficacy or result in plant-glycan specific immune responses that can accelerate protein clearance or cause potential adverse effects through
 25 immune complex formation. To overcome this challenge, glycosylation pathway in *N. benthamiana* and several other plants has been “humanized” through glycoengineering, for example, by genetically suppressing or eliminating enzymes for the biosynthesis of plant-specific glycans and by introducing glycoenzymes from mammalian cells (Bosch D, Castilho A, Loos A, Schots A, Steinkellner H (2013) N-Glycosylation of Plant-produced Recombinant Proteins. *Curr Pharm Des* 19: 5503-5512). These efforts have led to the creation of a portfolio of “humanized” plant lines that produce MABs that lack plant-specific glycans and produce glycoforms that are essentially mammalian (Bosch D, Castilho A, Loos A, Schots A, Steinkellner H (2013) N-Glycosylation of Plant-produced Recombinant Proteins. *Curr Pharm*

Des 19: 5503-5512). For example, a double knockout (Δ XF) *N. benthamiana* plant line, which was created by RNAi to suppress the expression of α -1,3-fucosyltransferase and β -1,2-xylosyltransferase, was used for production of an anti-Ebola MAb 13F6 (Zeitlin L, Pettitt J, Scully C, Bohorova N, Kim D, et al. (2011) Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. Proceedings of the National Academy of Sciences 108: 20690-20694; Strasser R, Stadlmann J, Schahs M, Stiegler G, Quendler H, et al. (2008) Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. Plant Biotechnol J 6: 392-402). 13F6 produced in the Δ XF plant line had no plant-specific N-glycans and 90% of 13F6 had the predicted mammalian glycoform GnGn (Zeitlin L, Pettitt J, Scully C, Bohorova N, Kim D, et al. (2011) Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. Proceedings of the National Academy of Sciences 108: 20690-20694). In contrast, mammalian cell-produced 13F6 had a mixture of 3-5 glycoforms with the most dominant glycoform (G0) ranging from 35-53%.

The high glycan homogeneity of plant-made 13F6 and the lack of core fucose increased its protection of mice against a lethal Ebola virus challenge compared to the mammalian cell produced 13F6. pHu-E16, pHu-E16 scFv- C_H^{1-3} , Tetra-E16, and other variants were expressed in these “humanized” *N. benthamiana* lines to produce highly uniform mammalian glycoforms. Indeed, these pHu-E16 variants were robustly produced in the Δ XF *N. benthamiana* plant line, yielding MAbs with the expected mammalian glycoform of GnGn with a 95% degree of uniformity.

The N-linked glycosylation pattern in the Fc region of an antibody affects its binding to Fc γ Rs and C1q, and consequently alters its effector function such as antibody-dependent cell mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Houde D, Peng Y, Berkowitz SA, Engen JR (2010) Post-translational modifications differentially affect IgG1 conformation and receptor binding. Mol Cell Proteomics 9: 1716-1728). The highly homogenous and defined nature of N-glycans displayed by plant-derived MAbs provides the opportunity to develop MAb glycoforms that enhance the efficacy through modulating their effector function. For example, plant-produced 13F6 has a higher affinity to Fc γ RIII than its mammalian cell-produced counterpart, and provided better protection against Ebola virus challenge in mice presumably via enhanced ADCC activity. Here, it was found that due to their distinct N-glycoforms, pHu-E16, pHu-E16scFv- C_H^{1-3} , and Tetra pHu-E16 exhibited differential binding to C1q and various Fc γ Rs including CD16V¹⁵⁸, CD16F¹⁵⁸ CD32aR¹³¹ and CD32aH¹³¹, CD32B, and CD64. For example, while pHu-E16 showed

reduced binding to all FcγRs and C1q, pHu-E16scFv-C_H¹⁻³ demonstrated slightly enhanced binding to the low-affinity (CD16F¹⁵⁸) isoform of CD16A and CD64, and maintained similar binding to the high-affinity (CD16V¹⁵⁸) CD16A isoform as mHu-E16. This diversity of binding affinity to C1q and various FcγRs by an individual MAb variant molecule will allow investigation of the impact of glycan moieties on MAb effector function and the development of MAb variants that are best suited for its efficacy or safety depending on the particular clinical application.

In addition to ADCC and CDC, the interaction between Fc and FcγRs can have pathogenic consequences. For example, one of the major impediments towards developing antibody-based therapeutics for certain flavivirus infections (e.g., Dengue virus) is the at least theoretical risk of ADE, which may render anti-flavivirus MAb treated subjects more susceptible to infection. ADE occurs because sub-neutralizing concentrations of antibodies (including therapeutic MAbs) and the infecting flavivirus form complexes that bind to FcγR-bearing cells, resulting in increased virus uptake and replication (Morens DM (1994)

Antibody-dependent enhancement of infection and the pathogenesis of viral disease. Clin Inf Dis 19: 500-512; Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM (2010) Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. Lancet Infect Dis 10: 712-722; Mehlhop E, Ansarah-Sobrinho C, Johnson S, Engle M, Fremont DH, et al. (2007) Complement protein C1q inhibits antibody-dependent enhancement of flavivirus infection in an IgG subclass-specific manner. Cell Host Microbe 2: 417-426). Thus, whether the unique binding of pHu-E16 variants to FcγRs affected ADE was investigated. The results demonstrated that all pHu-E16 variants lost ADE activity on CD32A+ K562 cells. Thus, plant complex-type N-glycans or the mammalian form of oligomannosidic glycans carried by plant-produced pHu-E16 variants cannot induce the development of ADE on cells bearing CD32A, consistent with their loss of binding *in vitro* by SPR. In a separate experiment, it was observed that ADE also was eliminated for a glycoengineered plant-derived pHu-E16 that carried the mammalian glycoform of GnGn. These results collectively demonstrate the potential of plant-made MAbs and their variants to be used as therapeutics against ADE-prone viruses. While it was previously reported that an aglycosylated version of a MAb can eliminate the risk of ADE (Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, et al. (2010) Lethal Antibody Enhancement of Dengue Disease in Mice Is Prevented by Fc Modification. PLoS Pathog 6: e1000790; Williams KL, Sukupolvi-Petty S, Beltramello M, Johnson S, Sallusto F, et al. (2013) Therapeutic efficacy of antibodies lacking Fcγ receptor binding against lethal

dengue virus infection is due to neutralizing potency and blocking of enhancing antibodies [corrected]. PLoS Pathog 9: e1003157), the complete removal of N-linked glycans and the resulting abolishment of binding to C1q also may compromise the efficacy of a MAb, which uses CDC activity. Plant-derived MAb glycolvariants that minimize ADE but retain C1q

5 binding might have better efficacy than their aglycosylated counterparts. While ADE is not a critical issue for WNV infection, it is highly relevant to its closely related Dengue virus, and some coronaviruses, paramyxoviruses, and lentiviruses (Santi L, Batchelor L, Huang Z, Hjelm B, Kilbourne J, et al. (2008) An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. Vaccine 26: 1846-1854).

10 Accordingly, a plant-derived anti-DENV MAb (E60) as a therapeutic candidate against all 4 serotypes of DENV was also developed. Our results demonstrated that E60 and its scFv-C_H¹⁻³ variants can be expressed at high levels in both WT and ΔXF *N. benthamiana* plants. These plant-derived E60s were easily purified to high homogeneity with scalable and cGMP compliant downstream processing scheme. Furthermore, they retained the specific

15 binding and the potent neutralizing activities as E60 produced in mammalian cells, but with greatly reduced or eliminated ADE activity.

In summary, it was demonstrated for the first time the ability of plant cells to express and assemble MAb and its variants against both WNV and DENV, including a large and complex IgG-like tetravalent MAb variant. The demonstration of producing MAb variants

20 with highly specific and homogeneous mammalian glycosylation patterns in plants supports the feasibility of designing MAbs with tailor-made N-glycosylation for their optimized safety or efficacy. The demonstration of anti-WNV and anti-DENV MAbs which retain their in *vitro* and *in vivo* therapeutic function, yet lacking ADE, may overcome the major impediment for the development of MAb-based therapeutics against flavivirus and lead to safer antibody-

25 based therapeutics against flavivirus and other viruses prone to ADE.

MATERIALS AND METHODS

Expression of pHu-E16 and E60 MAb variants in *N. benthamiana* leaves

A plasmid containing the pHu-E16 MAb sequence (Lai H, Engle M, Fuchs A, Keller

30 T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424) was used as template for the construction of the pHu-E16scFv. Primers H5 (5' GAATTCACAA TGGGATGG 3' (SEQ ID NO:9)) and H3 (5' CCTCCTGAAG TTGAACCAGA AGACACAGTA ACAGTAG 3' (SEQ ID NO:10)) were

used to amplify the pHu-E16 V_H, and primers L5 (5' GGTTCAACTT CAGGAGGAGG ATCAGGTGGT GGTTCAGGAG GTGGAGGATC TTCT GATATC GTTATGACAC AATC 3' (SEQ ID NO:11)) and L3 (5' TGCTAGCTT TGATCTCCAA CTTAGTTCC 3' (SEQ ID NO:12)) were used to amplify the pHu-E16 V_L. Subsequently, the V_H and V_L sequences were linked together by overlapping PCR. The product was cloned using EcoRI and NheI sites into a plasmid, which already contains the coding sequence of the C_H¹⁻³ or C_L to yield EcoRI-pHu-E16scFv-C_H¹⁻³-BamHI or EcoRI-pHu-E16scFv-C_L-BamHI constructs. pHu-E16scFv-C_H¹⁻³ and E16scFv-C_L coding sequences were then cloned into plant expression vector pICH11599 and pICH21595, respectively with EcoRI and BamHI. The construction of pHu-E16 HC and pHu-E16 LC in pICH11599 and pICH21595 has been described previously (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424). None of the pHu-E16 constructs contains the ER-retention signal KDEL (SEQ ID NO:8).

For E60, a plasmid containing the sequence of the HC of E60 (Williams, et al. (2013). "Therapeutic efficacy of antibodies lacking Fcγ receptor binding against lethal dengue virus infection is due to neutralizing potency and blocking of enhancing antibodies [corrected]." *PLoS Pathog* 9(2): e1003157) was used as template with primers 5' - TTGGTTGCAACAGCTACTGGTGTTCATTCTGAGGTCCAGGTGCAACAG - 3' (SEQ ID NO:13) and 5' - AGAATTCACAATGGGATGGTCTTGTATCATCCTTTTCTTGGTTGCAACAGC - 3' (SEQ ID NO:14) to generate the HC variable region (V_H) of E60. Similarly, the LC variable region (V_L) was amplified with primers 5' - TTGGTTGCAACAGCTACTGG TGTTCATTCTGA CATCCTG ATGACCCAATC - 3' (SEQ ID NO:15) and 5' - AGAATTCACAATGGGATGGTCTTGTATCA TCCTTT TCTTGG TTGCAACAGC - 3' (SEQ ID NO:16) from a plasmid containing the E60 LC gene (Williams, et al. (2013). "Therapeutic efficacy of antibodies lacking Fcγ receptor binding against lethal dengue virus infection is due to neutralizing potency and blocking of enhancing antibodies [corrected]." *PLoS Pathog* 9(2): e1003157). The V_H and V_L PCR products were then cloned using EcoRI and NheI sites into plasmids pIgG1-CH and pIgG-kCL (He, et al. (2014). "Generation and Analysis of Novel Plant-Derived Antibody-Based Therapeutic Molecules against West Nile Virus." *PLoS ONE* 9(3): e93541 DOI:93510.91371/journal.pone.0093541), respectively.

Plant expression vectors were individually transformed into *A. tumefaciens* GV3101 by electroporation as previously described (Santi L, Batchelor L, Huang Z, Hjelm B, Kilbourne J, et al. (2008) An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. *Vaccine* 26: 1846-1854). Wild-type *N.*

benthamiana plants were grown and Agroinfiltrated or co-Agroinfiltrated with GV3101 strains containing the pHu-E16scFv- C_H^{1-3} , pHu-E16scFv- C_H^{1-3} /LC, HC/pHu-E16scFv- C_L or pHu-E16scFv- C_H^{1-3} /pHu-E16scFv- C_L 3' modules along with their respective 5' modules and an integrase construct as described previously to express pHu-E16 variants (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2419-2424; Lai H, Chen Q (2012) Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. *Plant Cell Reports* 31: 573-584).

Extraction and purification of pHu-E16 and E60 variants from plant leaves

For evaluating the temporal pattern of pHu-E16 and E60 variant expression, Agroinfiltrated leaves were harvested 5, 6, 7, 8, 9, and 10 dpi. Leaves were harvested 9 dpi for other protein analysis. Extraction and purification of pHu-E16 or E60 variants from plant leaves were performed with a method previously reported for pHu-E16 MAbs (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of Sciences of the United States of America* 107: 2419-2424). Briefly, leaves were homogenized in extraction buffer (PBS, 1mM EDTA, 10 mg/ml sodium ascorbate, 10 µg/ml leupeptin, 0.3 mg/ml PMSF). The crude plant extract was clarified by centrifugation at 17,700 x g for 30 min at 4°C. pHu-E16 variants in clarified protein extract were purified by a two-step purification process comprised of ammonium sulfate precipitation and protein A affinity chromatography.

SDS-PAGE, Western blot, ELISA and flow cytometry with yeast surface display

Protein samples were subjected to 10% SDS-PAGE under reducing (5% v/v β-mercaptoethanol) or to 4-20% gradient SDS-PAGE under non-reducing conditions. Gels were stained with Coomassie blue or used to transfer proteins onto PVDF membranes. Horseradish peroxidase (HRP)-conjugated antibodies against human-kappa LC or gamma HC (Southern Biotech) were used for western blot analysis as previously described (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proceedings of the National Academy of*

Sciences of the United States of America 107: 2419-2424). pHu-E16 variant expression and antigen binding was examined with an ELISA as described (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424). Briefly, DIII (amino acids 296-415) of the New York 1999 strain of WNV purified from *E. coli* (Oliphant T, Nybakken GE, Austin SK, Xu Q, Bramson J, et al. (2007) Induction of epitope-specific neutralizing antibodies against West Nile virus. J Virol 81: 11828-11839) was immobilized on microtiter plates. After incubation with plant protein extract or purified pHu-E16 variants, an HRP-conjugated anti-human-gamma HC antibody (Southern Biotech) was used as the detection antibody. The plates were developed with TMB substrate (KPL Inc). mHu-E16 (Oliphant T, Engle M, Nybakken G, Doane C, Johnson S, et al. (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nature Medicine 11: 522-530) and pHu-E16 (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424) were used as reference standards. Yeast displaying WNV DIII or DENV DI-II on their surface were generated, stained with MAb variants, and analyzed with a Becton Dickinson FACSCalibur flow cytometer as described (Lai H, Engle M, Fuchs A, Keller T, Johnson S, et al. (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. Proceedings of the National Academy of Sciences of the United States of America 107: 2419-2424 Oliphant T, Engle M, Nybakken G, Doane C, Johnson S, et al. (2005) Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nature Medicine 11: 522-530).

N-linked glycan analysis

The N-linked glycosylation profile was determined by LC-ESI-MS as previously published (Stadlmann J, Pabst M, Kolarich D, Kunert R, Altmann F (2008) Analysis of immunoglobulin glycosylation by LC-ESI-MS of glycopeptides and oligosaccharides. Proteomics 8: 2858-2871). Briefly, purified MAb variants were separated by reducing SDS-PAGE, stained with Coomassie, and the HC-containing band was excised from the gel. Upon S-alkylation and tryptic or tryptic/GluC digestion, fragments were eluted from the gel with 50% acetonitrile and separated on a Reversed Phase Column (150 x 0.32 mm BioBasic-18, Thermo Scientific) with a gradient of 1%–80% acetonitrile. Glycopeptides were analyzed with a quadrupole time-of-flight (Q-TOF) Ultima Global mass spectrometer (Waters, Milford,

MA, USA). Spectra were summed and deconvoluted for identification of glycoforms. Glycans were annotated according to the ProGlycAn nomenclature (www.proglycan.com).

Surface plasmon resonance

Binding activity measurement of MAb variants for human C1q and FcγRs (CD16A-158^{val} (CD16V¹⁵⁸), CD16A-158^{phe} (CD16F¹⁵⁸), CD32A-131^{arg} (CD32aR¹³¹), CD32A-131^{his} (CD32aH¹³¹), CD32B, and CD64) was performed by SPR on a BIAcore 3000 biosensor (GE, Healthcare). WNV E protein was immobilized on the CM-5 sensor chip (~3000RU) by an amine coupling kit as recommended by the manufacturer. Hu-E16 variants were bound to the E protein surface at approximately 700RU followed by injection of C1q at 50 nmol/L or the soluble monomeric receptors CD16A-158^{val}, CD16A-158^{phe} at concentrations of 500 nmol/L, respectively, and flow rate of 30 μl/min for 60 sec with dissociation time 60 sec. Dimeric Fc-G2 (N297Q) fusions of human CD32B, CD32A-131^{arg}, and CD32A-131^{his}, or human soluble CD64 were injected at 200 nmol/L or at 50 nmol/L, respectively. Each receptor was injected in duplicate. Between experiments, the naked antigen surface was regenerated by pulse injection of 10 mM glycine pH 1.5. All binding experiments were performed in 10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% P20 surfactant.

Antibody-dependent Enhancement Assay

The enhancing activity of the MAbs was determined with CD32A⁺ K562 cells (Pierson TC, Xu Q, Nelson S, Oliphant T, Nybakken GE, et al. (2007) The Stoichiometry of Antibody-Mediated Neutralization and Enhancement of West Nile Virus Infection. Cell host & microbe 1: 135-145) in at least three independent experiments in triplicate using a high-throughput flow cytometry-based assay with GFP pseudo-infectious WNV replicon particles essentially as described (Pierson TC, Xu Q, Nelson S, Oliphant T, Nybakken GE, et al. (2007) The Stoichiometry of Antibody-Mediated Neutralization and Enhancement of West Nile Virus Infection. Cell host & microbe 1: 135-145). K562 erythroleukemic cells were obtained from the American Type Cell Collection (ATCC CCL-243).

WNV and DENV neutralization

A focus reduction neutralization assay was used to assess the neutralizing activity of pHu-E16 or E60 variants against WNV/DENV essentially as previously described (Fuchs A, Pinto AK, Schwaeble WJ, Diamond MS (2011) The lectin pathway of complement activation contributes to protection from West Nile virus infection. Virology 412: 101-109). Neutralization curves were generated using Prism software to express the percent reduction of spot numbers in samples pre-incubated with mAb compared to wells with virus pre-incubated with medium alone, and EC50 values calculated.

Efficacy of MAbs in vivo

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

5 Injections were performed under anesthesia that was induced and maintained with ketamine

hydrochloride and xylazine, and all efforts were made to minimize suffering. C57BL/6 mice
were housed in a pathogen-free mouse facility. Mice received a single dose of purified pHu-
E16scFv-CH¹⁻³, Tetra pHu-E16, or mHu-E16 by intraperitoneal injection the same day or four

days after footpad infection with 10² PFU of WNV strain 3000.0259. Kaplan-Meier analysis
of survival data was performed using the log-rank test. IC50 analyses were performed by

10 non-linear regression and statistical significances were determined using analysis of variance
(ANOVA) and F-tests.

All publications, patents and patent applications are incorporated herein by reference.

While in the foregoing specification this invention has been described in relation to certain

15 embodiments thereof, and many details have been set forth for purposes of illustration, it will

be apparent to those skilled in the art that the invention is susceptible to additional

embodiments and that certain of the details described herein may be varied considerably

without departing from the basic principles of the invention.

The use of the terms “a” and “an” and “the” and similar referents in the context of

20 describing the invention are to be construed to cover both the singular and the plural, unless

otherwise indicated herein or clearly contradicted by context. The terms “comprising,”

“having,” “including,” and “containing” are to be construed as open-ended terms (*i.e.*,

meaning “including, but not limited to”) unless otherwise noted. Recitation of ranges of

values herein are merely intended to serve as a shorthand method of referring individually to

25 each separate value falling within the range, unless otherwise indicated herein, and each

separate value is incorporated into the specification as if it were individually recited herein.

All methods described herein can be performed in any suitable order unless otherwise

indicated herein or otherwise clearly contradicted by context. The use of any and all

examples, or exemplary language (*e.g.*, “such as”) provided herein, is intended merely to

30 better illuminate the invention and does not pose a limitation on the scope of the invention

unless otherwise claimed. No language in the specification should be construed as indicating

any non-claimed element as essential to the practice of the invention.

Embodiments of this invention are described herein, including the best mode known

to the inventors for carrying out the invention. Variations of those embodiments may become

apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein.

Accordingly, this invention includes all modifications and equivalents of the subject matter

5 recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A plant-produced monoclonal antibody (MAb) specific for a target virus, the MAb comprising a defined and highly-uniform N-glycoform, wherein the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AGnF₆, GnAF₆, AAF₆, GnNa, NaGn, NaA, ANa, NaNa, GnNaF₆, NaGnF₆, NaAF₆, ANaF₆, NaNaF₆, GnGnbi, GnGnbiF₆, AGnbi, GnAbi, AAbi, NaGnbi, GnNabi, NaAbi, ANabi, NaNabi, AGnbiF₆, GnAbiF₆, AAbiF₆, NaGnbiF₆, GnNabiF₆, NaAbiF₆, ANabiF₆, NaNabiF₆ (GnGn)(GnGn), (GnGn)(GnGn)F₆, (AA)(AA)F₆, or (Na)(Na)F₆ as depicted in Figure 1,
 wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and
 wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.
2. The plant-produced Mab of claim 1, wherein the MAb comprises a heavy chain (HC) further comprising one or more amino acid mutations that affect Fc receptor or/and C1q binding.
3. The plant-produced MAb of claim 2, wherein the HC is a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with, these positions.
4. The plant-produced MAb of any one of claims 1-3, wherein the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AGnF₆, GnAF₆, or AAF₆.
5. The plant-produced MAb of any one of claims 1-3, wherein the N-glycoform is GnGnXF₃, Man5, Man7, Man8, or Man9.
6. The plant-produced MAb of any one of claims 1-3, wherein the MAb is anti-West Nile virus (WNV) MAb E16.

7. The plant-produced MAb of claim 4, wherein the MAb is pHu-E16.
8. The plant-produced MAb of any one of claims 1-3, wherein the MAb is a single chain variant of E16.
9. The plant-produced MAb of any one of claims 1-3, wherein the MAb is a single chain variant of E16 (scFv- C_H^{1-3} molecule) consisting of a single-chain variable fragment (scFv) of pHu-E16 fused to the heavy chain (HC) constant domains (C_H^{1-3}) of human IgG.
10. The plant-produced MAb of any one of claims 1-3, wherein the MAb is pHu-E16scFv- C_H^{1-3} .
11. The plant-produced MAb of any one of claims 1-10, wherein the N-glycoform selectively binds to Fc receptors or C1q.
12. The plant-produced MAb of any one of claims 1-11, wherein the MAb is a bivalent variant.
13. The plant-produced MAb of any one of claims 1-11, wherein the MAb is a tetravalent variant.
14. The plant-produced MAb of claim 13, wherein the MAb is Tetra pHu-E16.
15. The plant-produced MAb of any one of claims 1-3, wherein the MAb is an anti-Dengue virus (DV) MAb E60.
16. The plant-produced MAb of claim 15, wherein the MAb is a single chain variant of DV MAb E60 (scFv-Fc fusion molecule).
17. The plant-produced MAb of claim 15, wherein the MAb is a single chain variant of DV MAb E60 (scFv- C_H^{1-3}).

18. The plant-produced MAb of any one of claims 15-17, wherein the N-glycoform selectively binds to Fc receptors or C1q.
19. The plant-produced MAb of any one of claims 15-18, wherein the MAb is a bivalent or tetravalent variant.
20. The plant-produced MAb of any one of claims 1-3, wherein the MAb is specific for a flavivirus, coronavirus, paramyxovirus, lentivirus, or other virus that is prone to ADE.
21. A method of modulating antibody-dependent enhancement (ADE) of an antibody comprising contacting the antibody with an N-glycan.
22. The method of claim 21, wherein the N-glycan is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, or GnGnF6.
23. A plant-produced monoclonal antibody (MAb) specific for a target virus,
wherein the MAb is aglycosylated and comprises a heavy chain (HC) comprising one or more amino acid mutations that eliminate Fc receptor or/and C1q binding,
wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and
wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.
24. The plant-produced MAb of claim 23, wherein the MAb comprises two or more mutations.
25. The plant-produced MAb of claim 23 or 24, wherein an N-glycosylation site is mutated, and wherein the mutation results in aglycosylation of the MAb.

26. The plant-produced MAb of claim 25, wherein the N-glycosylation site consensus sequence N-X-S/T is mutated, wherein N is asparagine, X is any amino acid, and S/T is serine or threonine.
27. The plant-produced MAb of claim 26, wherein the HC is a human IgG1 isotype, and the mutation is N297Q, or an equivalent mutation that corresponds to, or aligns with, this position.
28. The plant-produced MAb of any one of claims 23-27, wherein the HC is a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.
29. The plant-produced MAb of claim of any one of claims 23-28, wherein the MAb is anti-West Nile virus (WNV) MAb E16.
30. The plant-produced MAb of claim of any one of claims 23-28, wherein the MAb is pHu-E16.
31. The plant-produced MAb of any one of claims 23-28, wherein the MAb is a single chain variant of E16.
32. The plant-produced MAb of any one of claims 23-28, wherein the MAb is a single chain variant of E16 (scFv- C_H^{1-3} molecule) consisting of a single-chain variable fragment (scFv) of pHu-E16 fused to the heavy chain (HC) constant domains (C_H^{1-3}) of human IgG.
33. The plant-produced MAb of any one of claims 23-28, wherein the MAb is pHu-E16scFv- C_H^{1-3} .
34. The plant-produced MAb of any one of claims of any one of claims 23-33, wherein the MAb is a bivalent or tetravalent variant.
35. The plant-produced MAb of claim 34, wherein the MAb is Tetra pHu-E16.

36. The plant-produced MAb of any one of claims 23-28, wherein the MAb is an anti-Dengue virus (DV) MAb E60.
37. The plant-produced MAb of claim 36, wherein the MAb is a single chain variant of DV MAb E60 (scFv-Fc fusion molecule).
38. The plant-produced MAb of claim 36, wherein the MAb is a single chain variant of DV MAb E60 (scFv-C_H¹⁻³).
39. The plant-produced MAb of any one of claims 36-38, wherein the MAb is a bivalent or tetravalent variant.
40. The plant-produced MAb of any one of claims 23-39, wherein the MAb is specific for a flavivirus, coronavirus, paramyxovirus, lentivirus, or other virus that is prone to ADE.
41. A plant-produced monoclonal antibody (MAb) specific for a target virus,
wherein the MAb comprises a heavy chain (HC) comprising one or more amino acid mutations that alter Fc receptor or/and C1q binding,
wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and
wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.
42. The plant-produced MAb of claim 41, wherein the heavy chain comprises two or three amino acid mutations.
43. The plant-produced MAb of claim 42, wherein the HC is a human IgG1 isotype and the mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.

44. The plant-produced MAb of any one of claims 41-43, wherein the MAb is anti-West Nile virus (WNV) MAb E16.
45. The plant-produced MAb of any one of claims 41-43, wherein the MAb is pHu-E16.
46. The plant-produced MAb of any one of claims 41-43, wherein the MAb is a single chain variant of E16.
47. The plant-produced MAb of any one of claims 41-43, wherein the MAb is a single chain variant of E16 (scFv-C_H¹⁻³ molecule) consisting of a single-chain variable fragment (scFv) of pHu-E16 fused to the heavy chain (HC) constant domains (C_H¹⁻³) of human IgG.
48. The plant-produced MAb of any one of claims 41-43, wherein the MAb is pHu-E16scFv-C_H¹⁻³.
49. The plant-produced MAb of any one of claims 41-48, wherein the MAb is a bivalent or tetravalent variant.
50. The plant-produced MAb of claim 49, wherein the MAb is Tetra pHu-E16.
51. The plant-produced MAb of any one of claims 41-43, wherein the MAb is an anti-Dengue virus (DV) MAb E60.
52. The plant-produced MAb of claim 51, wherein the MAb is a single chain variant of DV MAb E60 (scFv-Fc fusion molecule).
53. The plant-produced MAb of claim 51, wherein the MAb is a single chain variant of DV MAb E60 (scFv-C_H¹⁻³).
54. The plant-produced MAb of any one of claims 51-53, wherein the MAb is a bivalent or tetravalent variant.

55. The plant-produced MAb of any one of claims 41-54, wherein the MAb binds to Fc receptors and/or C1q with increased selectivity.
56. The plant-produced MAb of any one of claims 41-54, wherein the MAb binding to Fc receptors and/or C1q is reduced.
57. The plant-produced MAb of any one of claims 41-56, wherein the MAb is specific for a flavivirus, coronavirus, paramyxovirus, lentivirus, or other virus that is prone to ADE.
58. A method of modulating antibody-dependent enhancement (ADE) of an antibody comprising mutating the amino acid sequence of the antibody heavy chain.
59. The method of claim 58, wherein the antibody heavy chain is an IgG1 isotype and the amino acid sequence of the antibody heavy chain comprises mutations L234AL235A or L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.
60. A method of producing a MAb having a defined and highly-uniform N-glycoform, wherein the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGn F₆, AGnF₆, GnAF₆, AAF₆, GnNa, NaGn, NaA, ANa, NaNa, GnNaF₆, NaGnF₆, NaAF₆, ANaF₆, NaNaF₆, GnGnbi, GnGnbiF₆, AGnbi, GnAbi, AAbi, NaGnbi, GnNabi, NaAbi, ANabi, NaNabi, AGnbiF₆, GnAbiF₆, AAbiF₆, NaGnbiF₆, GnNabiF₆, NaAbiF₆, ANabiF₆, NaNabiF₆ (GnGn)(GnGn), (GnGn)(GnGn)F₆, (AA)(AA)F₆, or (Na)(Na)F₆ as depicted in Figure 1 comprising
 - (a) growing a plant comprising a nucleic acid encoding the MAb, and
 - (b) isolating the MAb,
 wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

61. The method of claim 60, wherein the nucleic acid encodes a MAb with one or more amino acid mutations in its heavy chain (HC) that affect Fc receptor and/or C1q binding.
62. The method of claim 61, wherein the HC is a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.
63. The method of any one of claims 60-62, wherein the plant is a wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produce N-glycoforms of GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AAF₆, NaNa, NaNaF₆, GnGnbi, or (GnGn)(GnGn) as depicted in Figure 1.
64. A method of producing a MAb having a defined and highly-uniform N-glycoform, wherein the N-glycoform is GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, or GnGn F₆ comprising
 - (a) growing a plant;
 - (b) transiently expressing MAb and a glyco-enzyme(s) in the plant by co-infiltrating the plant with *Agrobacterium* strains containing the genetic constructs of MAb HC, LC, and glycol-enzyme(s); and
 - (c) isolating the MAb,
 wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and
 wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity kinetics and *in vivo* therapeutic activity kinetics against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.
65. The method of claim 64, wherein the MAb HC construct encodes a HC comprising one or more amino acid mutations that affect Fc receptor and/or C1q binding.
66. The method of claim 65, wherein the MAb HC construct encodes a human IgG1 HC isotype and the one or more mutations are selected from L234AL235A and

L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.

67. The method of any one of claims claim 64-66, wherein the HC of a MAb is tagged with an endoplasmic reticulum (ER)-retention/retrieval signal.
68. The method of claim 67, wherein the ER-retention/retrieval signal is a peptide sequence containing the four amino acid H/KDEL (SEQ ID NO:7) or its variant attached to the carboxyl-terminus of the heavy chain of an antibody.
69. The method of any one of claims 64-68, wherein the plant is a wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produces N-glycoform of GnGn.
70. The method of claim any one of claims 64-69, wherein the glyco-enzyme is β 1,4-galactosyltransferase (GalT), α 1,6-fucosyltransferase (FUT8), human β 1,4-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIII), α 1,3-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIV), α 1,6-mannosyl- β 1,6-N-acetylglucosaminyltransferase (GnTV), UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE), N-acetylneuraminic acid phosphate-synthase (NANS), CMP-Neu5Ac transporter (CST), or α 2,6- or α 2,3-sialyltransferase.
71. The method of claim 70, wherein the genetic construct of one glycol-enzyme is co-infiltrated into a plant with those of MAb LC and HC.
72. The method of claim 70, wherein the genetic constructs of more than one glycol-enzymes are co-infiltrated into a plant with those of MAb LC and HC.
73. The method of any one of claims 64-72, wherein the plant is a wild-type *Nicotiana benthamiana* or a glycol-engineered *N. benthamiana* plant that produces N-glycoform of GnGn.
74. A method of producing an aglycosylated MAb comprising
 - (a) growing a plant comprising a nucleic acid encoding the MAb,

wherein the encoded MAb comprises a mutation that results in aglycosylation of the MAb (e.g, a mutation at an N-glycosylation site in the MAb heavy chain (HC)), and

(b) isolating the MAb,

wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

75. The method of claim 74, wherein a N-glycosylation site consensus sequence N-X-S/T in the MAb HC is mutated, and wherein N is asparagine, X is any amino acid, and S/T is serine or threonine.
76. The method of claim 75, wherein the HC is a human IgG1 isotype, and the mutation is N297Q, or an equivalent mutation that corresponds to, or aligns with this position.
77. The method of any one of claims 74-76, wherein the encoded MAb further comprises one or more amino acid mutations that eliminate Fc receptor or/and C1q binding,
78. The method of claim 77, wherein the HC is a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.
79. A method of producing an aglycosylated MAb comprising
- (a) growing a plant;
 - (b) transiently expressing the MAb in the plant by co-infiltrating the plant with *Agrobacterium* strains containing the genetic constructs of MAb HC and LC, wherein the genetic construct of the MAb HC comprises a mutation that results in aglycosylation of the MAb (e.g, a mutation altering an N-glycosylation site); and
 - (c) isolating the MAb,

wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity kinetics and *in vivo* therapeutic activity kinetics against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

80. The method of claim 79, wherein the genetic construct of the MAb HC comprises a mutation that alters an N-glycosylation site consensus sequence N-X-S/T, and wherein N is asparagine, X is any amino acid, and S/T is serine or threonine.
81. The method of claim 80, wherein the genetic construct of the MAb HC encodes a human IgG1 isotype comprising an N297Q mutation, or an equivalent mutation that corresponds to, or aligns with this position.
82. The method of any one of claims 79-81, wherein the MAb HC genetic construct encodes an HC comprising one or more mutations that eliminate Fc receptor or/and C1q binding.
83. The method of claim 82, wherein the genetic construct of the MAb HC encodes a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.
84. The method of any one of claims 74-83, wherein the plant is a wild-type *Nicotiana benthamiana* or any glyco-engineered *N. benthamiana* plants.
85. A method of producing a MAb having altered Fc receptor and/or C1q binding comprising
 - (a) growing a plant comprising a nucleic acid encoding the MAb, wherein the encoded MAb comprises one or more amino acid mutations in its heavy chain that alter Fc receptor and/or C1q binding,
 - (b) isolating the MAb,

wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity, and *in vivo* therapeutic activity against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

86. A method of producing a MAb having altered Fc receptor and/or C1q binding comprising

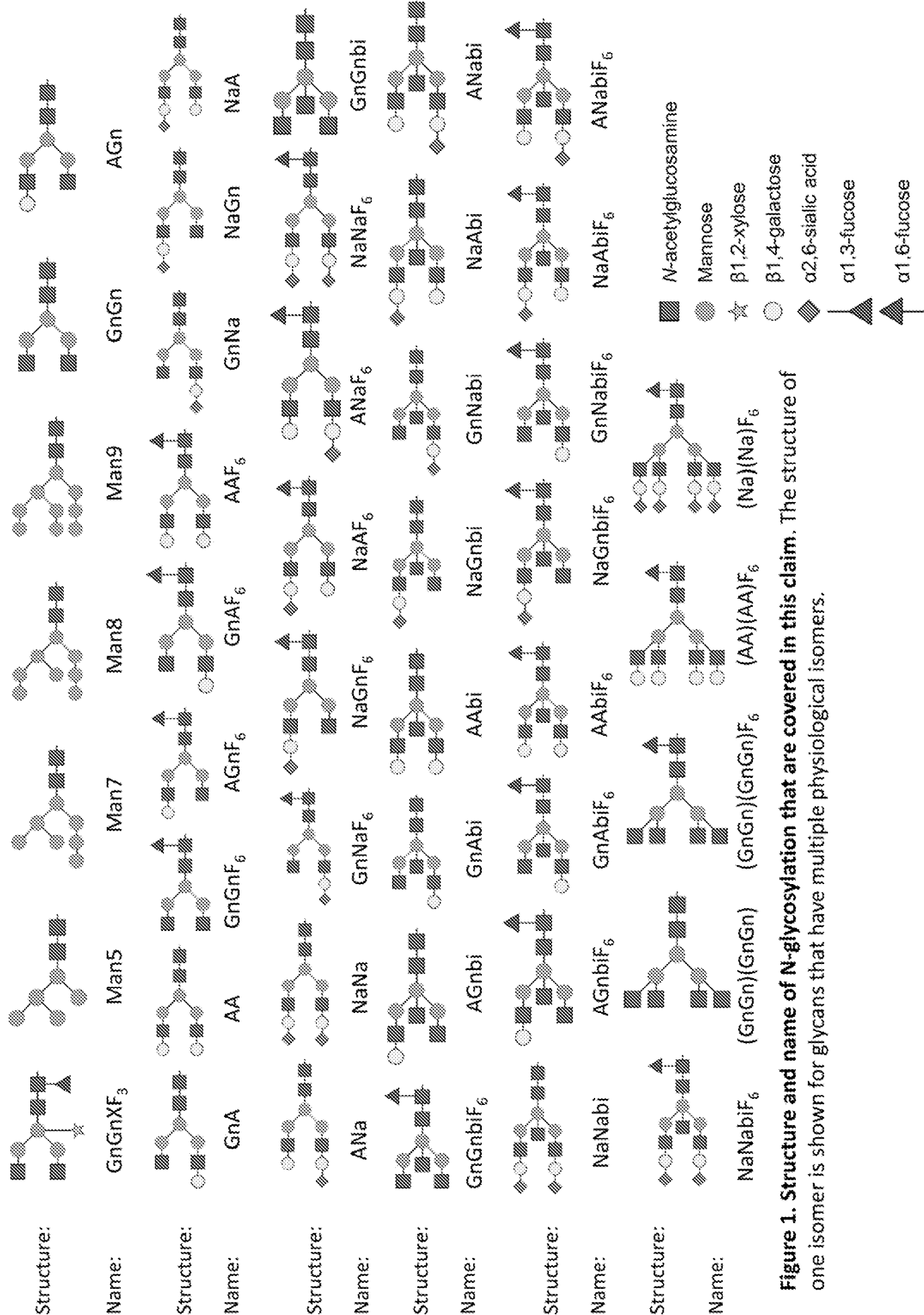
- (a) growing a plant;
- (b) transiently expressing MAb in the plant by co-infiltrating the plant with *Agrobacterium* strains containing the genetic constructs of MAb HC and LC, wherein the HC genetic construct encodes an HC comprising one or more mutations that alter Fc receptor and/or C1q binding; and
- (c) isolating the MAb,

wherein the MAb has reduced antibody-dependent enhancement (ADE) as compared to a mammalian-produced MAb, and

wherein the MAb has equivalent antigen binding affinity and kinetics, neutralization activity kinetics and *in vivo* therapeutic activity kinetics against a target virus infection as compared to an antibody produced from non-plant cells including mammalian, yeast and bacterial cells.

87. The method of claim 85 or 86, wherein genetic construct of the MAb HC encodes a human IgG1 isotype and the one or more mutations are selected from L234AL235A and L234AL235AG237A, or equivalent mutations that correspond to, or align with these positions.
88. The method of any one of claims 85-87, wherein the MAb binds to Fc receptors and/or C1q with increased selectivity.
89. The method of any one of claims 85-87, wherein the MAb binding to Fc receptors and/or C1q is reduced.

90. The method of any one of claims 86-89, further comprising transiently expressing a glyco-enzyme(s) in the plant by infiltrating the plant with an *Agrobacterium* strain(s) containing the genetic construct of a glycol-enzyme(s).
91. The method of claim 90, wherein the glyco-enzyme is β 1,4-galactosyltransferase (GalT), α 1,6-fucosyltransferase (FUT8), human β 1,4-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIII), α 1,3-mannosyl- β 1,4-N-acetylglucosaminyltransferase (GnTIV), α 1,6-mannosyl- β 1,6-N-acetylglucosaminyltransferase (GnTV), UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE), N-acetylneuraminic acid phosphate-synthase (NANS), CMP-Neu5Ac transporter (CST), or α 2,6- or α 2,3-sialyltransferase.
92. The method of claim 90 or 91, wherein the genetic construct of one glycol-enzyme is co-infiltrated into a plant with those of MAb LC and HC.
93. The method of claim 90 or 91, wherein the genetic constructs of more than one glycol-enzymes are co-infiltrated into a plant with those of MAb LC and HC.
94. The method of any one of claims 85-93, wherein the plant is a wild-type *Nicotiana benthamiana* or a glyco-engineered *N. benthamiana* plant that produce N-glycoforms of GnGnXF₃, Man5, Man7, Man8, Man9, GnGn, AGn, GnA, AA, GnGnF₆, AAF₆, NaNa, NaNaF₆, GnGnbi, or (GnGn)(GnGn) as depicted in Figure 1.
95. The method of any one of claims 85-93, wherein the plant is a wild-type *Nicotiana benthamiana* or a glyco-engineered *N. benthamiana* plant that produces N-glycoform of GnGn.



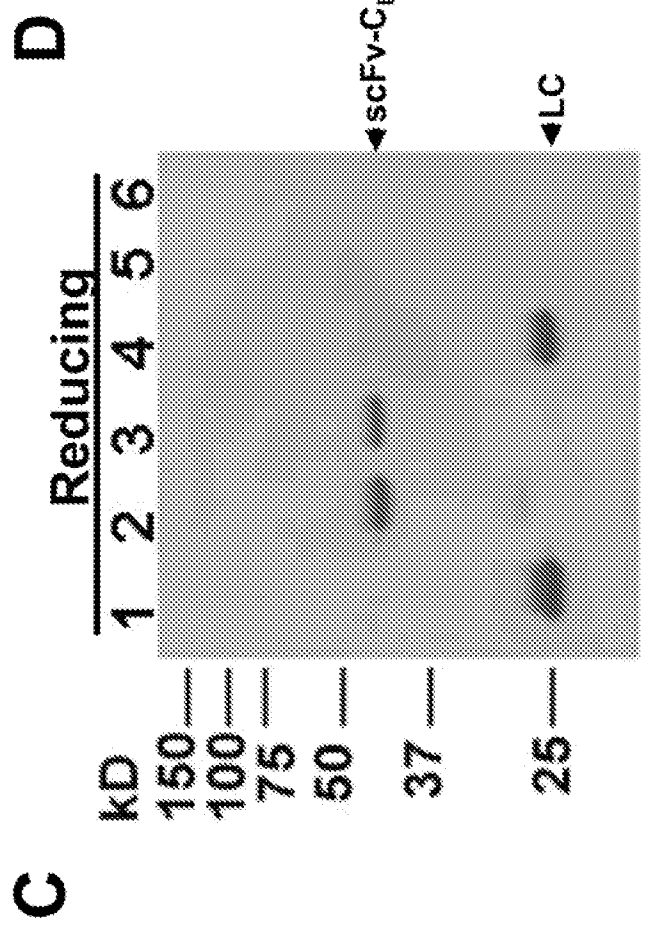


Figure 2

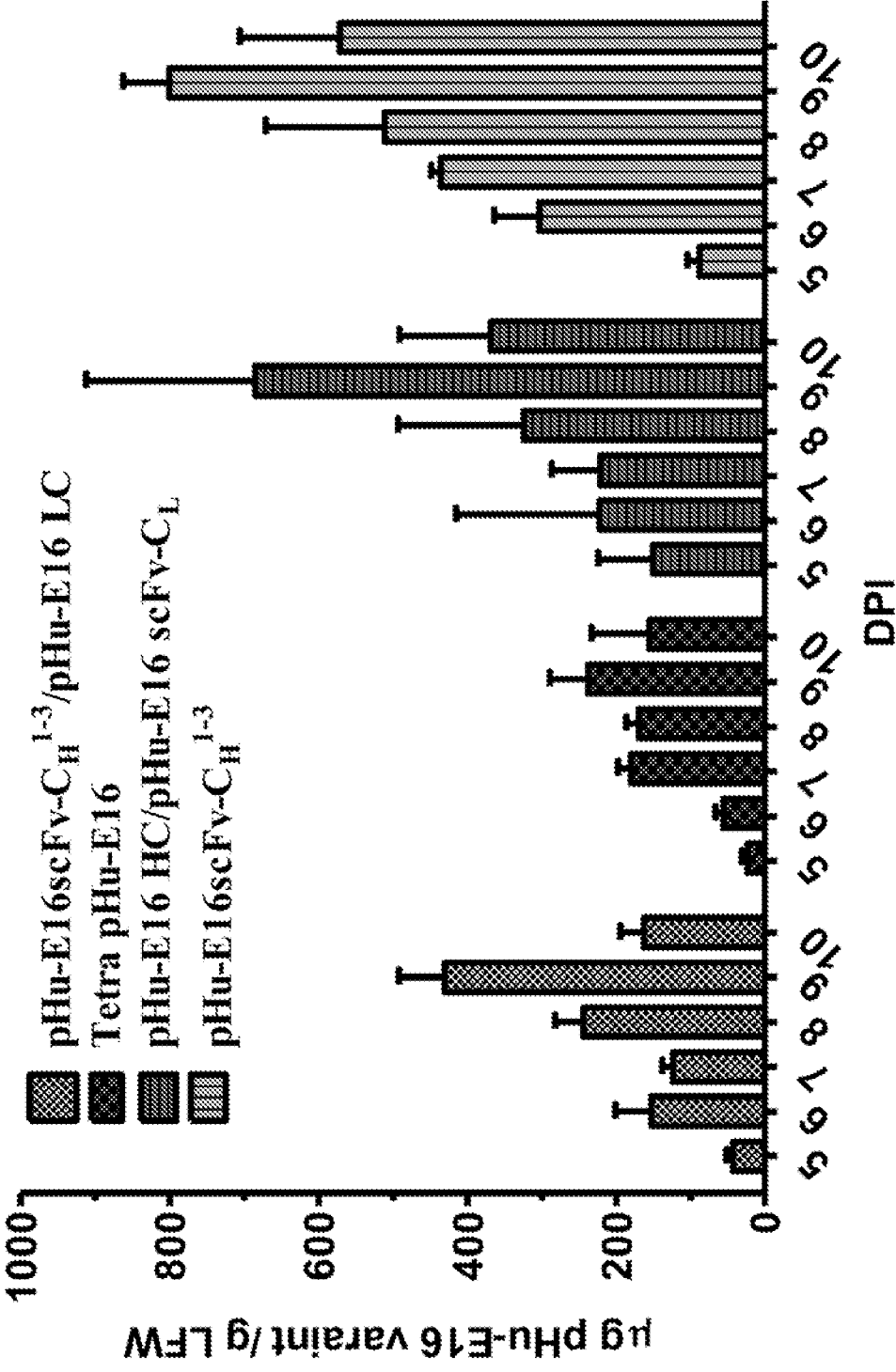


Figure 3

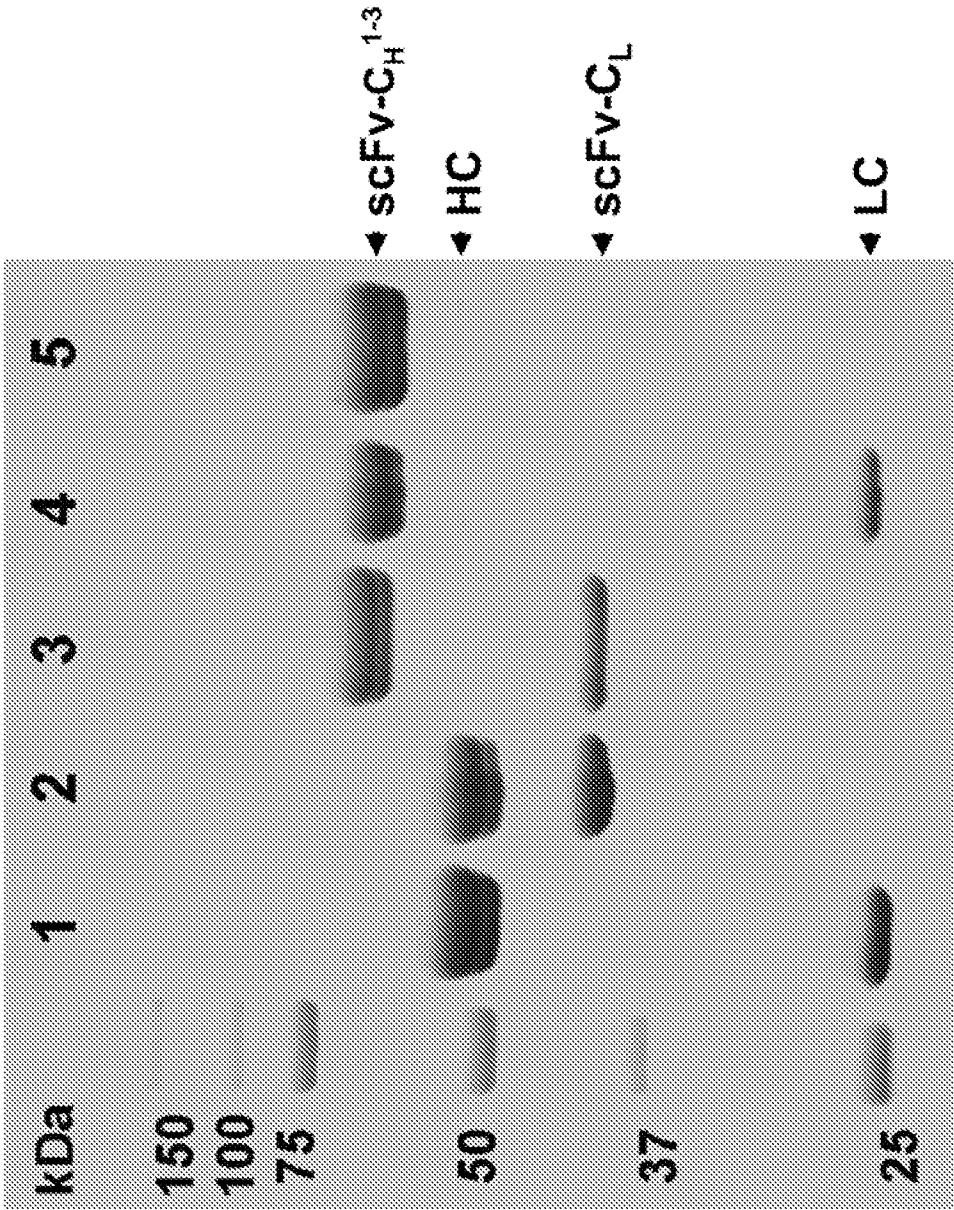


Figure 4

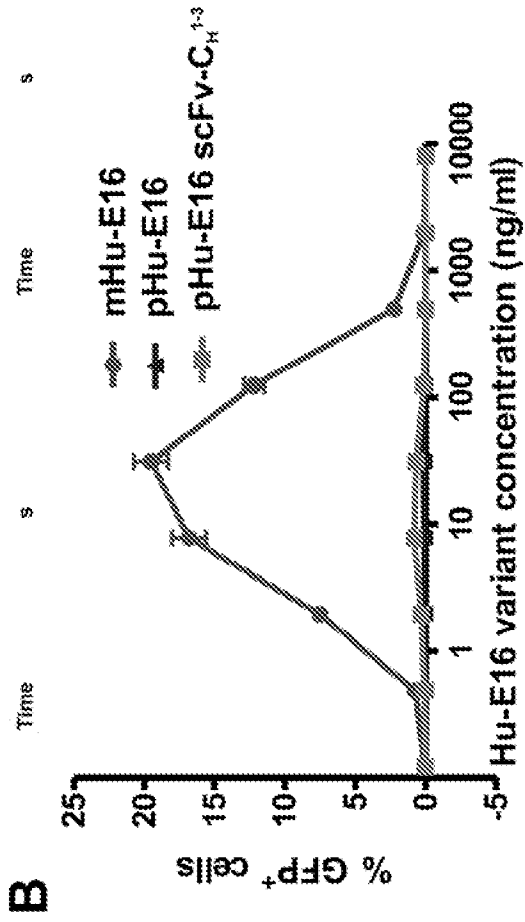
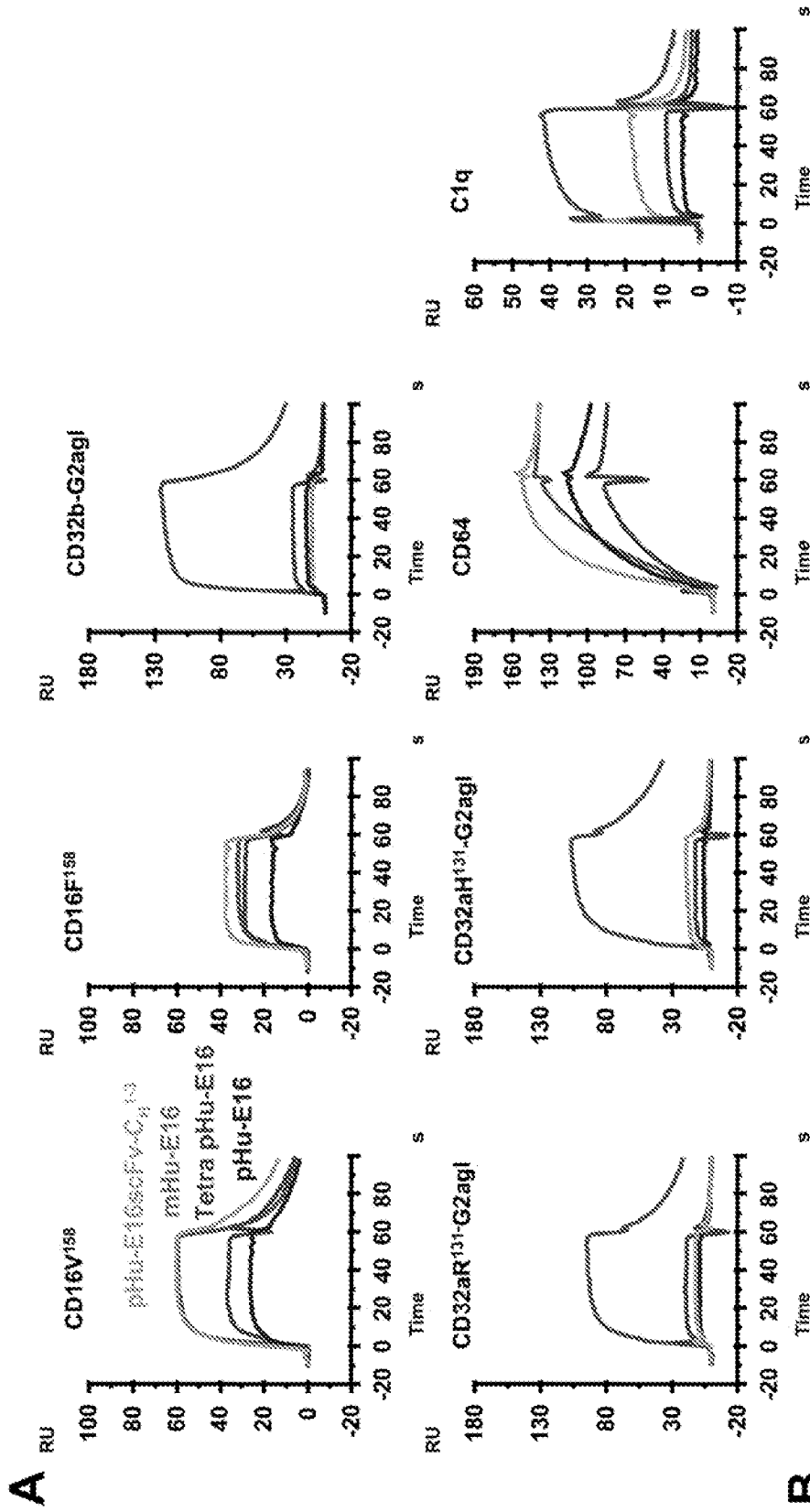
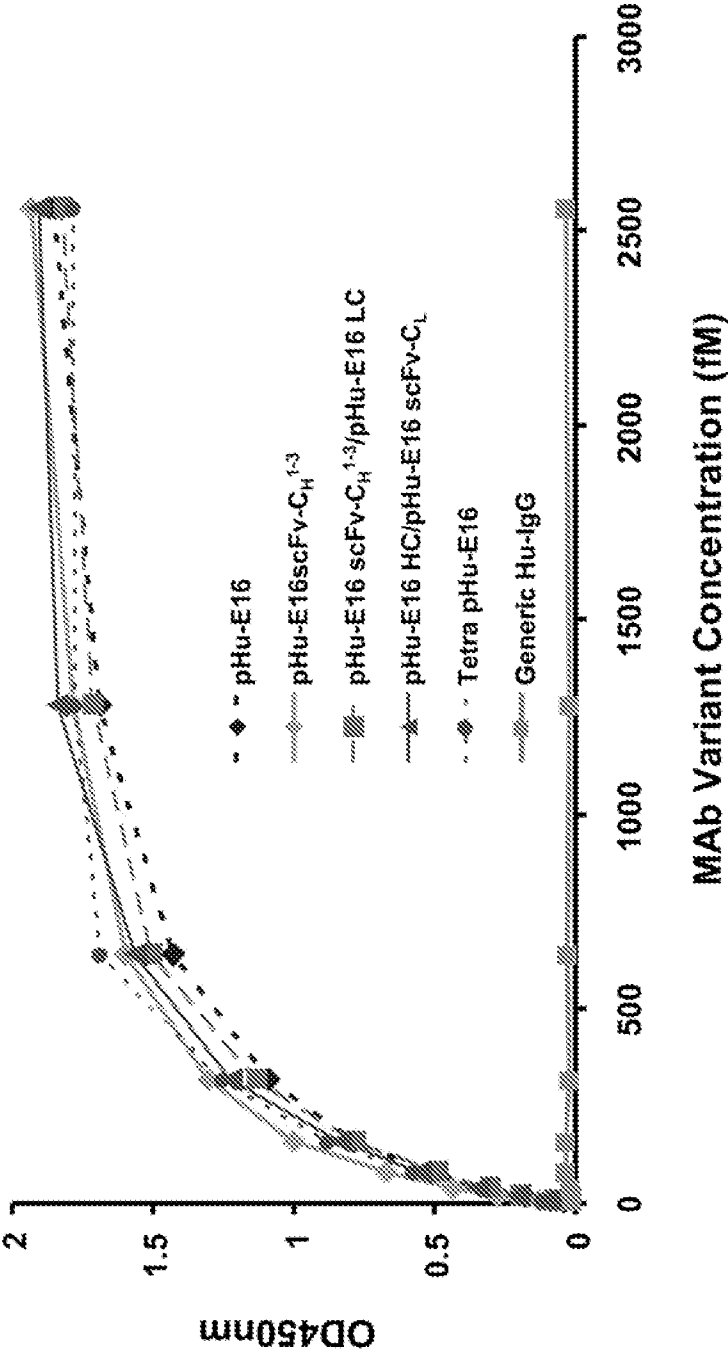


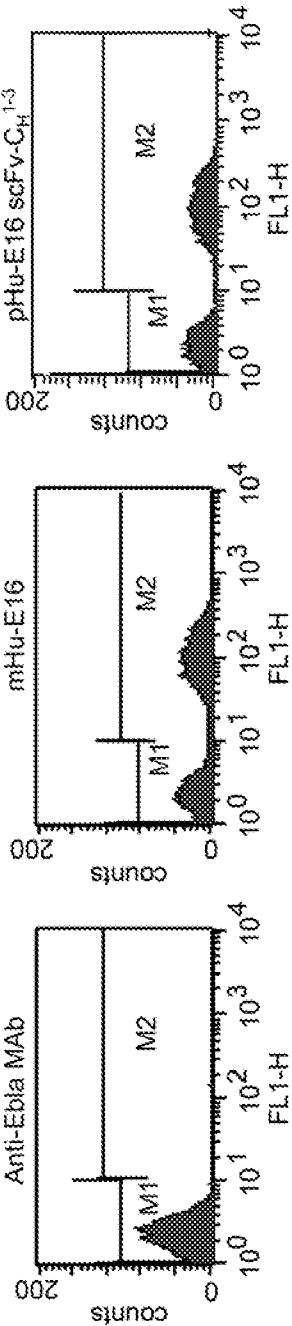
Figure 5

Figure 6

A



B



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Figure 7

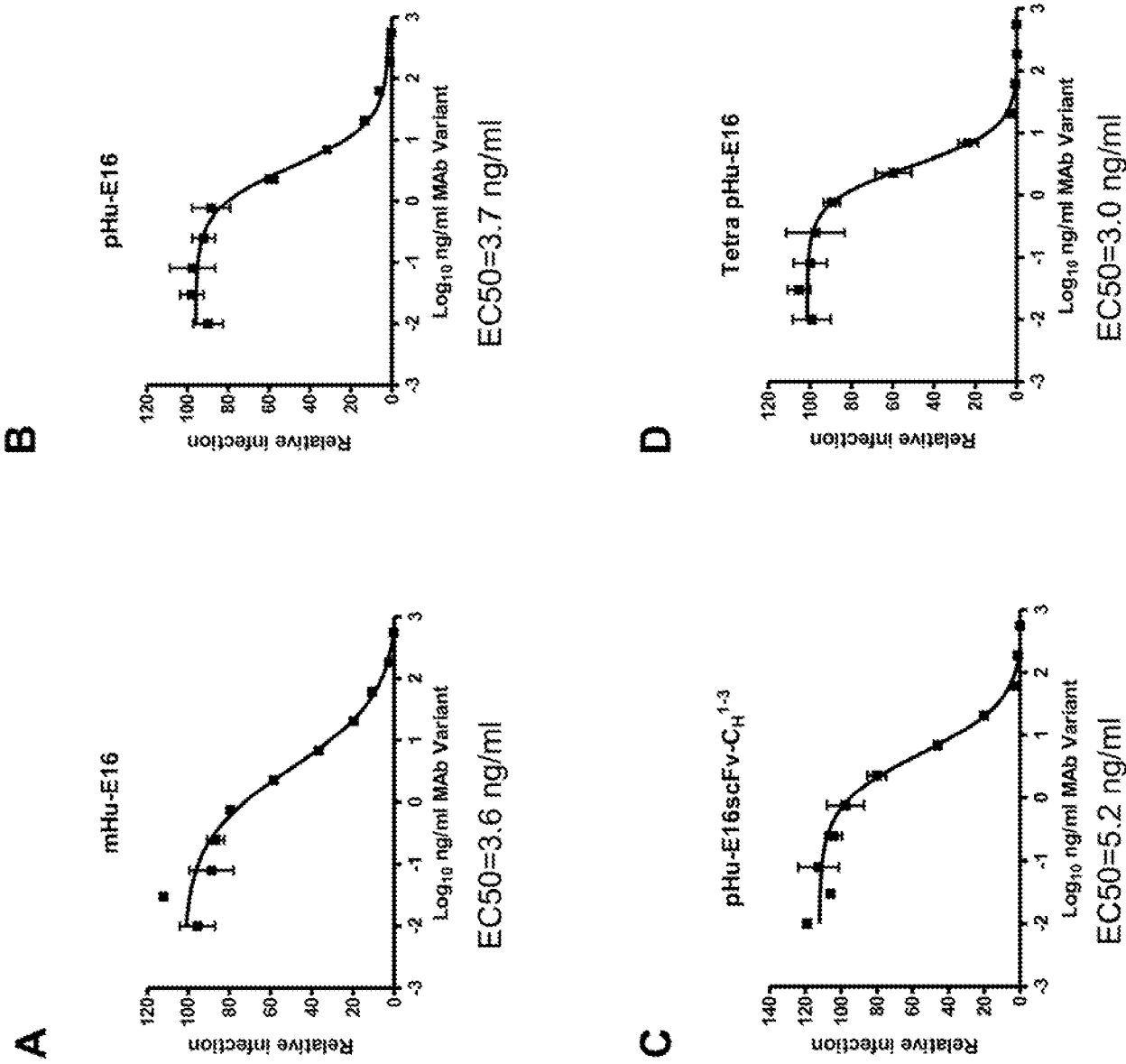
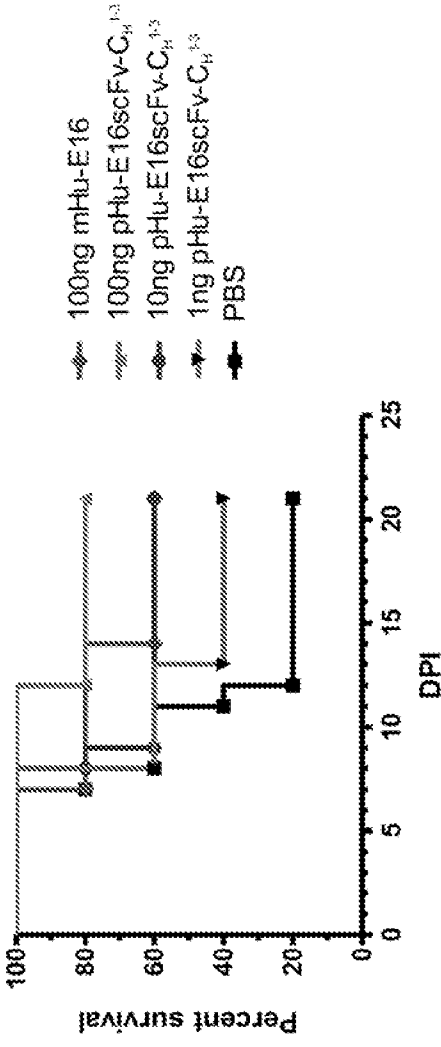


Figure 8

A: Pretreatment



B: Day + 4 therapeutic

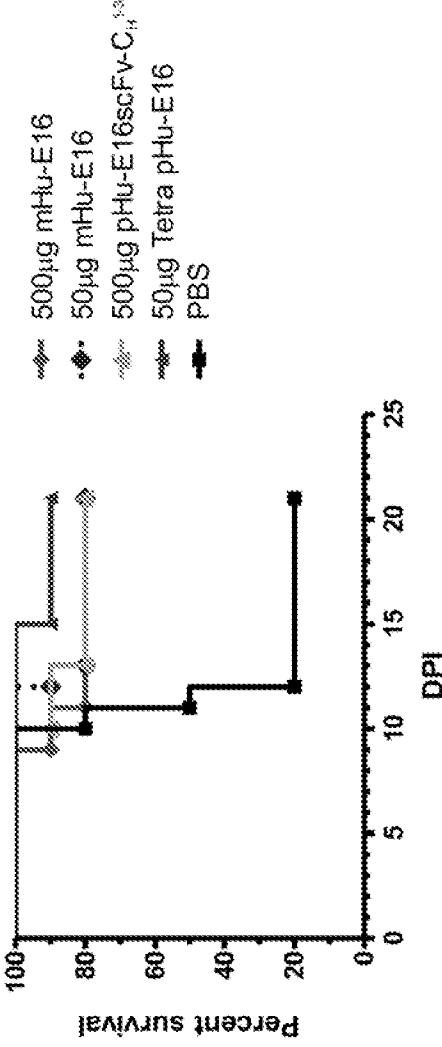
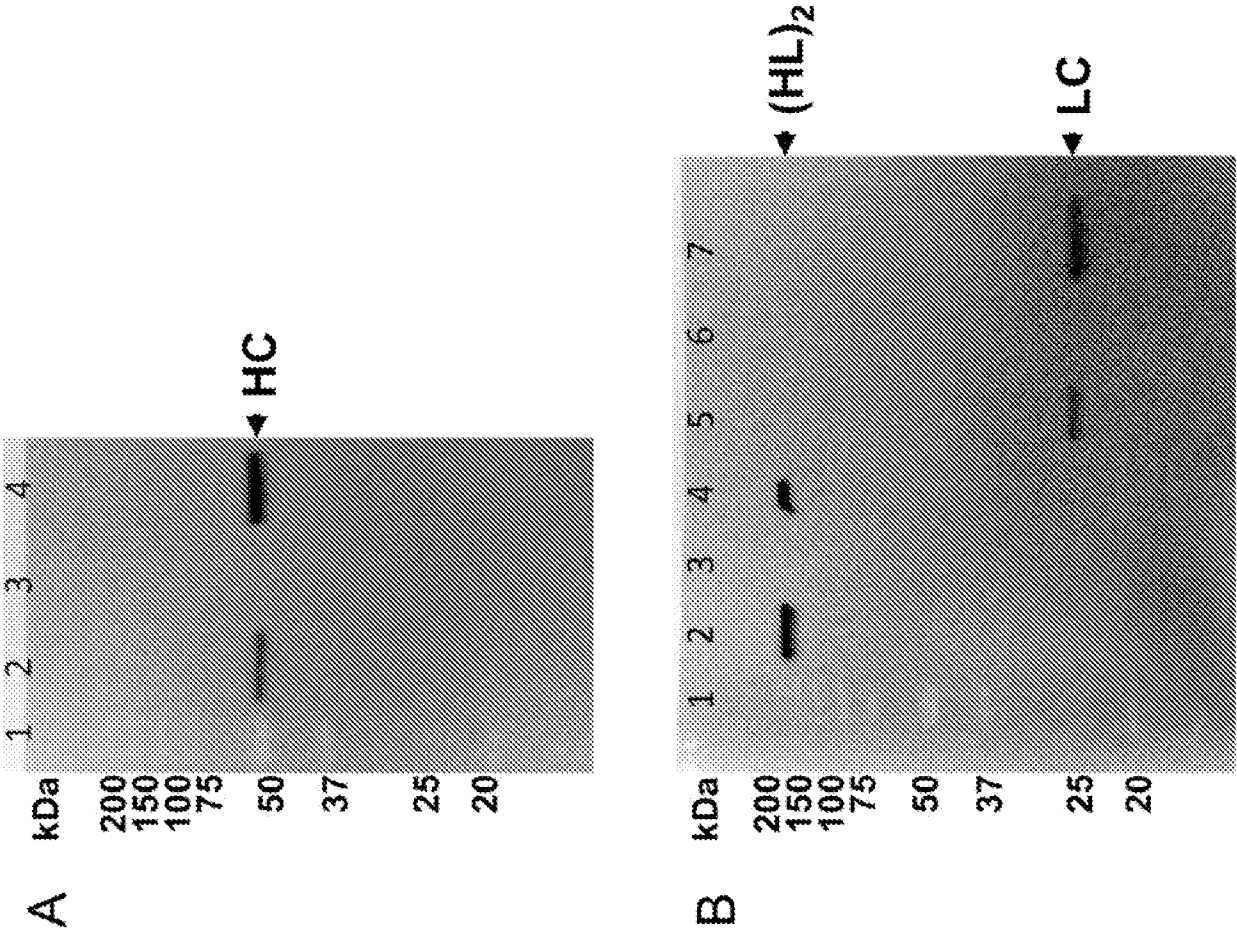


Figure 9



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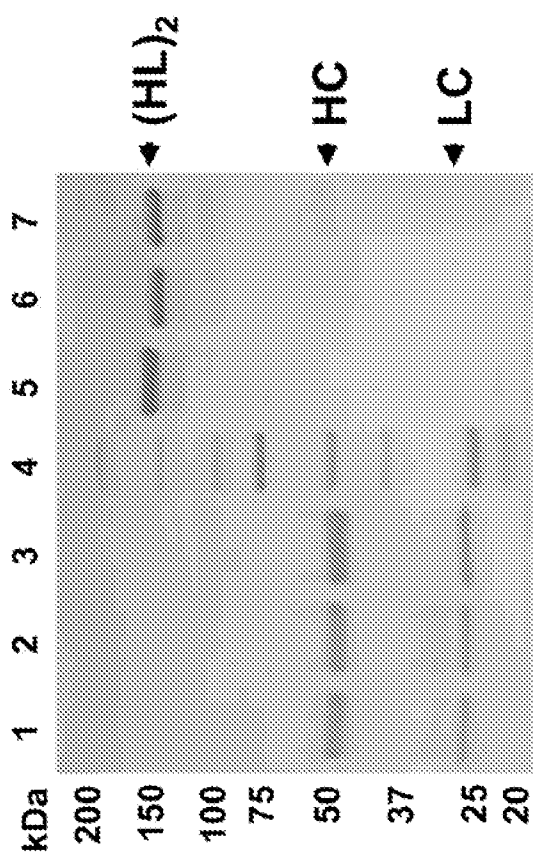


Figure 10

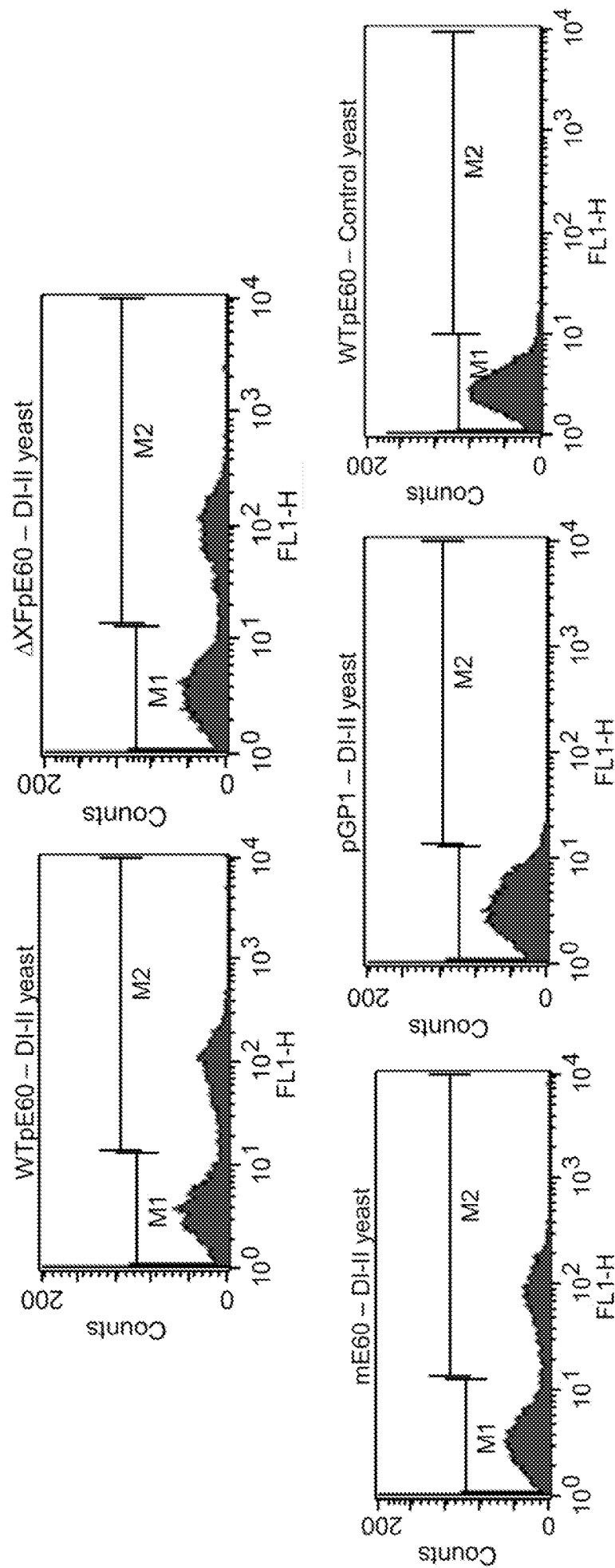


Figure 11

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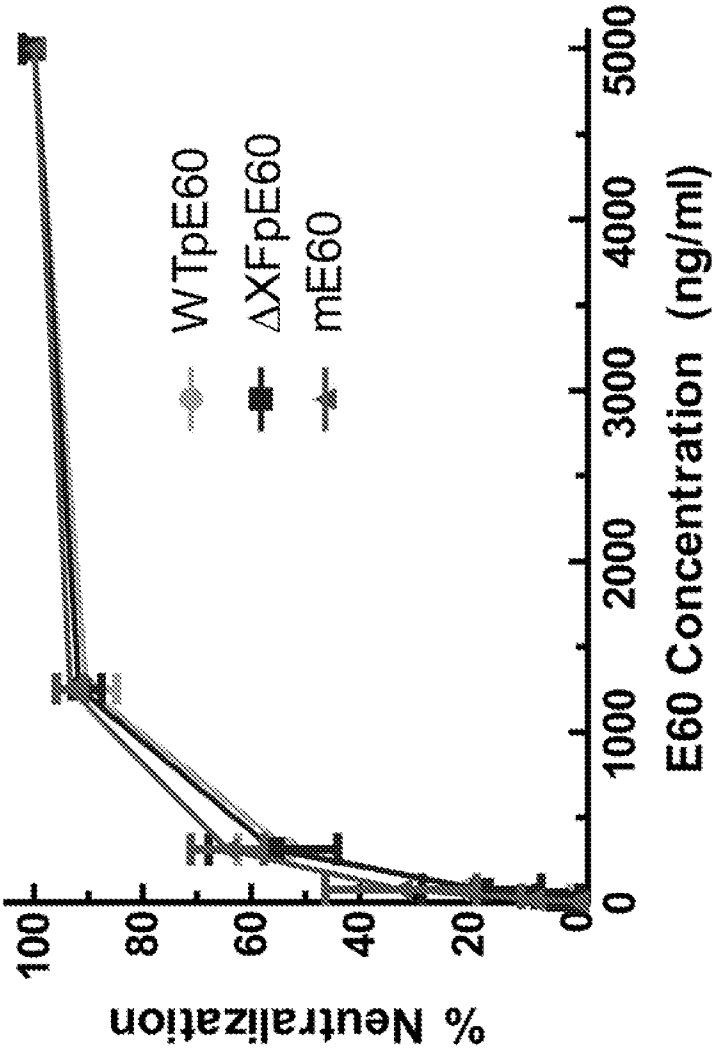
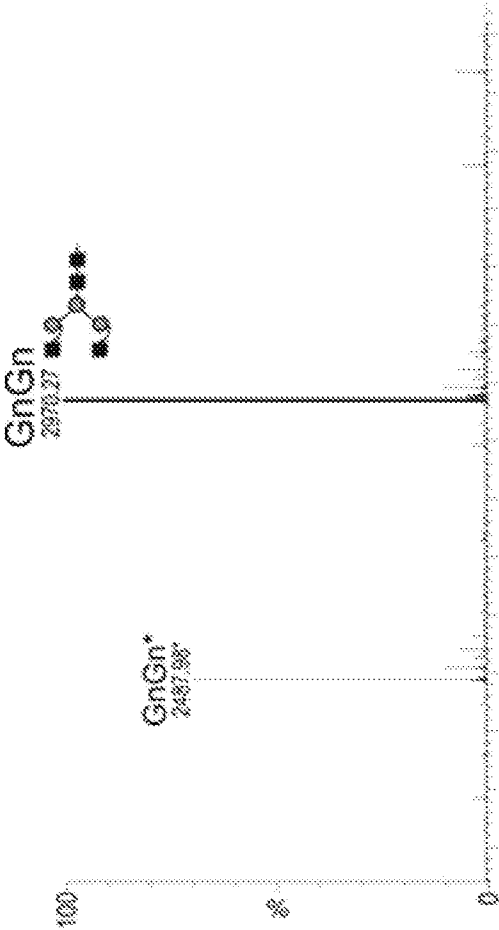


Figure 12

A



B

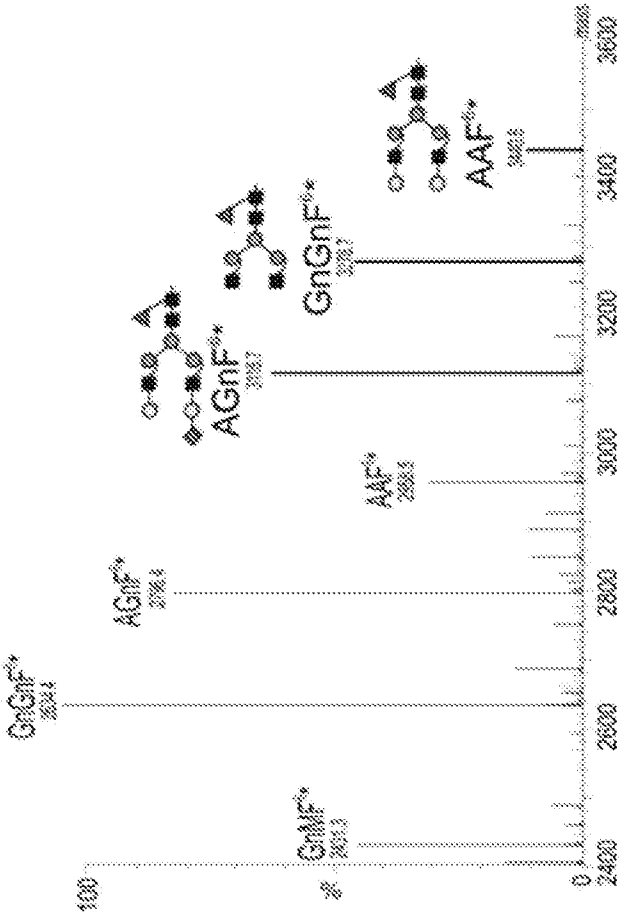


Figure 13

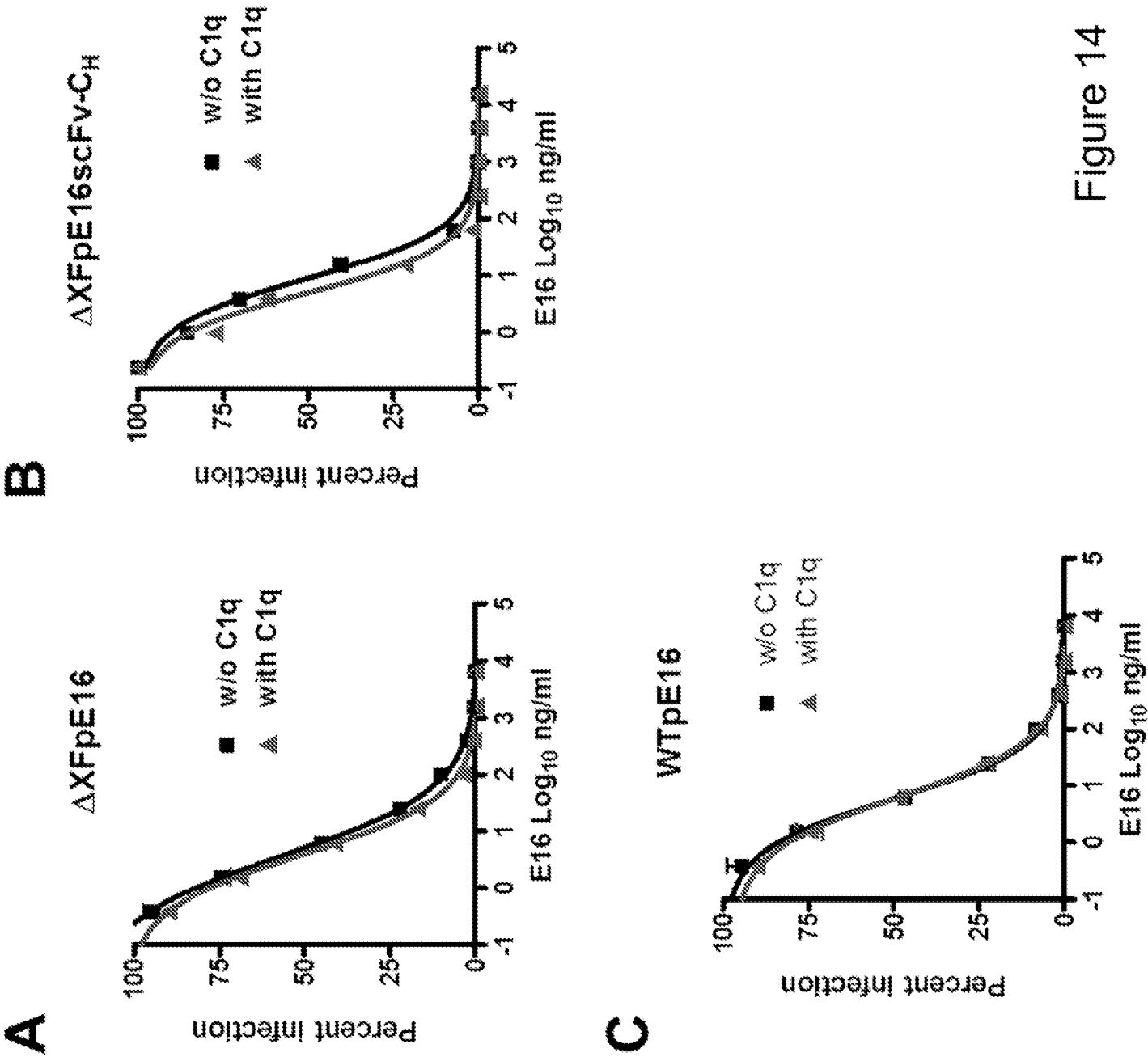
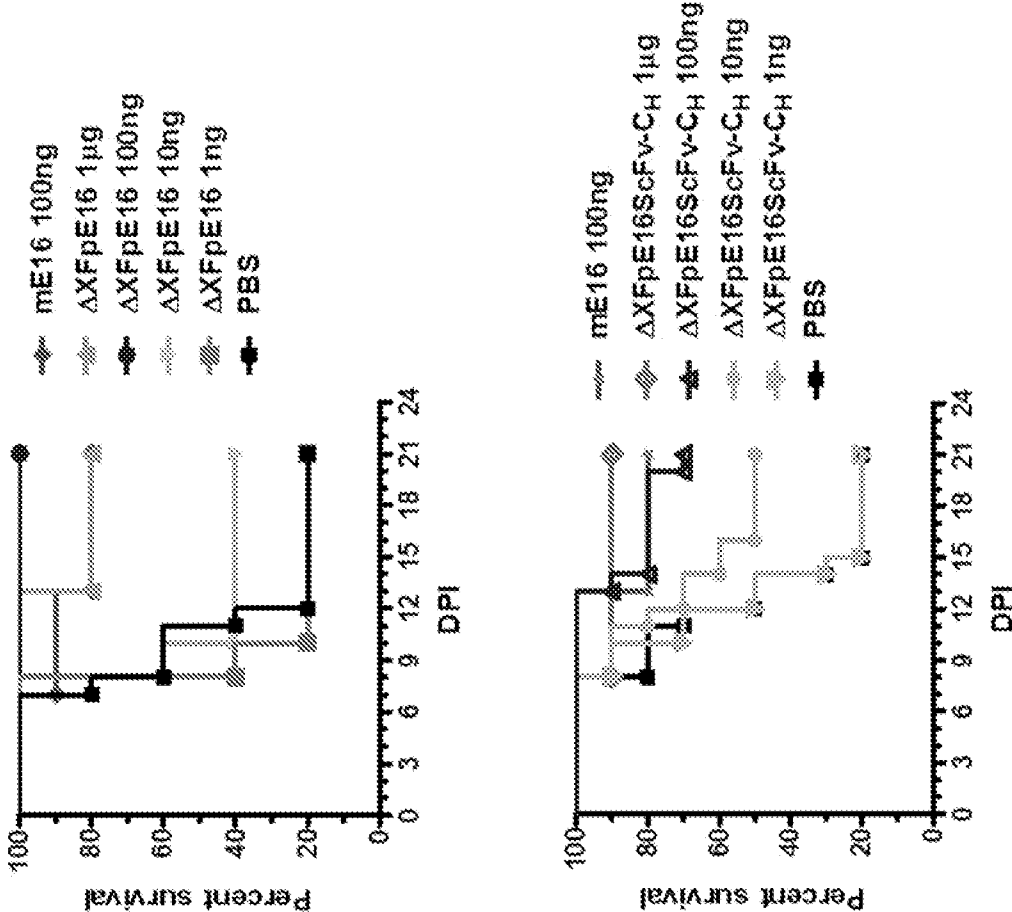


Figure 14

A: Prophylactic treatment



B: Post-exposure (D+4) treatment

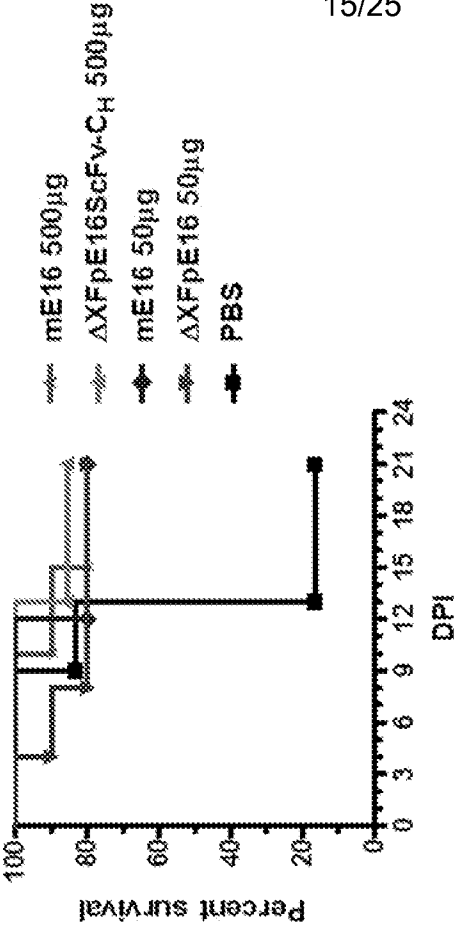


Figure 15

	A	B	C	D	E	F	G
Glycan Structure	pHu-E16	pHu-E16scFv-CH ^{1,2}	pHu-E16scFv-CH ^{1,2} / LC	HC/ pHu-E16scFv-CL	pHu-E16scFv-CH ^{1,2} / pHu-E16 scFv-CL	mHu-E16	mHu-E16scFv-CH ^{1,2}
GnGnXF	90		27	88	19		
∑ oligoman		90	66	8	72		10
GnGnF6						78	65
AGnF ⁶						15	20
∑ other	10	10	7		9	8	5

Figure 16. . Relative abundance in percentage of major glyco-structures detected on Hu-E16 variants.

∑ other complex: sum of glycoforms present at levels below 5 %. The glycan structures are assigned using the ProGlycan nomenclature (www.proglycan.com). HC: heavy chain, CH¹⁻³: the constant regions 1-3 of HC, LC: light chain, CL: Constant region of LC.

FIGURE 17-1**E60 WT Heavy Chain***Nucleic Acid Sequence*

gaattcacaatgggatggtcttgtatcatccttttcttggttgaacagctactggtgttcattctgaggtccaggtgcaacagctctggacctgaactggt
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ccgtgaaccacaagtgatcacacttctccatctcgcgatgaactgaccaagaaccaggtcagcttgacttgctggtgaaaggcttctatccctctga
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Amino Acid Sequence

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TKPREEQYNSTYRVSVLTIVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPKEPQVYTLPPSRDELTKNQVSL
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LSEGE (SEQ ID NO:18)

E60 Heavy Chain NQ mutant*Nucleic Acid Sequence*

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FIGURE 17-2

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Amino Acid Sequence

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 LSPCK (SEQ ID NO:20)

E60 Heavy Chain LALA mutant*Nucleic Acid Sequence*

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Amino Acid Sequence

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FIGURE 17-3

```

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E60 Heavy Chain LALAGA mutant*Nucleic Acid Sequence*

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Amino Acid Sequence

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LSPGK (SEQ ID NO:24)

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E60 scFv WT*Nucleic Acid Sequence*

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FIGURE 17-4

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Amino Acid Sequence

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 NYNQRFRCATILTVDRSSSTAYMEIRSLTSEDSAVYFCACTLYGYFFDFWQGTTLTVSSG3TSGG3SGGSGGGG
 SDILMTQSPSSMSVSLGDSVSLTCHASQGLISGNI GWLQKPKGSKXGLIYHGINLEEGVPERFSGSGSGADYSLTIS
 SLESSEPADYYCVQYQGFPTFGGGTKLEIKASTKGPSVFLAPSSKSTSGKTAALGCLVKDYFPELTVSWNSGAL
 TSCVHTFPAVLQSSGLYSLSSVTVTSSSLCTQTITCNVNHKPTENTKYOKYVEPKSCDKTHTCPPCPAPELLGGPSV
 FLPPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
 EYKCKVSNKALPAPTEKTSKAKGQPREPOVYTLPPSRDELTKNOVSLTCLVKGYFSGDIAVFESNGOPENNYKTT
 FPLVDSGG3FFLYSKLTVDKSRWQGNVFPSCVMHEALHNHYTOKSLSLSPGK (SEQ ID NO:26)

E60 scFv NQ Mutant*Nucleic Acid Sequence*

Gaattcacaatgggatggtcttctgtatcatccttttcttggttgaacagctactggtgttcattctgaggtccaggtgcaacagctctggacctgaactggt
 gacgcctggggcctcagtgaaagatatcctgcaagacttctggatacactttcactgaatataccgtccactgggtgaagcagagccatggaaagagcc
 ttgagtggattggaggcattaatctaccagtggtggtactaactacaaccagaggttcaggggcaaggccacattgactgtagacaggtcctccagc
 acagcctacatggagctccgcagcctgacatctgaggattctgcagctctattttgtgcaggaaacctctatggctacccttttgacttctggggccaag
 gcaccactctcacagctctctcaGTTCAACTTCAGGAGGAGGATCAGGTGGTGGTTCAGGAGGTGGAGGATCTTCTgaca
 tcctgatgacccaatctccatctccatgtctgtatctctgggagactcagtcagcatcacttgccatgcaagtcagggcattagcggaatatagggtg
 gttgcagcagaaaccagggaaatcatttaagggcctgatctatcatggaaccaacttggaagagggagttccatcaaggttcagtggcagtgatctg
 gagcagattattctctcaccatcagcagcctggagctgaagattttgcagactattactgtgtacagtatggtcagtttctccgacgttcggtggagg
 caccaagctggaatcaaagctagcaccaagggaccttctgtttttcacttgctccttcttaagctacttctggtggaactgctgcttgggttgttt
 ggtgaaagattactttctgagccagtgaccgtttcttggaactcaggtgctcttacatctggtgttcatactttccagctgttcttcaatcttcaggactt
 tactactttcttctgttaccgttcttctcaagcttgggcactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgacaa

FIGURE 17-5

gaaagttgagcccaagtccttgacaagactcatactgtccaccgtgccagcacctgaacttcttgaggaccgtcagtccttctgtttctccaaag
 cctaaggataccttgatgatctccaggactcctgaagtcacatgtgtagttgtggatgtgagccatgaagatcctgaggtgaagttcaactggtatgtg
 gatggtgtggaagtgcacaatgccaaagacaaagccgagagaggaacagtaccaaagcacgtacaggggtgtctcagttctcactgttctccatcaag
 attggttgaaaggcaagagtacaagtgcaaggtctccaacaaagccctcccagccccattgagaagaccatttccaaagcgaagggaaccccc
 gtgaaccacaagtgacacacttctccatctcgcgatgaactgaccaagaaccaggtcagcttgacttgccctggtgaaaggcttctatccctctgaca
 tagctgtagagtgaggagagcaatgggcaaccggagaacaactacaagactacacctcccgttctcgattctgacggctccttctctctacagcaag
 ctacagtgagacaagagcaggtggcaacaagggaatgtcttctcatgctccgtgatgcatgaggctcttcacaatcactacacacagaagagctctc
 cttgtctccgggtaaatgaggatcc (SEQ ID NO:27)

Amino Acid Sequence

MGWSCIILFLVATATGVHSEVQVQDSGPGLVTFGASVKISCKTSGYIFTEYTVHWVKQSHGKSLIEWIGGINFTSGG
 NYNQRFNGKATLTVDRSSSTAYMELPSLTSEDSAVYFCAGTLYGYIFDFWQGTTLTVSSGSTSGGGSGGGSGGGG
 SDILMTQSPFQSMGVSIGLSVSTTCHASQQLIGNIGWILQKFGKSFKLLTYHGTNLRGGVPSRPSGSGSGGADYSLTIS
 SLESDFADYYCWOYQQFPETFGGCKLEIKASTKGPSVFTFLAPSSKSTSGGTAALGCLVKDYTHETFTVSWNSGAL
 TSGVHTFPAYLQSSSLYGLSSVYTVESSELGTQTYICNVNKKFSTNTKVLKAVEPKKQDKTHKPTCPAPELLEGTSV
 FLFPFKKDTLMISRTPEVTCVVDVSHEDFEVKFNWYVDQVEVHNAAKTPREECYQSTYRVSVLTIVLHODWLNK
 SYKCVSNKALPAFIEKTIKAGQPREPOVYTLPPSPDELTKNOVSLTCLVKGFYPSDIAVEWESNGGFENNYKTI
 PPVLDSDGSFFLYSKLTVDKSRWOOQCNVTFSCVMHEALHNHYTQKSLSPGK (SEQ ID NO:28)

E60 scFv LALA Mutant*Nucleic Acid Sequence*

gaattcacaatgggatggtcttgtatcatcttttcttggttgaacagctactggtgttcattctgaggtccaggtgcaacagctctggacctgaactggt
 gacgcctggggcctcagtgaaatctcctgaagacttctggatacactttcactgaatataccgtccactgggtgaagcagagccatggaaagagcc
 ttgagtggttgaggcattaatctaccagtggtggtactaactacaaccagaggttcaggggcaaggccattgactgtagacaggtcctccagc
 acagcctacatggagctccgcagcctgacatctgaggattctgcagtcattttgtgcaggaaccctctatggctaccctttgacttctggggccaag
 gcaccactctcacagtcctctcaGGTTCAACTTCAGGAGGAGGATCAGGTGGTGGTTCAGGAGGTGGAGGATCTTCTgaca
 tcttgatgaccaatctccatctccatgtctgtatctctgggagactcagtcagcatcacttgccatgcaagtcagggcattagcggaatatagggtg
 gttgcagcagaaaccagggaatcatttaagggcctgatctatcatggaaccaacttggaagagggagttccatcaagggtcagtggcagtggtctg
 gagcagattattctctaccatcagcagcctggagctgaagattttgcagactattactgtgtacaglatggtcagtttctccgacgttcggtggagg
 caccaagctggaatcaaagctagcaccaaggaccttctgttttccacttgccttcttctaagtctacttctggtggaactgctgcttgggtgtt
 ggtgaaagattactttctgagccagtgaccgtttcttggaactcaggtgtcttcatctggtgttcatacttcccagctgttcttcaatcttcaggactt
 tactcactttcttctgttaccgttcttctcaagctgggactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgacaa
 gaaagttgagcccaagtccttgacaagactcatactgtccaccgtgccagcacctgaaGtGtGtgaggaccgtcagtccttctgttctccaa
 agcctaaggataccttgatgatctccaggactcctgaagtcacatgtgtagttgtggatgtgagccatgaagatcctgaggtgaagttcaactggtatg
 tggatggtgtggaagtgcacaatgccaaagacaaagccgagagaggaacagtacaacagcacgtacaggggtgtctcagttctcactgttctccatca
 agattggttgaaaggcaagagtacaagtgcaaggtctccaacaaagccctcccagccccattgagaagaccatttccaaagcgaagggaaccc
 ccgtgaaccacaagtgacacacttctccatctcgcgatgaactgaccaagaaccaggtcagcttgacttgccctggtgaaaggcttctatccctctga
 catagctgtagagtgaggagagcaatgggcaaccggagaacaactacaagactacacctcccgttctcgattctgacggctccttctctctacagca

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FIGURE 17-6

agctcacagtggacaagagcaggtggcaacaaggaatgtcttctcatgctccgtgatgcatgaggctcttcacaatcactacacacagaagagtctc
tccttgtctccgggtaaatgaggatcc (SEQ ID NO:29)

Amino Acid Sequence

MGWSEITLFLVATATGVHSEVQVQQSGPELVTEGASVKISCKTSGYTFTEYTVHWVKQSHGKSLEWIGGINPTSGCT
NYNQRFPGKATLTVDRESSTAYMELRSLTSEDNAVYFCAGTLYGYFFDFWQGTTLTVSSGSTSGGGSGGGSGGGG
SDILMTQSPSSMEVSLGDSVSTCHASQGISGNIQWLQOKPGKSPFKGLIYHGTNLEEGVPSRPSGGSGGADYSLTIS
SLESSEDFADYYCVQYQGFPTFGGGTKLEIKASTKGPSVFPPLAPSSKSTSGGTAALGCLVKDYTFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKVEPKSCDKTHTCTPPCPAPEAAGGPSV
FLFPPKPKDTLMISRTPEVTCVYVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYKVVSVLTLYLHQDWLNGK
EYKCKVSNKALPAPIEKTIISKAKQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSEFFLYSKLTVDKGRWQQGNVFNESCVMHHEALHNHYTQKSLSLSPGK (SEQ ID NO:30)

E60 scFv LALAGA Mutant*Nucleic Acid Sequence*

gaattcacatgggatgggtcttgtatcatccttttcttggttgaacagctactgggtgttcattctgaggtccaggtgcaacagtctggacctgaactgg
gacgcctggggcctcagtgaagatatcctgcaagacttctggatacacttactgaatataccgtccactgggtgaagcagagccatggaaagagcc
ttgagtggattggaggcattaatctaccagtggtggtactaactacaaccagaggttcaggggcaaggccacattgactgtagacaggtcctccagc
acagcctacatggagctccgcagcctgacatctgaggattctgcagtcctattttgtgcaggaaccctctatggctacccttttgacttctggggccaag
gcaccactctcacagtctcctcaGGTTCAACTTCAGGAGGAGGATCAGGTGGTGGTTCAGGAGGTGGAGGATCTTCTgaca
tcctgatgaccaatctccatctccatgtctgtatctctgggagactcagtcagcatcacttgccatgcaagtcagggcattagcggtaatatagggtg
gttgacgagaaaccagggaatcatttaaggccctgatctatcatggaaccaacttgaagaggagggttccatcaagggttcagtggtgagtgatcg
gagcagattattctctcaccatcagcagcctggagctgaagattttgcagactattactgtgtacagtatggtcagtttctccgacgttcggtggagg
caccaagctggaaatcaaaagctagcaccaagggaaccttctgttttccacttgctccttcttaagtctacttctgggtggaactgctgctttgggtgttt
gggtgaaagattacttctgagccagtgacgtttcttggaactcaggtgctcttcatctggtgttcatacttcccagctgttcttcaatcttcaggactt
tactacttttctgtgttaccgttcttcttcaagcttgggcactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgacaa
gaaagttgagcccaagcttctgacaagactcatagctgtccaccgtgccagcactgaaGCtGCTggagCaccgtcagcttcttgtttcctccaa
agcctaaggataccttgatgatctccaggactcctgaagtcacatgtgtagttgtggatgtgagccatgaagatcctgaggtgaagttcaactggtatg
tggatggtgtggaagtgcacaatgccaaagacaagccgagagaggaacagtacaacagcacgtacagggttgctcagttctcactgttctccatca
agattggtgaaatggcaaagagtacaagtgaaggttccaacaaagccctccagccccattgagaagaccatttcaaagcgaaagggaacc
ccgtgaaccacaagtgtacacacttctccatctcgcgatgaactgaccaagaaccagggtcagcttgacttgctggtgaaaggcttctatccctctga
catagctgtagagtgaggagagcaatgggcaaccggagaacaactacaagactacacctccgttctcgattctgacggctccttctctctacagca
agctcacagtggacaagagcaggtggcaacaaggaatgtcttctcatgctccgtgatgcatgaggctcttcacaatcactacacacagaagagtctc
tccttgtctccgggtaaatgaggatcc (SEQ ID NO:31)

Amino Acid Sequence

MGWSEITLFLVATATGVHSEVQVQQSGPELVTEGASVKISCKTSGYTFTEYTVHWVKQSHGKSLEWIGGINPTSGCT
NYNQRFPGKATLTVDRESSTAYMELRSLTSEDNAVYFCAGTLYGYFFDFWQGTTLTVSSGSTSGGGSGGGSGGGG
SDILMTQSPSSMEVSLGDSVSTCHASQGISGNIQWLQOKPGKSPFKGLIYHGTNLEEGVPSRPSGGSGGADYSLTIS
SLESSEDFADYYCVQYQGFPTFGGGTKLEIKASTKGPSVFPPLAPSSKSTSGGTAALGCLVKDYTFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKVEPKSCDKTHTCTPPCPAPEAAGGPSV

FIGURE 17-7

FLRPPTKFKTILMISPTPEVITCVVVDVSHEDPEVKKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTIVLHQDLWNGK
 SYKCKVSNKALPALEKTIISKAKQPREPQVYTLPPSRDELTKNOVSLTCLVKGCFYPSDIAVEWESNGQPENNYKT
 PPVLDSDGSPFTLYSKLTVDKSRWQCGNVFSCSVMHREALNNHYTQKSLSLSPGK (SEQ ID NO:32)

E16 WT Heavy Chain*Nucleic Acid Sequence*

Gaattcacaatgggatggtcttgtatcatccttttcttggttgaacagctactggtgttcattctcaagttcaattgggtgcagtcagggtgctgaggtgaa
 gaaaccagggtgcttcagttaaggtttcttgaaggcttctggttacacattcacagattattggattgaatgggtgagacaagctcctggtcagggtctt
 gagggtgagggagatattctttgtgaactggaagaactagatacaacgagaaacttaaggctagagttactatgactgctgatacctctacatctact
 gcttacatggaacttagatctttgagatcagatgacactgctgtgtactattgtgctagggtcagcttcttatggagactacgctgactattggggacaag
 gtactactgttactgtgtcttctgcttctaccaaggaccttctgttttccacttgctccttctctaagtctacttctggtggaactgctgctttgggttgtt
 ggtgaaagattactttctgagccagtgaccgtttcttgaactcagggtgctcttacatctggtgttcatacttcccagctgttcttcaatcttcaggactt
 tactcactttcttctgttaccgttcttcttcaagcttgggcactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgacaa
 gaaagttgagcccaagcttctgtacaagactcatagtgctccaccgtgccagcacctgaactcttggaggaccgtcagctcttctgttctcctcaag
 cctaaggataccttgatgatctccaggactcctgaagtcacatgtgtagttgtggatgtgagccatgaagatcctgaggtgaagttcaactggtatgtg
 gatggtgtggaagtgcacaatgccaagacaaagccgagagaggaacagtaaacagcacgtacaggggtgtctcagttctcactgttctccatcaag
 attggttgaatggcaagagtacaagtgcaaggtctccaacaaagccctccagccccattgagaagaccatttccaagcgaaagggcaacccc
 gtgaaccacaaggttacacacttctccatctcgcgatgaactgaccaagaaccagggtcagcttgacttgccctggtgaaaggcttctatcctctgaca
 tagctgtagagtgaggagcaatgggcaaccggagaaactacaagactacacctccggttctcgattctgacggctccttctcctctacagcaag
 ctcacagtggacaagagcaggtggcaacaaggaatgtcttctcatgctcctgatgcatgagggtcttcacaatcactacacacagaagagtctctc
 cttgtctccgggtaatgaggatcc (SEQ ID NO:33)

Amino Acid Sequence

MQWSCIILFLVATATSVHQQVQLVQSCAEVKKPGASVNVVSCAKSGYTFEDYWIENRQAPGQGLEWNGDILCOGT
 SYNEELKARVTMTADTSTSTAYMELRSLPSDDTAVYICARSASYGIYADYWGQCTVTVSSASTKGPSVTFELAPSSK
 STSGGTAAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNWVHKPENTK
 VDKKVEPKSCDKTHTCTPFCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKKFNWYVDGVEVHNA
 KTKPREEQYNSTYRVVSVLTIVLHQDLWNGKEYKCKVSNKALPALEKTIISKAKQPREPQVYTLPPSRDELTKNOV
 SLTCLVKGCFYPSDIAVEWESNGQPENNYKTPPVLDSDGSPFTLYSKLTVDKSRWQCGNVFSCSVMHREALNNHYTQK
 SLSPGK (SEQ ID NO:34)

E16 Heavy Chain NQ Mutant*Nucleic Acid Sequence*

Gaattcacaatgggatggtcttgtatcatccttttcttggttgaacagctactggtgttcattctcaagttcaattgggtgcagtcagggtgctgaggtgaa
 gaaaccagggtgcttcagttaaggtttcttgaaggcttctggttacacattcacagattattggattgaatgggtgagacaagctcctggtcagggtctt
 gagggtgagggagatattctttgtgaactggaagaactagatacaacgagaaacttaaggctagagttactatgactgctgatacctctacatctact
 gcttacatggaacttagatctttgagatcagatgacactgctgtgtactattgtgctagggtcagcttcttatggagactacgctgactattggggacaag
 gtactactgttactgtgtcttctgcttctaccaaggaccttctgttttccacttgctccttctctaagtctacttctggtggaactgctgctttgggttgtt

FIGURE 17-8

gggtgaaagattactttctgagccagtgaccgtttcttggaaactcaggtgctcttaccatctgggtgttcatactttccagctgttcttcaatcttcaggactt
tactcactttcttctgtgttaccgttcttcttcaagcttgggcactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgacaa
gaaagttgagcccaagtcttgtgacaagactcatactgtccaccgtgccagcactgaacttcttggaggaccgtcagttcttctgttctccaaag
cctaaggatacttgatgatctccaggactcctgaagtcacatgtgtagttgtggatgtgagccatgaagatcctgagggtgaagttcaactggtatgtg
gatgggtgtggaagtgcacaatgccaagacaaagccgagagaggaacagtacCaAagcacgtacagggtgtctcagttctcactgttctccatcaag
attggttgaaatggcaaagagtacaagtgaaggtctccaacaaagccctcccagccccattgagaagaccatttccaaagcgaaagggcaacccc
gtgaaccacaagtgtacacacttctccatctcgcgatgaactgaccaagaaccaggtcagcttgacttgccctggtgaaaggcttctatccctctgaca
tagctgtagagtgggagagcaatgggcaaccggagaacaactacaagactacacctccgttctcgattctgacggctccttctctctacagcaag
ctcacagtggacaagagcaggtggcaacaaggaatgtcttctcatgctccgtgatgcatgaggctcttcacaatcactacacacagaagagtctctc
cttgtctccgggtaaatgaggatcc (SEQ ID NO:35)

Amino Acid Sequence

MSWRCITLFLVATATGVNSQVQVQVNSCAEVNRPASVSVSKASSYTFDYWIENRNDAPGCGLEWNGDILCTGR
RYNEKLPKAVIMTADTSTSTAYMELSLRSDDTAVYYCARSAHYQDYADYWGQGTFTVYSSASTKSPVEFLAIPSSK
STSGCTAALGCLVNDYFHEPVTVSNHNGALTSCVHTFPAYVLOSGLYSLSSVCTVSSSLGTQTYICNVNKKPSNIE
YDKKVEPKSCDKHTCTCPPELIGGPRSVLETPPKPKDTLMISRTPEVTCVQVDSHEUPEVKTHRYVDQVEVHNA
KTKPREEQYQSTYRVVSVLTLYLHQDNINGKEYCKVSNKALPAPTEKTLISKAKGQPREPQVYTLPPSRDELTKNOVS
LTCILVYGTFPSDIAVEWESNGQPFENNYKTTPEVLDSGGSTFLYSKLTVQKSRWQXNVFSCDMHEALHNRYTQKSE
SLSPGX (SEQ ID NO:36)

E16 Heavy Chain LALA Mutant*Nucleic Acid Sequence*

Gaattcacaatgggatggtcttgtatcatcctttcttgggtgcaacagctactggtgttcattctcaagttcaattggtgcagtcagggtgctgaggtgaa
gaaaccaggtgcttcagtttaaggttcttctaaggcttctggttacacattcacagattattggattgaatgggtgagacaagctcctggtcagggtctt
gagtgatggatgggagatattcttcttggaaactggaagaactagatatacaacgagaaacttaaggctagagttactatgactgctgatacctctacatctact
gcttacatggaacttagatctttagatcagatgacactgctgtgtactattgtgctaggtcagcttcttatggagactacgctgactattggggacaag
gtactactgttactgtgtcttctgcttctaccaaggaccttctgtttttccacttgctccttcttctaagttacttctggttggaaactgctgcttgggtgtgtt
gggtgaaagattactttctgagccagtgaccgtttcttggaaactcaggtgctcttaccatctgggtgttcatactttccagctgttcttcaatcttcaggactt
tactcactttcttctgtgttaccgttcttcttcaagcttgggcactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgacaa
gaaagttgagcccaagtcttgtgacaagactcatactgtccaccgtgccagcactgaaGCtGtGtggaggaccgtcagttcttctgttctccaa
agcctaaggatacttgatgatctccaggactcctgaagtcacatgtgtagttgtggatgtgagccatgaagatcctgagggtgaagttcaactggtatg
tggatggtgtggaagtgcacaatgccaagacaaagccgagagaggaacagtacaacagcacgtacagggttgtctcagttctcactgttctccatca
agattggttgaaatggcaaagagtacaagtgaaggtctccaacaaagccctcccagccccattgagaagaccatttccaaagcgaaagggcaacc
ccgtgaaccacaagtgtacacacttctccatctcgcgatgaactgaccaagaaccaggtcagcttgacttgccctggtgaaaggcttctatccctctga
catagctgtagagtgggagagcaatgggcaaccggagaacaactacaagactacacctccgttctcgattctgacggctccttctctctacagca
agctcacagtggacaagagcaggtggcaacaaggaatgtcttctcatgctccgtgatgcatgaggctcttcacaatcactacacacagaagagtctc
tcttgtctccgggtaaatgaggatcc (SEQ ID NO:37)

FIGURE 17-9*Amino Acid Sequence*

MCWSCITLFLVATATGVHQQVQLVQSGAEVKKPGASVYSCKASGYTFTDYWIEWVRQAPGQGLEWMQDILCCTGRT
 RYNEKFKARVTMTAQTSTSTAYMEIRSLPSDDTAVYYCARASASYGIYADYWGQGTFTVTVSSASTKGPSTVTEFLAPSSK
 STSGGTAALGCLVFDYFFPEPTVSWNSGALTSGVHTFPAVLQSSGLYSLSEVTVTPSSSLGTOTYIDIVNHKPSNTK
 YDKKVEPKSCLKTHTCPPCPAPEAAGGPSVFLFPFKPDITLMISKRIPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA
 KTKPREEQYNSTYRVVSVLTIVLHODNLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYITLPPSSDELTKNQV
 LTCLVKGFYPSDIAVEWESNQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSL
 SLSPCK (SEQ ID NO:38)

E16 Heavy Chain LALAGA Mutant*Nucleic Acid Sequence*

Gaattcacaatgggatggtcttctatcatccttttcttggtgcaacagctactggtgttcattctcaagttcaattggtgcagtcaggtgctgaggtgaa
 gaaaccaggtgcttcagtttaaggttttctgtaaggcttctggttacacattcacagattattggtgaatgggtgagacaagctcctggtcaggtgctt
 gagtggatgggagatattctttgtggaactggaagaactagatacaacgagaaacttaaggctagagttactatgactgctgatacctctacatctact
 gcttcatatggaacttagatctttgagatcagatgacactgctgtgtactattgtgctaggtcagcttctfatggagactacgctgactattggggacaag
 gtactactgttactgtgtcttctgcttctaccaaggaccttctgttttccacttgctccttcttaagtctacttctggtggaactgctgcttgggtgttt
 ggtgaaagattacttctctgagccagtgaccgtttcttggaactcaggtgctcttacatctggtgttcatcttccagctgttctcaatcttcaggactt
 tactacttttcttctgttaccgttcttcttcaagcttgggcactcagacctacatctgcaatgtgaatcacaaccagcaacaccaaggttgaca
 gaaagttgagcccaagcttcttgacaagactcatactgtccaccgtgccagcactgaaGCTGCTggagCaccgtcagcttcttcttctctccaa
 agcctaaggataccttgatgatctccaggactctgaagtcacatgtgtagttgtgagtgtagccatgaagatcctgaggtgaagttcaactggtatg
 tggatggtgtggaagtgcacaatgccaaagacaaagccgagagaggaacagtacaacagcacgtacagggtgtctcagttctcactgttctccatca
 agattggttgaatggcaaagagtaacagtgcaaggttctcaacaaagccctcccagccccattgagaagaccatttccaaagcgaagggaacc
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Amino Acid Sequence

MCWSCITLFLVATATGVHQQVQLVQSGAEVKKPGASVYSCKASGYTFTDYWIEWVRQAPGQGLEWMQDILCCTGRT
 RYNEKFKARVTMTAQTSTSTAYMEIRSLPSDDTAVYYCARASASYGIYADYWGQGTFTVTVSSASTKGPSTVTEFLAPSSK
 STSGGTAALGCLVFDYFFPEPTVSWNSGALTSGVHTFPAVLQSSGLYSLSEVTVTPSSSLGTOTYIDIVNHKPSNTK
 YDKKVEPKSCLKTHTCPPCPAPEAAGGPSVFLFPFKPDITLMISKRIPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNA
 KTKPREEQYNSTYRVVSVLTIVLHODNLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYITLPPSSDELTKNQV
 LTCLVKGFYPSDIAVEWESNQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSL
 SLSPCK (SEQ ID NO:40)