

IgG Variants for Induction of Immune Response without Adjuvant

Related Applications

[0001] This application claims the benefit of U.S. provisional patent application 62/980,012, filed February 21, 2020 titled “IgG Variants for Induction of Neutralizing Immune Response Without Adjuvant,” the entirety of the disclosure of which is hereby incorporated by this reference.

Incorporation-By-Reference of Material Electronically Filed

[0002] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 416,572 byte ASCII (text) file named “SeqList” created on February 21, 2021.

Technical Field

[0003] The disclosure relates to immunoglobulin variants that induce potent immune responses without an adjuvant and vectors for producing such variants in plants.

Background

[0004] Subunit vaccines consisting of recombinant protein antigens are very promising due to their safety, ease of production, and capacity to elicit targeted immune responses tailored towards desired epitopes. When delivered by themselves, however, these antigens often fail to generate robust and long-lasting immune responses, necessitating strategies to enhance their immunogenicity. Accordingly, recombinant protein antigens have been candidates for therapeutic uses. Protein fusions to the immunoglobulin Fc domain have demonstrated tremendous potential as therapeutic candidates. Fusion of a protein of interest to Fc can enhance the solubility and stability of the fusion partner while also allowing simple and cost-effective purification via protein A/G affinity chromatography. Furthermore, by interacting with neonatal Fc receptors (FcRn) in the body, Fc-fusions can escape lysosomal degradation, thereby extending the serum half-life of the Fc-fusion.

[0005] While much of the work with Fc-fusions has focused on improving their therapeutic potential, fewer studies have investigated Fc-fusion as a strategy to enhance antigen immunogenicity. Antigen-presenting cells containing Fcγ receptors and the complement receptor

C1q can uptake and process IgG-bound antigen. However, these interactions require high avidity binding for activation, and thus monovalent Fc-antigen fusions cannot efficiently utilize these pathways. On the other hand, larger antigen-antibody immune complexes with multivalent Fc domains can cross-link Fc receptors and efficiently bind C1q, resulting in greatly improved uptake and presentation by dendritic cells, as well as improved activation of T-cells. Immune complexes generated by mixing antibody with antigen often yields inconsistent results: immune responses may be skewed towards favorable antigenic sites, but overall immunogenicity may not be markedly improved.

[0006] By contrast, recombinant immune complexes (RICs), have been used to produce vaccine candidates for *Clostridium tetani*, Ebola virus, *Mycobacterium tuberculosis*, dengue virus, and human papillomavirus. An RIC is an IgG genetically fused to its cognate antigen, which allow the formation of larger highly immunogenic antigen-antibody clusters that mimic those found during native infection.

Summary

[0007] This disclosure is directed to recombinant proteins that are immunoglobulin variants, for example immunoglobulin G (IgG) variants, and their methods of production and use. In some aspects, the IgG variants are based on the 6D8 antibody. In certain embodiments where the parts of antibody in the recombinant protein is the 6D8 antibody, the Fc fusion, which comprises the CH2 domain and the CH3 domain of the antibody, comprises the substitution mutations E345R, E430G, and S440Y.

[0008] In some embodiments, the recombinant protein comprises a unit of an antigen, a unit of a Fc fusion comprising a CH2 domain and a CH3 domain of an IgG, a unit of a variable heavy chain (VH) domain of the IgG, a unit of a CH1 domain of the IgG; and a unit of a light chain variable (VL) domain of the IgG. The unit of the antigen is not an epitope of the IgG, and it is linked to the unit of the VH domain of the IgG at the N-terminus or to the CH3 domain of IgG at the C-terminus. The unit of the CH1 domain of IgG is linked to the CH2 domain of the IgG, while the unit of the VL domain of the IgG is fused to the unit of the VH domain of the IgG and to the CH1 domain of the IgG. In some implementations, the recombinant protein further comprises a unit of an epitope tag, wherein the epitope tag is an epitope of the IgG.

[0009] In some aspects, the recombinant protein comprises two units of the antigen, two units of the Fc fusion, two units of the CH1 domain of the IgG, two units of the VH domain of the IgG, and two units of the VL domain of the IgG. A disulfide bond formed at the linkage of the CH2 domain and the CH1 domain of the IgG links the two units of the Fc fusion. In some aspects, the each unit of the VL domain of the IgG is linked to a unit of VH domain of the IgG and the CH1 domain of the IgG. In particular implementations, the recombinant protein does not comprise any light chain constant (CL) domain of the IgG. In some embodiments of the recombinant protein comprising two units of the antigen, two units of the Fc fusion, two units of the CH1 domain of the IgG, two units of the VH domain of the IgG, and two units of light chain variable (VL) domain of the IgG, the recombinant protein further comprises two units of an epitope tag, wherein the epitope tag is an epitope of the IgG. In some implementations, the two units of the epitope tag are linked to the two units of the Fc fusion at the C-terminus of the CH3 domain of the IgG, while the two units of the antigen are linked to the two units of the VH domain of the IgG. In other implementations, the two units of the epitope tag are linked to the two units of the antigen, while the two units of the antigen are linked to the two units of Fc fusion at the C-terminus of the CH3 domain of the IgG. In particular embodiments, the antibody is the 6D8 antibody and the epitope tag comprises the peptide sequence YKLDIS (SEQ ID NO. 1).

[0010] In other embodiments, the recombinant protein comprises a unit of an antigen, a unit of a Fc fusion comprising a CH2 domain and a CH3 domain of an IgG, a unit of a VH domain of the IgG, a unit of a CH1 domain of the IgG, a unit of a VL domain of the IgG; and a unit of a CL domain of the IgG. In such embodiments, the IgG is the 6D8 antibody, and the Fc fusion comprises the substitution mutations E345R, E430G, and S440Y. The unit of the antigen is not an epitope of the 6D8 antibody, and it is linked to the CH3 domain of IgG at the C-terminus. The unit of the VL domain of the IgG is linked to the unit of the CL domain of the IgG, and the unit of the CL domain of the IgG is linked to the CH1 domain of the IgG. The unit of the CH1 domain of IgG is then linked to the CH2 domain of the IgG. In some aspects, the recombinant protein further comprises an epitope tag for the 6D8 antibody, for example comprising the peptide sequence YKLDIS (SEQ ID NO. 1).

[0011] In some aspects, the epitope tag in the recombinant protein comprises the sequence VYKLDISEA (SEQ ID NO. 2). In other aspects, epitope tag consists of the sequence YKLDIS (SEQ ID NO. 1).

[0012] In still other embodiments of the recombinant protein, IgG variant comprises two units of an antigen, two units of the Fc fusion, two units of a VH domain of the IgG, and two unit of a CH1 domain of the IgG. The antigen is not an epitope of the IgG, and the two units of the antigen are linked to the two units of the VH domain of the IgG at the N-terminus. The two units of the CH1 domain of IgG are linked to the two units of the Fc fusion at the CH2 domain of the IgG. In some aspects, the recombinant protein does not comprise a CL domain of the IgG and does not comprise a VL domain of the IgG.

[0013] In certain implementations, the antigen of the recombinant protein is from Zika virus, for example, the unit of the antigen comprises K301-T406 of Accession No. AMC13911.1. In other implementations, the antigen of the recombinant protein is from norovirus, for example, the unit of the antigen comprises at least one portion from the major capsid protein of noroviruses and/or at least one portion from nonstructural protein 1 (NS1) of noroviruses. In some embodiments, the unit of the antigen comprises at least one 5- to 500-residue long portion from the protruding domain of the norovirus major capsid protein, the shell domain of the norovirus major capsid protein, or from NS1 of noroviruses. In some aspects, the antigen of the recombinant protein may comprise a plurality of antigenic peptides. For example, in some implementations, the unit of the antigen comprises at least one portion from the major capsid protein or at least one portion from NS1 of a plurality of norovirus strains or species, for example, from human norovirus virus GI.3, human norovirus virus GII.4, and murine norovirus (MNV). In some embodiments, the unit of the antigen comprises at least one sequence set forth in SEQ ID NOs. 34-72.

[0014] The methods of production described herein comprise expressing the recombinant protein in a plant. In other words, the recombinant protein is produced in a transgenic plant. In certain implementations, the recombinant protein is expressed in a transgenic plant silenced for xylosyltransferase and fucosyltransferase. In some aspects, the method of producing the recombinant protein described herein comprises introducing into agrobacteria a vector selected from the group consisting of: pBYR11eM-h6D8ZE3, pBYR11eMa-BAZE3-Hgp371, pBYR11eMa-BAZE3-H, pBYKEMd-HZE3, pBYKEMd-ZE3H, pBYKEMd-ZE3Hx, pBYKEMd-HVLZe, pBYKEMd2-HVL-Hx, pBYKEMd2-HVLZnt, pBYKEMd2-ZHVLnt, pBYKEMd2-ZHVLx, pBYKEAM-N12MHd, pBYKEAM-NPHd, pBYKEAM-NSHd, pBYKEAM-NTHd, pBYKEHM-CPHd, pBYKEHM-CSHd, and pBYKEHM-CTHd and infiltrating a plant part with agrobacteria containing the vector to produce

a transformed plant part. Crude protein is then extracted from the transformed plant followed by purification for the recombinant protein. For methods of production where the vector introduced into agrobacteria is selected from the group consisting of: pBYR11eM-h6D8ZE3, pBYR11eMa-BAZE3-Hgp371, pBYR11eMa-BAZE3-H, and pBYKEMd-HZE3, the method further comprises co-infiltration with agrobacteria containing pBYKEMd-6D8K and agrobacteria containing the vector.

[0015] The methods of use described herein include a method of inducing an immune response in a subject against the antigen in the recombinant protein. The method comprises administering the described recombinant protein to the subject. In certain embodiments, the method induces in the subject an immune response against Zika virus. In such embodiments, the recombinant protein comprises an antigen with an amino acid sequence comprising K301-T406 of Accession No. AMC13911.1. In other embodiments, the method induces in the subject an immune response against norovirus, including human norovirus and MNV. In such embodiments, the recombinant protein comprises an antigen from the VP1 of a norovirus, for example from its protruding domain or the shell domain, or from NS1 of a norovirus.

[0016] In some implementation, the subject is administering a composition comprising a recombinant protein with an antigen from VP1 of a plurality of noroviruses and a recombinant protein with an antigen from NS1 of a plurality of noroviruses. In still other implementations, the recombinant protein is produced in a transgenic plant. In some aspects, the transgenic plant is silenced for xylosyltransferase and fucosyltransferase.

Brief Description of the Drawings

[0017] Figs. 1A-B depict, in accordance with certain embodiments, IgG fusion constructs and a comparison of their respective ability to bind C1q. Fig. 1A is a schematic representation of certain IgG fusion constructs disclosed herein. As used in this figure, ZE3 refers to the Zika envelope domain III containing amino acids K301 to T406; e refers to an epitope tag with a peptide sequence consisting of VYKLDISEA (SEQ ID NO. 2), which is the 6D8 binding motif for recombinant immune complex (RIC) formation; e with lightning bolt refers to an epitope tag with a peptide sequence consisting of YKLDIS (SEQ ID NO. 1) to reduce RIC formation; VH refers to the variable heavy domain; VL refers to the variably light domain; H refers to the heavy chain constant CH1 domain; L refers to the light chain constant domain; Fc refers to a portion of

heavy chain constant region, which consist of the CH2 and CH3 domains; and Fc with lightning bolt refers to the same as Fc but with E345R, E430G, and S440Y mutations to induce hexamer formation. Fig. 1B depicts C1q binding ELISA of purified IgG fusion constructs where the IgG fusion constructs are built on the mAb 6D8 human IgG1 backbone. ELISA plates were coated with 10 µg/ml human C1q and incubated with 10 µg/ml each fusion, using 6D8 antibody with no fusion as a negative control. Constructs were detected using polyclonal goat anti-human IgG-HRP. Mean OD450 values from three samples are shown $\pm$ standard error with one star (*) indicating $p < 0.05$ and three stars (***) indicating $p < 0.001$ as measured by one-way ANOVA with comparisons between the indicated groups.

[0018] Figs. 2A-B depict, in accordance with certain embodiments, the expression of the IgG fusions built on 6D8 antibody backbone. Fig. 2A depicts the ELISA and gel quantification of IgG fusion construct expression. Clarified protein extracts from leaf spots agroinfiltrated with each IgG fusion construct were analyzed by ELISA or SDS-PAGE followed by gel quantification. For ZE3 ELISA, plates were coated with polyclonal mouse anti-ZE3, incubated with serial dilutions of extracts from each IgG fusion using purified HLZ as a standard, and probed with goat anti-human IgG-HRP. For IgG ELISA, plates were coated with serial dilutions of extracts or human IgG standard and probed with goat anti-human IgG-HRP. For gel quantification, ImageJ software was used to compare the IgG fusion band intensity visualized on stain-free polyacrylamide gels using purified 6D8 as standard. Columns represent means $\pm$ standard error from three independently infiltrated leaf samples. Fig. 2B depicts a representative gel image of clarified leaf extracts were separated by reducing SDS-PAGE. The band position corresponding to each respective heavy chain/ZE3 fusion is indicated “ZH/HZ.” The small shift in size in HLZe and HLZd is due to epitope tag presence. The large subunit of Rubisco “RbcL” along with the ZFc and Fc bands are indicated.

[0019] Fig. 3 depicts, in accordance with certain embodiments, the purification of IgG fusions built on a 6D8 antibody backbone. Agroinfiltrated leaf material from 1-3 plants per construct was homogenized, clarified, and purified by protein G affinity chromatography. The peak elutions were pooled and separated on nonreducing and reducing SDS-PAGE using stain-free polyacrylamide gels. Representative lanes for each construct are shown.

[0020] Figs. 4A-B depict, in accordance with certain embodiments, the sucrose gradient density centrifugation of IgG fusions built on a 6D8 antibody backbone. Purified IgG

fusions were separated by sucrose gradient centrifugation using 5/10/15/20/25% discontinuous sucrose layers. Gradient fractions were analyzed by SDS-PAGE and representative results are shown. The relative band intensity was quantified using ImageJ software and the peak band was arbitrarily assigned the value of 1.

[0021] Figs. 5A-B depict, in accordance with certain embodiments, the stability and C1q binding of purified IgG fusions built on a 6D8 antibody backbone. Samples of purified IgG fusions were frozen and thawed once after purification (initial) or additionally subjected to either additional 5 freeze/thaw cycles, incubation for 2 weeks at 4°C, or incubation for 2 weeks at room temperature. Fig. 5A shows the relative proportion of fully assembled product (analyzed using ImageJ software) for each treatment. After the treatments, samples were separated on reducing and nonreducing SDS-PAGE gels. Fig. 5B shows the C1q binding ELISA results of the IgG constructs after each treatment. Columns represent the mean OD₄₅₀ value $\pm$ standard error from three samples.

[0022] Figs. 6A-6B depict, in accordance with certain embodiments, the results of mouse immunization with the IgG fusions and the serum titers. BALB/c mice (6 per group) were immunized twice two weeks apart subcutaneously with a dose that would deliver 8 μ g ZE3 for each IgG fusion or with PBS as a control. Mouse serum samples were collected two weeks after the final dose. For Fig. 6A, serially diluted mouse serum was analyzed for total IgG production by ELISA. The endpoint was taken as the reciprocal of the greatest dilution that gave an OD₄₅₀ reading at least twice the background. Three stars (***) indicates $p < 0.01$ by ANOVA comparing the indicated columns to ZE3. For Fig. 6B, mouse serum samples were diluted 1:100 and analyzed for IgG2a production by ELISA. (**) indicates $p < 0.05$ and (***) indicates $p < 0.01$ by ANOVA comparing the indicated columns to HLZ.

[0023] Fig. 7 depicts, in accordance with certain embodiments, a single-chain antibody (HVL) and HVL-related constructs. The single-chain antibody shown contains the 6D8 antibody variable heavy region linked to a variable light chain region that is directly fused to the 6D8 antibody heavy chain. As used in this figure, ZE3 refers to the Zika envelope domain III containing amino acids K301 to T406; e refers to an epitope tag with a peptide sequence consisting of VYKLDISEA (SEQ ID NO. 2), which is the 6D8 binding motif for RIC formation; VH refers to the variable heavy domain from the 6D8 antibody; VL refers to the variable light domain from the 6D8 antibody; H refers to the heavy chain constant region CH1 domain from the 6D8 antibody; Fc refers to the heavy chain constant region consisting of the CH2 and CH3 domains from the 6D8

antibody; and Fc with lightning bolt refers to the same as Fc but with E345R, E430G, and S440Y mutations to induce hexamer formation.

[0024] Fig. 8 depicts, in accordance with certain embodiments, the purification and western analysis of HVL and HVL-fusion constructs. After protein G purification of the HVL, HVLZe, and ZHVLHx constructs, samples from the peak elutions were separated on a 4-15% polyacrylamide, stain-free gel. Analysis of the HVLHX and HVLZnt constructs was conducted through a western blot containing small-scale leaf samples that had been clarified through centrifugation. The samples were separated on 4-15% polyacrylamide gel, transferred to a PVDF membrane, and detected with HRP-labeled goat anti-human IgG antibody.

[0025] Fig. 9 depicts, in accordance with certain embodiments, the C1q binding comparison of IgG fusion constructs produced in wildtype and glycoengineered plants. ELISA plates were coated with 10 µg/ml human C1q and incubated with 5 µg/ml each purified construct. Constructs were detected using polyclonal goat anti-human IgG-HRP. Mean OD450 values from three replicates are shown ± standard error with two stars indicating $p < 0.01$ as measured by one-way ANOVA with comparisons between the indicated groups. As used in this figure, wt refers to constructs made in wildtype *Nicotiana benthamiana* plants, while GnGn refers to constructs made in glycoengineered plants silenced for xylosyltransferase and fucosyltransferase.

[0026] Fig. 10 depicts, in accordance with certain embodiments, C1q binding comparison between IgG fusions built on a 6D8 antibody backbone. ELISA plates were coated with 10 µg/ml human C1q and incubated with 10-fold serial dilutions of each purified construct starting at 50 µg/ml. Constructs were detected using polyclonal goat anti-human IgG-HRP. Mean OD450 values from three replicates are shown. As used in this figure, wt refers to constructs made in wildtype *N. benthamiana* plants; GnGn, refers to constructs made in glycoengineered plants silenced for xylosyltransferase and fucosyltransferase; Ag refers to dengue consensus dengue E protein domain III (cE) tagged with the 6D8 epitope; and ZHL refers to the same as HL but with ZE3 fused to the N-terminus of the 6D8 heavy chain.

[0027] Fig. 11 depicts, in accordance with certain embodiments, epitope binding of single-chain antibody built on a 6D8 antibody bone (HVL) was compared to that of wildtype 6D8 antibody. ELISA plates coated with 900 ng of purified epitope-tagged protein were incubated with serial dilutions of either HVL (single-chain antibody with the variable heavy chain linked to the variable light chain region that is fused to the 6D8 heavy chain) or full-length 6D8 antibody.

[0028] Fig. 12 depicts, in accordance with certain embodiments, representative SDS-PAGE demonstrating RIC insolubility. Protein was extracted from leaves of *N. benthamiana* agroinfiltrated with the indicated constructs and separated by SDS-PAGE followed by western blotting under nonreducing conditions. The constructs were detected using goat anti-human IgG-HRP. Soluble (S) refers to the clarified crude leaf extract, while insoluble (IS) refers to the pellet following clarification that had been resuspended in SDS sample buffer.

[0029] Figs. 13A-C depict, in accordance with certain embodiments, studies on the solubility and binding of 6D8 epitope tag mutants. For Figs. 13A-B, protein was extracted from leaves of *N. benthamiana* agroinfiltrated with the indicated constructs and separated under nonreducing conditions by SDS-PAGE followed by western blotting using goat anti-human IgG-HRP as probe. Soluble refers to the clarified crude leaf extract, while insoluble refers to the pellet following clarification that had been resuspended in SDS sample buffer. The epitope mutant designated “a” has a peptide sequence consisting of VYKLDISEA (SEQ ID NO. 2). The epitope mutant designated “b” has a peptide sequence consisting of VYKLDISE (SEQ ID NO. 3). The epitope mutant designated “c” has a peptide sequence consisting of YKLDISE (SEQ ID NO. 4). The epitope mutant designated “d” has a peptide sequence consisting of YKLDIS (SEQ ID NO. 1). For Fig. 13C, ELISA plates coated with serial dilutions of purified heavy chains containing the indicated epitope tag mutants were probed with full-size 6D8 followed by goat anti-human kappa-HRP. Mean OD450 values are shown from three replicates $\pm$ standard error.

[0030] Figs. 14A and 14B depict, in accordance with certain embodiments, a schematic of recombinant immune complex (RIC) constructs targeting norovirus (NoV) and a SDS-PAGE of the purified RIC constructs, respectively. In Fig. 14B, RIC constructs targeting conserved epitopes of VP1 protruding domain are labeled as P-RIC (the unit of the antigen comprises tandem-linked sequences of SEQ ID NOs. 34-42); VP1 shell domain, labeled as S-RIC (the unit of the antigen comprises tandem-linked sequences of SEQ ID NOs. 45-53); T-cell epitopes, labeled as T-RIC (the unit of the antigen comprises tandem-linked sequences of SEQ ID NOs. 58-72); and containing a unit of an antigen comprising epitopes from nonstructural protein 1 (NS1) of human norovirus GI.3, human norovirus GII.4, and murine norovirus (MNV), labeled as N-RIC (the unit of the antigen comprises tandem-linked sequences of SEQ ID NOs. 55-57). The RICs were purified, separated by SDS-PAGE under reducing conditions, and visualized under UV via stain-

free 2,2,2-trichloroethanol (TCE) imaging. “+Inhibitors” refers to the addition of plant protease inhibitor cocktail to reduce degradation of N-RIC.

[0031] Fig. 15 depicts, in accordance with certain embodiment, a SDS-PAGE (left) and western blot (right) of purified RIC constructs targeting norovirus. The purified RICs were separated by SDS-PAGE under nonreducing (NR) or reducing (R) conditions and visualized under UV via stain-free 2,2,2-trichloroethanol (TCE) imaging (left). Western blot was performed with anti-murine norovirus 1 (MNV) NS1/2 antibodies, or anti-MNV capsid antibodies. P, P-RIC; S, S-RIC; T, T-RIC; N-RIC.

[0032] Fig. 16 depicts, in accordance with certain embodiments, serum binding to murine norovirus. Serum from mice immunized with 3 doses of 2 µg total antigen delivered via RIC constructs or with human norovirus GI.I (Norwalk virus)/ human norovirus GII.4 (Minerva virus) VLPs mixed (labeled as “VLP”) were tested for binding to murine norovirus 1 (MNV-1) virus by ELISA. Polystyrene 96-well plates were coated with rabbit anti-MNV capsid, blocked with 5% PBSTM, and then incubated with lysate from RAW267.4 cells infected with MNV-1. After incubation, serial dilutions of mouse sera were added to the plate, and then detected with goat anti-mouse IgG-HRP conjugate. Mean OD₄₅₀ values ± standard error from pooled mouse sera containing two technical replicates are shown. Abbreviations: P-RIC: serum from mice immunized with P-RIC; S-RIC: serum from mice immunized with S-RIC; T-RIC: serum from mice immunized with T-RIC; N-RIC: serum from mice immunized with N-RIC, PSTN-RIC: serum from mice immunized with P-RIC, S-RIC, T-RIC, and N-RIC but with a quarter of the dose used for the individual experiments.

[0033] Fig. 17 depicts, in accordance with certain embodiments, serum binding to GI.I VLPs. Serum from mice immunized with 3 doses of 2 µg total antigen delivered via RIC constructs were tested for binding to GI.I norovirus VLPs by ELISA. Polystyrene 96-well plates were incubated with plant-made Norwalk virus VLPs, blocked, and then incubated with serial dilutions of mouse sera. Bound antibodies were detected with goat anti-mouse IgG-HRP conjugate. Mean OD₄₅₀ values ± standard error from six samples containing two technical replicates are shown. Abbreviations: P-RIC: serum from mice immunized with P-RIC; S-RIC: serum from mice immunized with S-RIC; T-RIC: serum from mice immunized with T-RIC; N-RIC: serum from mice immunized with N-RIC, PSTN-RIC: serum from mice immunized with P-RIC, S-RIC, T-RIC, and N-RIC but with a quarter of the dose used for the individual experiments.

[0034] Fig. 18 depicts, in accordance with certain embodiments, serum binding to human norovirus GII.2 VLPs. Serum from mice immunized with 3 doses of 2 µg total antigen delivered via RIC constructs were tested for binding to human norovirus GII.2 (Snow Mountain virus) VLPs by ELISA. Polystyrene 96-well plates were incubated with plant-made GII.2 VLPs, blocked, and then incubated with serial dilutions of mouse sera. Bound antibodies were detected with goat anti-mouse IgG-HRP conjugate. Mean OD₄₅₀ values ± standard error from pooled serum samples containing two technical replicates are shown. Abbreviations: P-RIC: serum from mice immunized with P-RIC; S-RIC: serum from mice immunized with S-RIC; T-RIC: serum from mice immunized with T-RIC; N-RIC: serum from mice immunized with N-RIC, PSTN-RIC: serum from mice immunized with P-RIC, S-RIC, T-RIC, and N-RIC but with a quarter of the dose used for the individual experiments.

[0035] Fig. 19 depicts, in accordance with certain embodiments, IFN-γ production from mice immunized with the VLP or RIC constructs targeting norovirus. Splenocytes were harvested from immunized mice and stimulated with their corresponding antigen for 72 hours. Supernatants from stimulated and unstimulated splenocytes were collected and assayed IFN-γ production by ELISA. Polystyrene 96-well plates were coated with serially diluted splenocyte supernatants or recombinant mouse IFN-γ standard, blocked with 5% PBSTM, and then probed with rat anti-mouse IFN-γ and goat anti-rat IgG horseradish peroxidase conjugate. OD₄₅₀ values were used to construct a standard curve, and the mean IFN-γ production ± standard error from six samples each containing two technical replicates are shown. Abbreviations: P-RIC: serum from mice immunized with P-RIC; S-RIC: serum from mice immunized with S-RIC; T-RIC: serum from mice immunized with T-RIC; N-RIC: serum from mice immunized with N-RIC, PSTN-RIC: serum from mice immunized with P-RIC, S-RIC, T-RIC, and N-RIC but with a quarter of the dose used for the individual experiments.

[0036] Figs. 20-39 depict maps of pBYR11eM-h6D8ZE3, pBYR11eMa-BAZE3-Hgp371, pBYR11eMa-BAZE3-H, pBYKEMd-HZE3, pBYKEMd-ZE3H, pBYKEMd-ZE3Hx, pBYKEMd-HVLZe, pBYKEMd2-HVL-Hx, pBYKEMd2-HVLZnt, pBYKEMd2-ZHVLnt, pBYKEMd2-ZHVLe, pBYKEMd2-ZHVLhx, pBYKEMd-6D8K, pBYKEAM-N12MHd, pBYKEAM-NPHd, pBYKEAM-NSHd, pBYKEAM-NTHd, pBYKEHM-CPHd, pBYKEHM-CSHd, and pBYKEHM-CTHd.

Detailed Description

[0037] Detailed aspects and applications of the disclosure are described below in the following drawings and detailed description of the technology. Unless specifically noted, it is intended that the words and phrases in the specification and the claims be given their plain, ordinary, and accustomed meaning to those of ordinary skill in the applicable arts.

[0038] In the following description, and for the purposes of explanation, numerous specific details are set forth in order to provide a thorough understanding of the various aspects of the disclosure. It will be understood, however, by those skilled in the relevant arts, that embodiments of the technology disclosed herein may be practiced without these specific details. It should be noted that there are many different and alternative configurations, devices and technologies to which the disclosed technologies may be applied. The full scope of the technology disclosed herein is not limited to the examples that are described below.

[0039] The singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a step” includes reference to one or more of such steps.

[0040] As used herein, the term “6D8 antibody” refers to a monoclonal antibody against the GP1 protein of Ebola virus (described in Wilson et al., 2000; plant optimized sequence described in Huang et al., 2010). In some embodiments, the CH1 domain of the 6D8 antibody refers to a peptide sequence comprising TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS WNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC (SEQ ID NO. 5) or a peptide sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence similarity. In some embodiments, the CH2 domain of the 6D8 antibody refers to a peptide sequence comprising DKTHTCPPCPAPELLGGPSVFLFPPKP KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEHNAKTKPREEQYNSTYRVVSVLTVLHQD WLNQKEYKCKVSNKALAPIEKTISKAKG (SEQ ID NO. 6) or a peptide sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence similarity. In some embodiments, the CH3 domain of the 6D8 antibody refers to a peptide sequence comprising QPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSF LYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO. 7). In some embodiments, the variable heavy chain domain of the 6D8 antibody refers to a peptide sequence comprising GenBank Accession No. AEB96146 or a peptide sequence having at least 80%, at least

85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence similarity. In some embodiments, the variable light chain domain of the 6D8 antibody refers to a peptide sequence comprising GenBank Accession No. AEB96146 or a peptide sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence similarity. In some aspects, nucleic acid encoding the light chain variant domain is set forth in GenBank Accession No. HQ407546. In some aspects, the nucleic acid encoding the heavy chain variant domain is set forth in GenBank Accession No. AEB96148.

[0041] As used herein, the term “linked” when used to describe a protein structure or configuration of protein domains refers to a linkage between two domains or structural portions, preferably where there are no other intervening domains or structural portions except a linker. In some aspects, the term “linker” as used herein refers to an amino acid sequence between protein domains or structural portions where its only function is to link the protein domains or structural portions. For example, the linker is sequence consisting of glycine, valine, and/or threonine residues. In some embodiments, the linker is sequence consisting of glycine, valine, threonine, alanine, and/or serine residues. In some aspects, the linker consists of 1 to 50 amino acids in length, for example, 3, 4, 6, 10, 12, 14, or 16 amino acids in length. In certain embodiments, the linker is a flexible linker.

[0042] As used herein, the term “unit” refers to a single peptide that is a functional fragment of a larger multi-component protein. The single peptide may be an antigenic fragment or a domain of a protein, for example a domain of an immunoglobulin. Accordingly, as used herein, the description of a recombinant protein comprising two units of a peptide refers to the recombinant protein having two such functional fragments that may or may not be linked together, for example, a dimer of the peptide.

[0043] As used herein, the term “immune complex” refers to a complex comprising immunoglobulin molecules or fragments thereof bound to its cognate antigen. As used herein, the term “recombinant immune complex” or “RIC” refers to an immune complex that is not produced by the original species that naturally produces the immunoglobulin molecule in the immune complex. For example, an exemplary recombinant immune complex comprises human immunoglobulin, which is synthesized in plants.

[0044] The disclosure relates to immunoglobulin variants. In particular preferred embodiments, the disclosure is directed to recombinant proteins that are variants of

immunoglobulin G, also referred to herein as IgG fusions. In spite of the deviation from the natural structure of immunoglobulins, the described recombinant proteins do have immunogenic potential. In fact, the immune response induced by the recombinant protein is the same kind of immune response expected of the antigen in the recombinant protein. The recombinant proteins described herein are suitable for creating vaccines for targeting a variety of pathogens, for example Zika virus and norovirus.

[0045] Regardless of the variations in immunoglobulin structure, glycosylation state of the Fc strongly controls its function. By modulating the stability, conformation, and aggregation of the Fc, glycosylation can enhance or inhibit binding to Fcγ receptors, FcRn, and C1q. These alterations result in important differences in antibody effector functions, including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), and antibody-dependent enhancement of viral infection (ADE). Advances in glycoengineering have allowed targeted optimization of the Fc glycosylation state in a variety of recombinant expression systems. Using glycoengineered plants lacking core xylose and fucose N-glycans, an anti-CD20 antibody was produced with improved binding to FcγRI, FcγRIIIa, and C1q. The antibody had enhanced ADCC and CDC compared to a commercial anti-CD20 antibody produced in mammalian cells. Similarly, anti-DENV antibodies produced in glycoengineered plants have been shown to forgo their ADE activity and, consequently, have superior efficacy and safety profiles than their mammalian cell-produced counterparts. Antibody therapeutics made in glycoengineered plants have been used to treat rhesus macaques and humans with Ebola, HIV, and Chikungunya virus disease.

[0046] Mutations in the Fc region have been identified that confer desirable properties to antibodies. Introduction of M252Y, S254T, and T256E mutations increased the serum half-life of an anti-respiratory syncytial virus antibody from 20 days to 60 days in humans by improving interactions with FcRn under low pH conditions. An H237Y mutation reduced detrimental cleavage of the hinge region while improving FcγRIII binding and ADCC activity. Engineering additional disulfide bonds has been reported to prevent unfolding and aggregation of Fc-fusions. S239D and I332E mutations improved FcγRIII binding and ADCC activity of a CD37 antibody. Replacing the IgG1 hinge region with the longer hinge region from IgG3 or from a camelid antibody increased ADCC of an epidermal growth factor receptor antibody. An IgG1 with E345R, E430G, and S440Y mutations formed hexamers that had greatly enhanced C1q binding and

complement activation. The mutations T437R and K248E also promoted multimerization and improved effector functions of an OX40 receptor antibody.

[0047] The IgG variants described herein can be properly assembled with human-like glycosylation and expressed at very high levels in plants. These constructs can be efficiently made in plants, assemble appropriately, and purified via protein G column chromatography. As shown in the examples, the purified recombinant proteins are potently immunogenic.

[0048] As shown in the examples described herein, optimizing various aspects of the antibody design, such as complex size, to produce the described immunoglobulin variants resulted in optimized expression levels in plant and improved stability, solubility, and immune receptor binding. In certain embodiments where the immunoglobulin variants are designed with a portion of the Zika virus envelope protein domain III as the antigen, total IgG titers raised by the administration of the immunoglobulin variant was 150-fold higher compared to the total IgG titer raised by administration of the antigen alone. In fact, the endpoint titers was >1:500,000 with administration of only two doses of the immunoglobulin variant without any adjuvant. The recombinant proteins could be produced at levels exceeding the estimated thresholds for commercial viability of antibody therapeutics.

Recombinant Protein

[0049] The recombinant proteins described herein are variant immunoglobulin structures comprising a unit of an antigen, a unit of a Fc fusion, a unit of a variable heavy chain (VH) domain of the IgG, and a unit of a CH1 domain of the IgG. The Fc fusion comprises a CH2 domain and a CH3 domain of an IgG. Protein fusions to the immunoglobulin Fc domain are highly successful therapeutics. They can enhance the solubility and stability of the fusion partner, while also providing a means for simple and cost-effective purification. Furthermore, by interacting with neonatal Fc receptors (FcRn) in the body, Fc-fusions can escape lysosomal degradation, thereby extending the serum half-life of the Fc-fusion. The recombinant protein induces a greater immune response against the antigen attached to the recombinant protein than the antigen alone. Thus, the recombinant protein increases the immunogenicity of the antigen.

[0050] The unit of the antigen is not an epitope of the IgG. In certain embodiments, the unit of the antigen is from a different organism than the epitope of the IgG. The amino acid sequence of the unit of the antigen is between 5 and 500 residues long. In certain embodiments,

the unit of the antigen is between 5 and 400 residues long. For example, the unit of the antigen is between 5 and 300 residues, between 5 and 250 residues, between 5 and 200 residues, between 5 and 100 residues, between 9 and 300 residues, between 9 and 250 residues, between 9 and 200 residues, between 9 and 100 residues, between 10 and 300 residues, between 10 and 250 residues, between 10 and 200 residues, between 10 and 100 residues, between 20 and 300 residues, between 20 and 250 residues, between 20 and 200 residues, between 20 and 100 residues, between 30 and 300 residues, between 30 and 250 residues, between 30 and 200 residues, between 30 and 100 residues, between 50 and 300 residues, between 50 and 250 residues, between 50 and 200 residues, or between 50 and 100 residues in length. In some aspects, the unit of the antigen comprises tandem-linked epitopes.

[0051] In some aspects, the tandem-linked epitopes comprise different epitopes, which may or may not be from the same or similar antigen protein. For example, a recombinant protein that targets a plurality of norovirus can comprise epitopes on the same target antigen from the plurality of noroviruses (see recombinant immune complexes studied in Figs. 15-19) to address the diversity genotypes of noroviruses. Thus, the tandem-linked epitopes may comprise epitopes from the same antigenic protein. In other aspects, the tandem-linked epitopes are repetitions of a single epitope.

[0052] The unit of the antigen is linked to the unit of the VH domain of the IgG at the N-terminus or to the CH3 domain of IgG at the C-terminus. The unit of the CH1 domain of IgG is linked to the CH2 domain of the IgG. In some aspects, the unit of an antigen, the unit of the Fc fusion, the unit of the VH domain of the IgG, and the unit of a CH1 domain of the IgG forms half of the recombinant protein. For example, the recombinant protein self assembles upon production wherein a disulfide bond is formed at the linkage of the CH2 domain and the CH1 domain to link two units of the Fc fusion (see Figs. 1A and 7). Accordingly in some embodiments, the recombinant protein comprises two units of the antigen, two units of the Fc fusion, two units of the VH domain of the IgG, and two units of the CH1 domain of the IgG. In certain embodiments, the antibody is the 6D8 antibody.

[0053] In certain embodiments, the recombinant protein comprises a unit of an antigen, a unit of a Fc fusion comprising a CH2 domain and a CH3 domain of an IgG, a unit of a VH domain of the IgG, a unit of a CH1 domain of the IgG; and a unit of a light chain variable (VL) domain of the IgG. The unit of the antigen is not an epitope of the IgG, and it is linked to the unit

of the VH domain of the IgG at the N-terminus or to the CH3 domain of IgG at the C-terminus. The unit of the CH1 domain of IgG is linked to the CH2 domain of the IgG, while the unit of the VL domain of the IgG is fused to the unit of the VH domain of the IgG and to the CH1 domain of the IgG.

[0054] In some aspects, the recombinant protein is self-assembled upon production, and a disulfide bond formed at the linkage of the CH2 domain and the CH1 domain of the IgG links the two units of the Fc fusion. Accordingly, the recombinant protein comprises two units of the antigen, two units of the Fc fusion, two units of the CH1 domain of the IgG, two units of the VH domain of the IgG, and two units of the VL domain of the IgG. In some aspects, the each unit of the VL domain of the IgG is linked to a unit of VH domain of the IgG and the CH1 domain of the IgG. In particular implementations, the recombinant protein does not comprise any light chain constant (CL) domain of the IgG. The recombinant protein of such embodiments is a single-chain antibody variant (see for example, Fig. 7). As shown in Fig. 8, the single-chain antibody variant can be produced in plants and efficiently purified. The single-chain antibody variant with the IgG being the 6D8 antibody also retains its ability to bind the 6D8 epitope (Fig. 11).

[0055] In some embodiments of the recombinant protein comprising two units of the antigen, two units of the Fc fusion, two units of the CH1 domain of the IgG, two units of the VH domain of the IgG, and two units of the VL domain of the IgG, the recombinant protein further comprises two units of an epitope tag, wherein the epitope tag is an epitope of the IgG. In some implementations, the two units of the epitope tag are linked to the two units of the Fc fusion at the C-terminus of the CH3 domain of the IgG, and the two units of the antigen are linked to the two units of the VH domain of the IgG. In other implementations, the two units of the epitope tag are linked to the two units of the antigen, and the two units of the antigen are linked to the two units of Fc fusion at the C-terminus of the CH3 domain of the IgG. Where the antibody is the 6D8 antibody, the epitope tag comprises the peptide sequence YKLDIS (SEQ ID NO. 1). In some aspects, the epitope tag comprises the sequence VYKLDISEA (SEQ ID NO. 2). In other aspects, the epitope tag consists of the sequence YKLDIS (SEQ ID NO. 1).

[0056] In other embodiments, the recombinant protein comprises a unit of an antigen, a unit of a Fc fusion comprising a CH2 domain and a CH3 domain of an IgG, a unit of a VH domain of the IgG, a unit of a CH1 domain of the IgG, a unit of a VL domain of the IgG; and a unit of a CL domain of the IgG. In such embodiments, the IgG is the 6D8 antibody, and the Fc

fusion comprises at least one substitution mutation selected from the group consisting of: E345R, E430G, and S440Y. In certain embodiments, the Fc fusion comprises the substitution mutations E345R, E430G, and S440Y. The unit of the antigen is not an epitope of the 6D8 antibody, and it is linked to the CH3 domain of IgG at the C-terminus. The unit of the VL domain of the IgG is linked to the unit of the CL domain of the IgG, and the unit of the CL domain of the IgG is linked to the CH1 domain of the IgG. The unit of the CH1 domain of IgG is then linked to the CH2 domain of the IgG. In some aspects, the recombinant protein further comprises an epitope tag for the 6D8 antibody, for example comprising the peptide sequence YKLDIS (SEQ ID NO. 1). In some aspects, the epitope tag in the recombinant protein comprises the sequence VYKLDISEA (SEQ ID NO. 2). In other aspects, epitope tag consists of the sequence YKLDIS (SEQ ID NO. 1).

[0057] In still other embodiments of the recombinant protein, IgG variant comprises two units of an antigen, two units of the Fc fusion, two units of a VH domain of the IgG, and two unit of a CH1 domain of the IgG. The antigen is not an epitope of the IgG, and the two unit of the antigen are linked to the two units of the VH domain of the IgG at the N-terminus. The two units of the CH1 domain of IgG are linked to the two units of the Fc fusion at the CH2 domain of the IgG. In some aspects, the recombinant protein does not comprise a CL domain of the IgG and does not comprise a VL domain of the IgG.

Zika Virus

[0058] Zika virus (ZIKV) is a substantial global health threat that lacks safe, affordable, and efficacious vaccines. In addition to their widespread therapeutic value, IgG fusions are promising vaccine candidates due to their safety and self-adjuvating nature.

[0059] In exemplary embodiments, some IgG variants were designed to generate an immune response against ZIKV. Accordingly, the antigen in the recombinant protein is an antigen targeting ZIKV. In some aspects, the antigen targets Zika virus envelope domain III (ZE3). These IgG variants were highly stable and highly expressed in plants. Accordingly, the recombinant proteins described herein are vaccine candidates, for example against ZIKV.

[0060] In some aspects, the antigen targeting ZE3 comprises K301-T406 of Accession No. AMC13911.1. In other aspects, the antigen targeting ZE3 comprises a peptide sequence having at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% sequence similarity to K301-T406 of Accession No. AMC13911.1. In still other aspects, the

antigen targeting ZE3 comprises a sequence that is a functionally equivalent version of corresponding regions of GenBank Accession No. AMC13911 from other strains of Zika virus, for example, the corresponding sequences of ZE3 in GenBank Accession Nos. AY632535, KU321639, KJ776791, KF383115, KF383116, KF383117, KF383118, KF383119, KF268948, KF268949, KF268950, EU545988, KF993678, JN860885, HQ234499, KU501215, KU501216, KU501217.

[0061] The recombinant proteins described herein targeting ZIKV elicited strong immune responses against ZIKV without adjuvants and only two doses (Figs. 6A, 6B). Many of the recombinant proteins were produced at high levels in plants (0.5-1.5 mg/g LFW) (Fig. 2) and were stable upon repeated freeze-thaw and storage (Fig. 5). Compared to ZE3 antigen alone, the best IgG fusion groups produced over 100-fold higher antibody responses. This is in agreement with previous studies showing that ZE3 or ZIKV E are weakly immunogenic on their own, requiring an adjuvant and 3-4 doses.

[0062] Constructs expected to form polymeric structures were the most resistant to degradation and had the highest overall yield. By optimizing intermolecular binding of RIC, smaller complexes were generated that had greatly improved solubility, yielding 1.5 milligrams IgG fusion per gram leaf fresh weight (mg/g LFW), and equivalent or improved immunogenic properties. When used to immunize mice, the IgG fusions elicited high titers of Zika-specific antibodies using only two doses without adjuvant, exceeding the titers produced by ZE3 alone by 20-fold to 150-fold, and potently neutralized Zika virus. The IgG fusions were also found to strongly enhance IgG2a production compared to unfused ZE3 antigen in a manner that correlated with C1q binding. These findings demonstrate the excellent potential of IgG fusions as self-adjuvanting subunit vaccines for Zika virus that can be made efficiently in plants.

[0063] C1q binding of the IgG fusions targeting ZIKV was enhanced by ZE3 fusion to the IgG N-terminus. The removal of the IgG light chain (HVL and HVL-related constructs) or Fab regions also enhanced C1q binding. The addition of hexamer-inducing mutations in the IgG Fc region enhanced C1q binding as well. C1q binding was also enhanced by adding a self-binding epitope tag to create RIC or producing IgG fusions in plants that lack plant-specific β 1,2-linked xylose and α 1,3-linked fucose N-glycans (for example, by silencing xylosyltransferase and fucosyltransferase, see Figs. 9 and 10). Both the RIC construct (HLZe) and the modified immune complex based on the single-chain antibody variant (HVLZe) have significantly improved C1q

binding compared to uncomplexed IgG (Figs. 1, 9, and 10). The results after immunization showed that the HVLZe construct produced antibody titers similar to that of the traditional RIC containing the full heavy and light chains (Figs. 6A and 6B).

[0064] In some aspects, the RIC construct is not preferred, because the large complex size renders them poorly soluble upon extraction (Figs. 2A and 10) and high concentrations may precipitate during storage. Very large RIC may also be too big to efficiently drain to lymph nodes from the injection site, which favor particles < 200 nm. Hexamer-sized IgG have been found be the optimal substrate for efficient C1q binding. Mutating the 6D8 epitope tag (construct HLZd) could also mitigate problems with the RIC construct. Notably, compared to the RIC, HLZd was significantly more soluble (Fig. 13B), had intermediate density (Fig. 4A), showed higher C1q binding (Fig. 1B), reached higher expression levels (1.5 mg/g LFW) (Fig. 2A), and elicited higher IgG2a titers (Figs. 6A and 6B). These improvements to the design of RIC vaccine candidates are applicable to other antigens.

[0065] The HVLZe fusion had slightly higher C1q binding than traditional RIC (Fig. 1B) and had high stability at the tested conditions (Fig. 5A). Since this single chain configuration reduced binding to the epitope tag when compared with the full 6D8 antibody, the HVLZe formed smaller complexes (sucrose gradient data showing reduced density compared to HLZe in Fig. 4A). However, some very dense material remained unlike with construct HLZd. Solubility could be improved by reducing the epitope binding of HVLZe.

[0066] Table 1 summarizes characteristics of the recombinant protein, an antigen targeting ZIKV. For expression, only the yield (mg/g LFW) of the fully assembled product is shown. A greater number of “+” symbols indicates either a statistically significant increase in the mean value for that property (for C1q, IgG, and IgG2a), or a repeatably observed difference (for density). For ZHx (*), peaks of both low-density and of high-density material were observed.

Table 1.

Construct	Expression	Solubility	Density	Stability	C1q	IgG	IgG2a
ZE3	N.D.	N.D.	N.D.	N.D.	N.D.	+	+
HLZ	0.17 mg/g	High	+	Medium	+	+++	++
HLZe	0.22 mg/g	Low	++++	High	++	+++	++
HLZd	1.50 mg/g	High	++	High	+++	+++	+++
ZH	0.83 mg/g	High	+	Medium	++	+++	+++
ZHx	0.21 mg/g	Low	++++*	High	+++	+++	+++
VHLZe	<0.1 mg/g	Low	++++	High	+++	+++	+++
ZFc	0.57 mg/g	High	+	Low	+++	+++	+++

Norovirus

[0067] The widespread occurrence of human norovirus (HNoV) infections causes tremendous economic damage and disease burden, however no approved vaccines or specific treatments are available. Efforts to control HNoV are hampered by the large genetic diversity of these viruses, which continually evolve to evade natural immunity. A wealth of published antibody mapping and T-cell data demonstrates the existence of broadly conserved HNoV B-cell and T-cell epitopes which, crucially, are targetable by the human immune system. Accordingly, it would be possible to design recombinant proteins for the purpose of eliciting immune responses against broadly conserved HNoV antigenic targets.

[0068] In exemplary embodiments, IgG variants were designed to generate an immune response against norovirus (NoV). In some aspects, the antigen of the recombinant protein comprises epitopes from a plurality of noroviruses, for example from human norovirus GI.3, human norovirus GII.4, and/or murine norovirus (MNV). The antigen in the recombinant protein targeting noroviruses comprises at least one epitope from the major capsid protein of NoV (VP1) or the nonstructural protein of NoV. In some aspects, the antigen targets the protruding domain or the shell domain of VP1. Such recombinant proteins are vaccine candidates against NoV.

[0069] In certain implementations, the recombinant proteins targeting NoV may be used in combination as a vaccination composition. As shown in the examples and Figs. 16-19, these antigens have been successfully used to produce immunoglobulin variants that are capable of broadly binding, blocking, and neutralizing a panel of diverse NoVs from different genogroups and genotypes.

[0070] In some aspects, the antigen targeting VP1 comprises 5- to 500-residue-long portion of VP1 and/or NS1. In some embodiments, the unit of the antigen comprises at least one sequence, at least three sequences, at least six sequences, at least nine sequence, at least twelve sequences, or at least fifteen sequences set forth in SEQ ID NOs. 34-72. For example, the unit of the antigen in recombinant proteins targeting VP1 comprises at least one sequence, at least two sequences, at least three sequences, at least four sequences, at least five sequences, at least six sequences, at least seven sequences, at least eight sequences, or at least nine sequences set forth in SEQ ID NOs. 34-54. In a particular embodiment, the unit of the antigen targeting the protruding domain of VP1 comprises at least one sequence, at least two sequences, at least three sequences,

at least four sequences, at least five sequences, at least six sequences, at least seven sequences, at least eight sequences, or at least nine sequences selected from SEQ ID NOs. 34-44. In certain embodiments, the unit of the antigen of a recombinant protein targeting norovirus comprises the sequences set forth in SEQ ID NOs. 34-42. In another particular embodiment, the unit of the antigen of a recombinant protein targeting the shell domain of VP1 comprises at least one sequence, at least two sequences, at least three sequences, at least four sequences, at least five sequences, at least six sequences, at least seven sequences, at least eight sequences, or at least nine sequences selected from SEQ ID NOs. 45-54. In some aspects, the unit of the antigen of a recombinant protein targeting norovirus comprises the sequences set forth in SEQ ID NOs. 45-53. In still another particular embodiment, the unit of the antigen of a recombinant protein targeting NS1 comprises at least one sequence or at least two sequences selected from SEQ ID NOs. 55-57. In some aspects, the unit of the antigen of a recombinant protein targeting norovirus comprises the sequences set forth in SEQ ID NOs. 55-57. In yet another particular embodiment, the unit of the antigen of a recombinant protein designed to generate a T-cell mediated immune response comprises at least one sequence, at least two sequences, at least three sequences, at least four sequences, at least five sequences, at least six sequences, at least seven sequences, at least eight sequences, or at least nine sequences selected from SEQ ID NOs. 58-72, for example, the peptide sequence of the unit of the antigen comprises the sequences of SEQ ID NOs. 58-72.

Method of Production

[0071] Plant recombinant expression systems have inherent safety, high scalability, and low production costs compared to mammalian cell systems, making them particularly well suited to make IgG fusions vaccines. While previous work has shown that some IgG fusion vaccines can enhance antigen immunogenicity, the many fusion strategies that have been developed have not been directly compared, making it difficult to determine the key properties involved in creating an optimal vaccine candidate.

[0072] The methods of production described herein comprise expressing the recombinant protein in a transgenic plant. In certain implementations, the recombinant protein is expressed in a transgenic plant silenced for xylosyltransferase and fucosyltransferase. In some aspects, for example the recombinant protein is a RIC, it is preferable to produce the proteins using plants with xylosyltransferase and fucosyltransferase silenced. The method comprises introducing

into agrobacteria a binary vector that expresses a unit of the antigen, a unit of the Fc fusion, a unit of the VH domain of the IgG, and a unit of the CH1 domain of the IgG. Accordingly, this binary vector encodes the heavy chain domains of the IgG. Next, a plant part is infiltrated with agrobacteria containing the binary vector to produce a transformed plant part. Crude protein from the plant part is extracted and then purified for the recombinant protein. In some aspects, the recombinant protein is purified using methods well-established in the art, for example, protein G affinity chromatography or metal affinity chromatography.

[0073] Where the recombinant protein comprises a unit of the VL domain of the IgG and a unit of the CL domain of the IgG, the method further comprises introducing into agrobacteria a different binary vector that encodes light chain domains of the IgG, namely the unit of the VL domain of the IgG and the unit of the CL domain of the IgG. The transformation of the plant part comprises co-infiltrating the plant part with agrobacteria containing the binary vector encoding the heavy chain domains of the IgG and agrobacteria containing the binary vector encoding the light chain domains of the IgG.

[0074] In particle implementations, the method of producing the recombinant protein described herein comprises introducing into agrobacteria a vector selected from the group consisting of: pBYR11eM-h6D8ZE3, pBYR11eMa-BAZE3-Hgp371, pBYR11eMa-BAZE3-H, pBYKEMd-HZE3, pBYKEMd-ZE3H, pBYKEMd-ZE3Hx, pBYKEMd-HVLZe, pBYKEMd2-HVL-Hx, pBYKEMd2-HVLZnt, pBYKEMd2-ZHVLnt, pBYKEMd2-ZHVLLe, pBYKEMd2-ZHVLhx, pBYKEAM-N12MHd, pBYKEAM-NPHd, pBYKEAM-NSHd, pBYKEAM-NTHd, pBYKEHM-CPHd, pBYKEHM-CSHd, and pBYKEHM-CTHd and infiltrating a plant part with agrobacteria containing the vector to produce a transformed plant part. Crude protein is then extracted from the transformed plant followed by purification for the recombinant protein. For methods of production where the vector introduced into agrobacteria is selected from the group consisting of: pBYR11eM-h6D8ZE3, pBYR11eMa-BAZE3-Hgp371, pBYR11eMa-BAZE3-H, and pBYKEMd-HZE3, the method further comprises co-infiltrating with agrobacteria containing pBYKEMd-6D8K and agrobacteria containing the vector.

Illustrative, Non-Limiting Example in Accordance with Certain Embodiments

[0075] The disclosure is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents, and published patent applications

cited throughout this application, as well as the Figures, are incorporated herein by reference in their entirety for all purposes.

A. Design and C1q Binding of IgG-Fusions with ZIKV Envelope Domain III

[0076] ZE3 is a promising subunit vaccine candidate, however it is not strongly immunogenic on its own, necessitating high antigen doses with adjuvant and repeated immunizations. A panel of human IgG1 variants was designed based on the humanized Ebola monoclonal antibody 6D8 fused to ZE3 (Fig. 1A). A RIC construct was created by fusing ZE3 via flexible linker to the C-terminus of the 6D8 heavy chain. ZE3 was tagged with the 6D8 epitope binding site to allow immune complex formation. The construct is referred to as “HLZe” as it contains the Heavy chain, Light chain, C-terminal ZE3 fusion, and epitope tag. As a control for immune complex formation, an otherwise identical construct was created without the epitope tag (construct “HLZ”) and thus would not be expected to form immune complexes. First, C1q binding by IgG variants was studied. To improve immune receptor binding, all constructs were expressed in glycoengineered *Nicotiana benthamiana*. 6D8 antibody or RIC made in glycoengineered plants showed highly improved C1q binding compared to constructs expressed in wildtype plants (Fig. 9). RIC are thought to engage C1q with high avidity due to densely clustered antigen-antibody complexes, and as expected, HLZe showed greatly improved C1q binding compared to HLZ ($p < 0.001$) (Fig. 1B). A small increase in C1q binding was also noted with HLZ compared to HL, perhaps suggesting low-level aggregation of the construct ($p < 0.05$) (Fig. 1B).

[0077] It has been reported that the antibody Fab arms may play a regulatory role in complement activation by inhibiting C1q binding unless cognate antigens are bound by the antibody. In agreement with these data, the addition of soluble antigen carrying the 6D8 epitope tag improved C1q binding (Fig. 10, compare HL vs HL + Ag), and C1q binding was greatly enhanced either by removal of the 6D8 light chain or, interestingly, by antigen fusion to the 6D8 heavy chain N-terminus (Fig. 10, compare HL, H, and ZHL). Therefore, a construct with ZE3 fused to the N-terminus of 6D8 (construct “ZH”) in the absence of the light chain was created and found to efficiently bind C1q (Fig. 1B). To further improve interaction with C1q, a construct was created that was identical to ZH except that E345R, E430G, and S440Y mutations were introduced to the 6D8 Fc region, which favor formation of hexamers. This construct (ZHx) had further improved C1q binding compared to ZH ($p < 0.001$) (Fig. 1B). Interestingly, while IgG1-Fc fusions

have been shown to have variable C1q binding, ZE3 fused to the 6D8 Fc (construct “ZFc”) showed very strong C1q binding (Fig. 1B). To build a simplified RIC that also lacked the light chain constant region, first a construct was made such that the variable light (VL) domain of 6D8 was inserted between the variable heavy (VH) and CH1 domains of 6D8, yielding construct HVL. This construct was found to bind antigen tagged with the 6D8 epitope, albeit at a somewhat reduced level compared to unaltered 6D8 (Fig. 11). This configuration was inserted into HLZe to create the single chain RIC HVLZe, which displayed potent C1q binding (Fig. 1B).

[0078] It is interesting to note that the theoretically monomeric construct HLZ (consisting of ZE3 fused to the C-terminus of 6D8 without any epitope tag) produced a small but repeatable improvement in C1q binding (Fig. 1B). This may suggest some low-level aggregation due to the ZE3 fusion (Fig. 4A, compare HL and HLZ). Furthermore, it has been reported that Fab-mediated events impact C1q binding, as deletion of the IgG Fab arms induced the formation of oligomers and further enhanced C1q binding via an unknown mechanism. The expression of HLZ was generally low, suggesting C-terminal ZE3 fusion may interfere with folding or otherwise cause instability (Fig. 2A). Many degradation products are visible by SDS-PAGE following purification (Fig. 3) and, interestingly the C1q binding of HLZ continued to increase upon further degradation by incubation for 2 weeks at RT or repeated freeze-thaw cycles (Fig. 5B). These data suggest that the degradation products may be more highly immunogenic than the original construct, a finding that may explain the relatively high total IgG titers produced by HLZ (Fig. 6A). However, HLZ IgG2a titers were reduced compared to the other constructs (Fig. 6B). As IgG2a is a general indicator of a Th1 response, HLZ may still have impaired effector functions that lead to T-cell activation, such as reduced complement activation. Notably, all IgG fusions strongly enhanced IgG2a production compared to unfused ZE3 (Fig. 6B). While human antibodies have recently been shown to have similar binding affinities to mouse Fc receptors compared to mouse antibodies, human IgG1 has reduced binding to low affinity mouse FcγRIII/CD16. It was also shown that a polymeric dengue IgG fusion vaccine showed enhanced immunogenicity in human adenotonsillar tissue compared to a monomeric form of the same IgG fusion, however both showed equivalent immunogenicity in mice expressing human FcγRI/CD64. Therefore, further testing in other animal models is needed to more fully evaluate the immunogenicity of these constructs.

[0079] Antigen binding may induce conformational changes in the IgG, which may improve C1q binding. Mixing 6D8 with an antigen containing the 6D8 epitope produced only a small increase in C1q binding (Fig. 10) and was found to be poorly immunogenic compared to RIC constructs (data to be presented elsewhere). However, N-terminal antigen fusion had a more pronounced effect on C1q binding (Fig. 1B), perhaps by strongly inducing conformational changes similar to antigen binding. In general, antigen fusion to the 6D8 N-terminus greatly enhances C1q binding for a variety of large and small antigens. Furthermore, deletion of the 6D8 light chain (construct ZH) also substantially enhanced C1q binding (Fig. 1B). These findings agree with the hypothesis that the Fab portions of the antibody plays a regulatory role in C1q binding. Additionally, removal of the light chain may also impact glycosylation and thus C1q binding. Construct ZHx synergistically combines the effects of light chain removal and N-terminal ZE3 fusion with hexamer-inducing mutations, resulting in an even more potent enhancement of C1q binding (Fig. 1B) and high total IgG and IgG2a titers (Fig. 6B). Despite its strong immunogenicity, ZHx had reduced yield compared to ZH (Fig. 2A). Additionally, ZHx appeared to have some very large material found in the very bottom of the sucrose gradients that was not found in construct ZH (Fig. 4B), suggesting the hexamer mutations may contribute to aggregation. Further work is needed to optimize the solubility of these constructs.

[0080] N-terminal ZE3 fusion to the 6D8 CH1 domain (construct ZFc) greatly improved C1q binding (Fig. 1B), elicited very high antibody titers (Fig. 6A). Many properties of Fc fusions, including C1q binding, seem to vary based on the individual fusion partner and thus must be determined empirically for each fusion. Interestingly, some Fc fusion constructs have been shown to form hexamers, which may explain the strong immunogenicity of ZFc observed here. Despite its strong immunogenicity, ZFc was remarkably unstable: 50% of the ZE3 was cleaved off before or during extraction (Fig. 2A, 2B) even when directly extracted in SDS sample buffer, and less than 25% of full-size ZFc molecules remained intact upon repeated freeze-thaw or storage (Fig. 5A). Unlike HLZ, this degradation was associated with a loss in C1q binding (Fig. 5B), suggesting impairment of the Fc receptor binding domains. ZFc has fewer disulfide bonds than whole IgG, and in general Fc fusions often suffer from instability or undesirable aggregation. While ZFc elicited high titers, to deliver 8 µg ZE3 (discounting the weight of the IgG fusion partner), the total amount of purified sample used for immunization had to be nearly tripled to account for the high degradation and resulting loss of the ZE3 fusion. Whether this increased

delivery of unfused Fc as well as other possible degradation products may have contributed to the high immunogenicity is unknown. By contrast, the larger and more highly oligomeric constructs HLZe, HLZd, ZHx, and HVLZe (Figs. 4A and 4B) were more stable both prior to and during extraction, as well as upon storage (Fig. 5A). Consistent with their inaccessibility to antibody probes during ELISA (Fig. 2A), the intramolecular and intermolecular associations of these constructs may protect them from proteolysis or other degradative processes.

B. Expression and Characterization of HVL Fusion Constructs

[0081] RICs are known to be immunogenic; however, they require the co-expression of a heavy and light chain in order to form the fully assembled product. This process can be simplified by creating a single chain antibody fusion. The benefit of single-chain antibody fusions is that the entire antibody-antigen fusion can be produced in a single coding sequence, thereby eliminating the need for co-expression of heavy and light chains. In addition, the new construct will still retain the variable regions of both the heavy and light chain. The core single-chain antibody contains the variable heavy regions linked to a variable light chain region that is directly fused to an antibody heavy chain (Fig. 7) While a similar construct expressed in *N. benthamiana* plants has been previously characterized and published, there is very little work that explores the potential of single-chain antibody fusions, in which the core single-chain antibody is fused to an antigen of interest. Several single-chain antibody fusions were created and tested (Fig. 7). The results from the purified constructs can be expressed in plants and appear at the correct size when run under non-reducing conditions on an SDS-PAGE gel.

C. Expression of IgG Fusion Constructs

[0082] RIC suffer from low yield of soluble product. Addition of the 6D8 epitope tag to the C-terminus of 6D8 renders the antibody mostly insoluble; however this is prevented by removal of the light chain, suggesting the insolubility arises from large complexes of antibody bound to the epitope tag (Fig. 12). To improve RIC solubility, the 6D8 epitope tag was mutated to reduce antibody binding. Reducing the epitope tag on HLZe to the minimal reported binding region for 6D8 (Wilson et al., 2000) (construct “HLa,” epitope sequence VYKLDISEA, SEQ ID NO. 2) showed no improvement in solubility, nor did removal of a single amino acid from the 3' end (construct “HLb,” VYKLDISE, SEQ ID NO. 3) (Fig. 13A). However, further removal of a single

amino acid from the 5' end (construct "HLc," YKLDISE, SEQ ID NO. 4) and additional truncation in construct "HLd" (YKLDIS, SEQ ID NO. 1) resulted in greatly improved solubility (Fig. 13B). This mutation was introduced to HLZe (construct HLZd) and characterized. Despite reducing epitope binding by approximately 25-fold by ELISA (Fig. 13C), HLZd still maintained very strong C1q binding (Fig. 1B), suggesting efficient complex forming activity remained.

[0083] To measure the yield of fully assembled IgG fusions, an ELISA assay was employed that first captured ZE3 and then detected human IgG. To detect cleavage of ZE3, an ELISA measuring only total IgG was also used as a comparison. When probed for both ZE3 and IgG, the highly soluble monomeric construct ZH yielded 0.83 mg fully formed product per gram leaf fresh weight (mg/g LFW), and the similar ZFc yielded 0.58 mg/g LFW (Fig. 2A). However, when measuring the total IgG content, the yield of ZH was roughly 20% higher and, for ZFc, 2.5-fold higher, which suggests that ZH and especially ZFc are susceptible to proteolytic cleavage, probably of the ZE3 linker (Fig. 2A). This finding which was confirmed by visualization of the ZFc cleavage products on SDS-PAGE (Fig. 2B). HLZ accumulated only 0.17 mg/g LFW fully assembled product, with roughly 40% ZE3 lost to cleavage, suggesting general instability of the construct (Fig. 2A, 2B). By ELISA, the RIC constructs seemingly performed worse than the monomeric constructs: HLZe and HLZd only accumulated 0.04 mg/g LFW and 0.30 mg/g LFW respectively, and the hexameric ZHx accumulated only 0.08 mg/g LFW (Fig. 2A). However, when visualized under reducing SDS-PAGE conditions, HLZd was the highest expressing construct, accumulating an estimated 1.5 mg/g LFW (Fig. 2A, 2B). This discrepancy may arise due to complexed HLZd being rendered inaccessible to the antibody probe by ELISA. Similarly, the total yield of HLZe and ZHx was also higher when measured by gel quantification (Fig. 2A, 2B). Despite its low solubility, HLZe had similar yields to HLZ, and HLZd greatly exceeded the yield of HLZ. This finding suggests complex formation may protect HLZe and HLZd either inside the cell or during extraction, though the possibility that the short epitope tag plays some other role in enhancing stability of HLZe and HLZd cannot be excluded. HVLZe yielded only 0.02 mg/g LFW soluble product by ELISA and was not detectable by SDS-PAGE (Fig. 2A); however it accumulated very high levels when extracted with 7.5 M urea, which suggests insolubility resulted in the low yield.

D. Purification, Aggregation, and Stability of IgG Fusions

[0084] IgG fusions were purified to >95% homogeneity using a simple one-step purification via protein G affinity chromatography. In agreement with the expression data, the more highly oligomeric constructs showed less degradation than the other constructs, and the ZFc fusion had particularly high levels of degradation (Fig. 3). To investigate the aggregation characteristics of each construct, purified IgG fusions were analyzed by sucrose gradient centrifugation. Consistent with the formation of large immune complexes, HLZe and HVLZe were found mostly in the bottom of the gradient while HLZ, ZH, and ZFc were found mostly at the top of the gradient (Figs. 4A and 4B). Solutions of ZHx contained both low-density material as well as some very high-density material (Fig. 4B). Solutions of HLZd showed intermediate density compared to HLZ and HLZe (Fig. 4A) which, when taken together with the expression data and C1q binding, are consistent with HLZd forming smaller, more soluble immune complexes compared to HLZe.

[0085] The stability of each construct was analyzed by comparing fully formed products and degradation products on SDS-PAGE after treatment with various temperature conditions. After five freeze-thaw cycles or two weeks at 4°C, small amounts of degradation were observed with all constructs (Fig. 5A). High concentrations (>1 mg/ml) of HLZe and HVLZe were found to precipitate after several days at 4°C. At room temperature, most constructs had 10-15% degradation after two weeks (Fig. 5A). Overall, the oligomerizing constructs retained the highest stability, while the monomeric constructs, and especially the Fc fusion, were more rapidly degraded (Fig. 5A). Most constructs retained strong C1q binding after storage, however after room temperature storage or repeated freeze-thaw cycles but not 4°C storage, construct HLZ displayed increased C1q binding (Fig. 5B). This may be due to degradation of the Fab regions or loss of light chain, as degradation products are visible on SDS-PAGE (Fig. 3), or aggregation. Conversely, construct ZFc lost C1q binding ability as it became more degraded, probably due to degradation of the C1q binding regions (Fig. 5B).

E. Mouse immunizations, titers, IgG2a, cytokines

[0086] To investigate the immunogenicity of the IgG fusions, BALB/c mice (n = 6) were immunized subcutaneously without adjuvant with two doses of each IgG variant such that the total dose of ZE3 delivered was 8 µg. As a control, mice were also immunized with 8 µg

unfused plant-expressed ZE3. All IgG fusions very strongly enhanced the production of ZE3-specific antibodies, producing 20-fold to 150-fold higher total IgG titers than ZE3 alone (Fig. 6A, $p < 0.01$ compared to ZE3). All IgG fusions significantly enhanced the production of IgG2a compared to ZE3 alone (Fig. 6B, $p < 0.01$ compared to ZE3). While no significant differences in total titer production were observed between the IgG fusions, the construct HLZ had a significantly reduced production of IgG2a compared to all other fusions except for HLZe (Fig. 6B, $p < 0.05$ compared to HLZ). The constructs ZHx and ZFc produced the highest levels of total IgG and IgG2a (Fig. 6A, Fig. 6B).

F. Design, Expression, and Purification of IgG-Fusions with Norovirus Epitopes

[0087] The norovirus (NoV) capsid protein VP1 consists of an inner shell domain (S), which forms the core surrounding the viral genome, and a protruding domain (P), which is further subdivided into P1 and P2 subdomains. Traditional human norovirus (HNoV) vaccine approaches, as well as natural infection, elicit immune responses primarily targeting immunodominant epitopes on the viral capsid that are poorly conserved, resulting in limited protection against novel strains and against related viruses from different genotypes. Nearly all HNoV vaccine candidates have focused on virus-like particles (VLPs) made from the VP1 capsid protein. Based on sequence divergence of the major capsid protein VP1, ten genogroups (GI-GX) which contains over forty genotypes of NoVs have been identified. Of these ten genogroups, GI and GII cause the most human disease. Yet, even the best attempts at generating conserved VLPs have produced only modest reductions of disease severity in human clinical trials and generally struggled to induce broadly protective immunity.

[0088] One broadly reactive antibody mapped to a strongly conserved epitope at the base of P1: LPQEWVQYFYQEAPPA (SEQ ID NO. 34). Critically, this antibody binds linear epitopes in P1, and thus the native VP1 conformation is not necessary to elicit functional antibodies. Antibodies targeting this epitope bind with high affinity to VLPs from 8 GI, 13 GII, and 1 GIV genotype. Antibodies directed against a second broadly reactive, linear epitope (ALLRFVNPDGTGRVLFE, SEQ ID NO. 37) bind VLPs from at least 3 GI and 7 GII genogroups. A third broadly conserved linear epitope at the base of the P1 domain is DSWVNQFYTLAP (SEQ ID NO. 41). The S domain is the most highly conserved region of VP1. Antibodies targeting the strongly conserved linear epitopes QNVIDPWIRNNF (SEQ ID NO. 45), QAPGGFTVSPRNAPGE

(SEQ ID NO. 46), and KVIFAAVPP (SEQ ID NO. 47) have been identified with broad cross-reactivity to NoV genogroups. Conserved HNoV T-cell epitopes were identified in humans¹³, namely the epitope TMFPHIIVDV (SEQ ID NO. 54) in the VP1 S domain elicited broad T-cell activation against both GI and GII HNoV. In addition, a dominant T-cell epitope (SWVSRFYQL, SEQ ID NO. 64) in the P1 C-terminal domain that is highly conserved between all NoV genogroups, including HNoV, was critical for viral clearance in NoV-infected mice. This region plays a vital role in capsid assembly, and thus structural constraints limit the potential for mutational escape. The nonstructural protein NS1, which is proteolytically cleaved from NS1-2, is secreted from cells infected with murine NoV (GV) or transfected with HuNoV NS1-2. Secreted NS1 suppresses IFN- λ production in the intestinal tract of mice. Importantly, vaccination with NS1 alone protected mice better than vaccination with P-domain, highlighting NS1 as a vaccine target. Vaccination with NS1-2 yielded antibodies directed against NS1.

[0089] For the development of antigens to make immunoglobulin variants targeting norovirus, epitopes from the protruding domain of VP1 of human norovirus GI.3, human norovirus GII.4, and murine norovirus (MNV) have been identified (SEQ ID NOs. 34-44). Also identified are epitopes from the shell domain of VP1 of GI.3, GII.4, and MNV (SEQ ID NOs. 45-54). The NS1 epitopes of MNV is set forth in SEQ ID NO. 55. The NS1 epitope of GI.3 is set forth in SEQ ID NO. 56. The NS1 epitope of GII.4 is set forth in SEQ ID NO. 57. The T-cell response epitopes are set forth in SEQ ID NOs. 58-72.

[0090] Accordingly, RIC constructs targeting conserved epitopes of the NoV protruding domain (P-RIC), shell domain (S-RIC), T-cell epitopes (T-RIC), or containing tandem linked nonstructural protein 1 (NS1) from murine norovirus (N-RIC) are designed and produced. The unit of the antigen for P-RIC is formed from nine tandem-linked epitopes from the protruding domain of VP1 from GI.3, GII.4, and MNV. The unit of the antigen for S-RIC is formed from nine tandem-linked epitopes from the shell domain of VP1 of GI.3, GII.4, and MNV. The unit of the antigen for T-RIC is formed from fifteen tandem-linked epitopes that have been found to cause a T-cell mediated immune response against GI.3, GII.4, and MNV. The unit of the antigen for N-RIC is formed from tandem-linked epitopes from NS1 of GI.3, GII.4, and MNV. The plant expression vector for producing these RIC constructs are

pBYKEAM-N12MHd, pBYKEAM-NPHd, pBYKEAM-NSHd, pBYKEAM-NTHd, pBYKEHM-CPHd, pBYKEHM-CSHd, and pBYKEHM-CTHd.

[0091] For each epitope, RIC constructs where the epitopes are linked to at the N-terminus or the C-terminus could be successfully expressed in plants (Fig. 14A). All of these RIC constructs could be purified (Fig. 14B and Fig. 15). Serum from mice immunized with these RIC constructs could bind to mouse norovirus (Fig. 16) and VLPs modeling GI.3 and GII.2 noroviruses (Figs. 17 and 18). Increase increased IFN- γ production by splenocytes of mice immunized with the RICs (Fig. 19) suggest that RICs successfully induced immunity norovirus.

G. Methods

1. Vector Construction

[0092] The construction of a BeYDV plant expression vector for ZE3, as well as its fusion to the 6D8 C-terminus (pBYR11eM-h6D8ZE3), referred to here as construct “HLZe”) or N-terminus with epitope tag (pBYR11eMa-BAZE3-Hgp371) or without epitope tag (pBYR11eMa-BAZE3-H) was described in Diamos et al., 2019. A vector pBYKEMd2-6D8 expressing the full 6D8 mAb without ZE3 fusion (construct “HL”) was also described in Diamos et al., 2019. To create a vector expressing only the light chain of 6D8, pBYKEMd2-6D8 was digested with XhoI and the vector was self-ligated to yield pBYKEMd-6D8K. A vector expressing only the heavy chain of 6D8 (construct “H”) was created by digesting pBYKEMd2-6D8 with SacI and self-ligating the vector, to yield pBYKEMd-6D8H. The 6D8 epitope binding tag was added to pBYKEMd-6D8H by digesting pBYR11eMa-BAZE3-Hgp371 with BsaI-SacI and inserting the tag-containing fragment into pBYKEMd-6D8H digested with BsaI-SacI, yielding pBYKEMd-6D8Hgp371 (construct “HLe” when coexpressed with light chain). To remove the epitope tag from HLZe, pBYR11eM-h6D8ZE3 was digested with BamHI-SacI and ligated with a fragment containing ZE3 obtained via amplification with primers ZE3-Bam-F (5'-gcgggatccaaggcgctgtcactacc, SEQ ID NO. 8) and ZE3-Sac-R (5'-acagagctcttaagtgtaccactcctgtg, SEQ ID NO. 9) and subsequent digestion with BamHI-SacI. The resulting vector, pBYKEMd-HZE3, was coinfiltrated with pBYKEMd-6D8K to produce construct “HLZ.” To produce ZE3 fused to the 6D8 N-terminus without light chain, pBYR11eMa-BAZE3-H was digested with SacI and the vector was self-ligated, yielding pBYKEMd-ZE3H (construct “ZH”). To introduce hexamer mutations, a region of the 6D8 heavy chain constant region was synthesized (Integrated

DNA Technologies, Iowa, USA) containing the E345R, E430G, and S440Y mutations, then digested with BsaI-SacI and used to replace the BsaI-SacI region of 6D8 in pBYKEMd-ZE3H, yielding pBYKEMd-ZE3Hx (construct “ZHx”). RIC epitope tag mutant “a” was generated by annealing oligos 6D89-F (5'-ctagtgtttacaagctggacatatctgaggcataagagct, SEQ ID NO. 10) and 6D89-R (5'-cttatgcctcagatatgtccagcttgtaaaca, SEQ ID NO. 11) and ligating them into pBYR11eM-h6D8ZE3 digested SpeI-SacI; mutant “b” was generated by first amplifying mutant “a” with primers gpDISE-Sac-R: (5'-tttgagctcttactcagatatgtccagcttgtaaaca, SEQ ID NO. 12) and 35S-F (5'-aatcccactatccttcgc, SEQ ID NO. 13), then digesting the product with SpeI-SacI and ligating it into pBYR11eM-h6D8ZE3 digested with SpeI-SacI. Mutants “c” and “d” were created similarly to mutant “a” using overlapping oligos 6D87-F (5'-ctagttacaagctggacatatctgagtaagagct, SEQ ID NO. 14) and 6D87-R (5'-cttactcagatatgtccagcttgtaa, SEQ ID NO. 15) for “c” and 6D86-F (5'-ctagttacaagctggacatatcttaagagct, SEQ ID NO. 16) and 6D86-R (5'-cttaagatatgtccagcttgtaa, SEQ ID NO. 17).

[0093] In order to make a construct in which the variable heavy (VH) domain is linked to a variable light chain (VL) domain that, in turn, is directly fused to the constant region of the 6D8 antibody, the variable regions were first obtained through PCR amplification and end-tailoring of segments from pBYR11eM-h6D8ZE3. For the VH domain, the primers LIR-H3A (5'-aagcttggtgtgtgactccgag, SEQ ID NO. 18) and 6D8VH-Spe-R (5'-cggactagtagtggaagacactgtgac, SEQ ID NO. 19) were used. The VL region was obtained through PCR amplification of pBYR11eM-h6D8ZE3 with primers 35S-F (5'-aatcccactatccttcgc, SEQ ID NO. 13) and 6D8VK-Nhe-R (5'-cgtgctagccttgatctccacttggtc, SEQ ID NO. 20). In order to fuse VL region to the constant region of a human IgG antibody, a subclone was created by digesting the PCR fragment with XhoI-NheI and inserting it into a vector, pKS-HH-gp371, that contained the 6D8 heavy chain. This subclone was named pKS-VL. Next, pBYKEM-6D8K was digested with SbfI-SacI, the PCR product that amplified the variable heavy chain fragment was digested SbfI-SpeI, and the variable light chain subclone was digested SpeI-SacI. These fragments were assembled to create pBYKEMd2-VHLVK (HVL). Finally, this construct was used to create pBYKEMd2-HVLZe by a two-fragment ligation. The pBYKEMd2-VHLVK construct was digested with BsaI and SacI to obtain the vector fragment along with the variable regions of the heavy and light chains. To obtain the ZE3 antigen segment and the epitope tag, pBYR11eM-h6D8ZE3 was also digested BsaI-SacI. The resulting construct, which was used to produce HVLZe, was named pBYKEMd-HVLZe.

[0094] The HVL panel of constructs were created as follows. HVL-Hx, a construct containing a single-chain antibody with three point mutations in the Fc region in order to facilitate formation of single-chain hexamers, was created by a two-fragment ligation. The backbone was derived by a BsaI-SacI digest of pBYKEMd2-VHLVK and the insert containing the mutations for hexamer formation was derived from a BsaI-SacI digest of pBYKEMd-ZE3Hx. The final construct was named pBYKEMd2-HVL-Hx.

[0095] HVLZnt, a single-chain fusion with the ZE3 antigen on the C-terminus but no epitope tag, was created by ligating a BsaI-SacI digested backbone from pBYKEMd2-VHLVK to an insert derived from pBYKEMd-HZE3. This construct was named pBYKEMd2-HVLZnt

[0096] ZHVLnt, a single-chain RIC with the ZE3 antigen linked to the antibody N-terminus and no epitope tag, was created by the following ligation. The insert fragment containing the ZE3 antigen and the VH segment was amplified from pBYR11eMa-BAZE3-Hgp371 using the 35S-F and VH-BsaS-R primers. The VH-BsaS-R primer end-tailored the 5' end to include a BsaI site that would result in a SpeI overhang upon digestion with BsaI. This PCR fragment was digested XhoI-BsaI and ligated with a backbone XhoI-SpeI fragment obtained from pBYKEMd2-VHLVK. The final construct was named pBYKEMd2-ZHVLnt.

[0097] ZHVLe, a single-chain antibody RIC with an N-terminally fused ZE3 antigen and an epitope tag was created by a two-fragment ligation. pBYKEMd2-ZHVLnt was digested BsaI-SacI to produce the vector segment containing the N-terminal ZE3 antigen and the HVL construct. The insert containing the 6D8 epitope tag was derived from a BsaI-SacI digest of pBYR11eMa-BAZE3-Hgp371. The final construct was named pBYKEMd2-ZHVLe.

[0098] ZHVLhx, a single-chain antibody with a N-terminally fused ZE3 antigen and point mutations to facilitate hexamer formation, was obtained by a two-fragment ligation. The vector fragment was obtained by a BsaI-SacI digest of pBYKEMd2-ZHVLnt and the insert fragment was derived from a BsaI-SacI digest of pBYKEMd2-HVL-Hx. The final construct was named pBYKEMd2- ZHVLhx.

[0099] The specific constructs described herein listed below:

- pBYR11em-h6D8ZE3 (sequence set forth in SEQ ID NO. 21)
- pBYR11eMa-BAZE3-Hgp371 (sequence set forth in SEQ ID NO. 22)
- pBYR11eMa-BAZE3-H (sequence set forth in SEQ ID NO. 23)
- pBYKEMd-HZE3 (sequence set forth in SEQ ID NO. 24)

- pBYKEMd-ZE3H (sequence set forth in SEQ ID NO. 25)
- pBYKEMd-ZE3Hx (sequence set forth in SEQ ID NO. 26)
- pBYKEMd-HVLZe (sequence set forth in SEQ ID NO. 27)
- pBYKEMd2-HVL-Hx (sequence set forth in SEQ ID NO. 28)
- pBYKEMd2-HVLZnt (sequence set forth in SEQ ID NO. 29)
- pBYKEMd2-ZHVLnt (sequence set forth in SEQ ID NO. 30)
- pBYKEMd2-ZHVLLe (sequence set forth in SEQ ID NO. 31)
- pBYKEMd2-ZHVLhx (sequence set forth in SEQ ID NO. 32)
- pBYKEMd-6D8K (sequence set forth in SEQ ID NO. 33)

2. Agroinfiltration of *Nicotiana benthamiana* Leaves

[0100] Binary vectors were separately introduced into *Agrobacterium tumefaciens* EHA105 by electroporation. The resulting strains were verified by restriction digestion or PCR, grown overnight at 30°C, and used to infiltrate leaves of 5- to 6-week-old *N. benthamiana* maintained at 23-25°C. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000 g and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM MgSO₄) to OD₆₀₀ = 0.2, unless otherwise described. The resulting bacterial suspensions were injected by using a syringe without needle into leaves through a small puncture (Huang and Mason, 2004). To evaluate the effects of glycosylation, transgenic plants silenced for xylosyltransferase and fucosyltransferase were employed. Plant tissue was harvested at 5 DPI.

3. Protein Extraction and Purification

[0101] Crude protein was extracted by homogenizing agroinfiltrated leaf samples with 1:5 (w:v) ice cold extraction buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 3 mM EDTA, 0.1% Triton X-100, 10 mg/mL sodium ascorbate, 0.3 mg/mL phenylmethylsulfonyl fluoride) using a Bullet Blender machine (Next Advance, Averill Park, NY) following the manufacturer's instruction. Homogenized tissue was rotated at room temperature or 4°C for 30 min. The crude plant extract was clarified by centrifugation at 13,000g for 15 min at 4°C and the supernatant was analyzed by SDS-PAGE or ELISA. Alternatively, to evaluate solubility of proteins in the original homogenate, the pellet was designated the insoluble fraction and treated with SDS sample buffer at 100°C for 10 min before loading on SDS-PAGE.

[0102] IgG variants, including HVLZe, were purified by protein G affinity chromatography. Agroinfiltrated leaves were blended with 1:3 (w:v) ice cold extraction buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 3 mM EDTA, 0.1% Triton X-100, 10 mg/mL sodium ascorbate, 0.3 mg/mL phenylmethylsulfonyl fluoride), stirred for 30 min at 4°C, and filtered through miracloth. To precipitate endogenous plant proteins, the pH was lowered to 4.5 with 1 M phosphoric acid for 5 min while stirring, then raised to 7.6 with 2 M tris base. Following centrifugation for 20 min at 16,000 g, the clarified extract was loaded onto a Pierce Protein G column (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Purified proteins were eluted with 100 mM glycine, pH 2.5, directly into collection tubes containing 1 M Tris-HCl pH 8.0 to neutralize the elution buffer. The HVL and ZHVLhx constructs were purified in a similar fashion; however, the acid precipitation step was skipped.

[0103] ZE3-His expressed from pBYe3R2K2Mc-BAZE3 was purified by metal affinity chromatography. Protein was extracted as described above, but without acid precipitation. The clarified extract was loaded onto a column containing TALON Metal Affinity Resin (BD Clontech, Mountain View, CA) according to the manufacturer's instructions. The column was washed with PBS and eluted with elution buffer (PBS, 150 mM imidazole, pH 7.4). Peak ZE3 elutions were pooled, dialyzed against PBS, and analyzed by SDS-PAGE and western blot.

4. SDS-PAGE and Western Blot

[0104] Plant protein extracts or purified protein samples were mixed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) and separated on 4-15% stain-free polyacrylamide gels (Bio-Rad, Hercules, CA, USA). For reducing conditions, 0.5M DTT was added, and the samples were boiled for 10 min prior to loading. Polyacrylamide gels were visualized and imaged under UV light, then transferred to a PVDF membrane. For IgG detection, the protein transferred membranes were blocked with 5% dry milk in PBST (PBS with 0.05% tween-20) overnight at 4°C and probed with goat anti-human IgG-HRP (Sigma-Aldrich, St. Louis, MO, USA diluted 1:5000 in 1% PBSTM). Bound antibody was detected with ECL reagent (Amersham, Little Chalfont, United Kingdom).

5. C1q Binding

[0105] 96-well high-binding polystyrene plates (Corning Inc, Corning, NY, USA) were coated with 15 µg/ml human complement C1q in PBS for 2h at 37°C. The plate was washed 3 times with PBST, and then blocked with 5% dry milk in PBST for 15 minutes. After washing 3 times with PBST, purified human IgG (Southern Biotech, Birmingham, AL, USA) and purified IgG-ZE3 fusions were added at 0.1 mg/ml with 10-fold serial dilutions and incubated for 1.5h at 37°C. After washing 3 times with PBST, bound IgG was detected by incubating with 1:1000 polyclonal goat anti human IgG-HRP (Southern Biotech, Birmingham, AL, USA) for 1h at 37°C. The plate was washed 4 times with PBST, developed with TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA), stopped with 1M HCl, and the absorbance was read at 450nm.

6. 6D8 Epitope Binding

[0106] To test the ability of HVL to bind to the 6D8 epitope tag, 900 ng of purified dengue consensus envelope domain III tagged with 6D8 epitope were bound to 96-well high-binding polystyrene plates (Corning Inc, Corning, NY, USA). After a 1-hour incubation at 37°C, the plate was washed thrice with PBST and blocked with 5% dry milk in PBST for 30 minutes. Then, the plate was washed thrice with PBST and various dilutions of either purified HVL or full-length 6D8 antibody were added to the plate. The plate was incubated at 37°C for 1-hour, washed thrice with PBST and detected with HRP-conjugated mouse anti-human IgG (Fc only) (Southern Biotech, Birmingham, AL, USA) antibody at a 1:2000 dilution. Then, the plate was thoroughly washed with PBST and developed with TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA). The absorbance was read at 450nm.

7. Sucrose gradient density centrifugation

[0107] Purified samples of each IgG fusion (100 µl) were loaded onto discontinuous sucrose gradients consisting of 350 µl layers of 5, 10, 15, 20, and 25% sucrose in PBS in a 2.0 ml microcentrifuge tube and centrifuged at 21,000 g for 16 h at 4°C. Fractions were collected and analyzed by SDS-PAGE followed by visualization on stain-free gels (Bio-Rad, Hercules, CA, USA). The relative band intensity of each fraction was determined using ImageJ software, with the peak band arbitrarily assigned the value of 1.

8. Immunization of mice and sample collection

[0108] All animals were handled in accordance to the Animal Welfare Act and Arizona State University IACUC. Female BALB/C mice, 6-8 weeks old, were immunized subcutaneously with purified IgG fusion variants. In all treatment groups, the total weight of antigen was set to deliver an equivalent 8 µg of ZE3. Doses were given on days 0 and 14. Serum collection was done as described in Santi et al., 2008 by submandibular bleed on days 0, 14, and 28.

9. Antibody Measurements

[0109] Mouse antibody titers were measured by ELISA. Plant-expressed 6-His tagged ZE3 at 50ng/well was bound to 96-well high-binding polystyrene plates (Corning Inc, Corning, NY, USA), and the plates were blocked with 5% nonfat dry milk in PBST. After washing the wells with PBST (PBS with 0.05% Tween 20), the mouse sera were diluted with 1% PBSTM (PBST with 1% nonfat dry milk) and incubated. Mouse antibodies were detected by incubation with polyclonal goat anti-mouse IgG-horseradish peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA). The plate was developed with TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA), stopped with 1M HCl, and the absorbance was read at 450 nm. Endpoint titers were taken as the reciprocal of the lowest dilution which produced an OD450 reading twice the background produced using PBS as the sample. IgG2a antibodies were measured from sera diluted 1:100 in 1% PBSTM and detected with IgG2a horseradish peroxidase conjugate (Santa Cruz Biotechnology, Dallas, TX, USA).

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