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(54) Title: NOVEL MALARIA VACCINE COMPRISING AMA1 AND RON2 ANTIGENS

(57) Abstract: Apical membrane antigen 1 (AMA1) is a key malaria vaccine candidate and target of neutralizing antibodies. AMA1 binds to a loop in rhoptry neck protein 2 (RON2L) to form the moving junction during parasite invasion of host cells, and this complex is conserved among apicomplexan parasites. AMA1-RON2L complex immunization achieves higher growth inhibitory activity than AMA1 alone and protects mice against *Plasmodium yoelii* challenge. Here, three single-component AMA1-RON2L immunogens were designed that retain the structure of the two-component AMA1-RON2L complex: one structure-based design (SBD1) and two insertion fusions. All immunogens elicited high antibody titers with potent growth inhibitory activity, yet these antibodies did not block RON2L binding to AMA1. The SBD1 immunogen induced significantly more potent strain-transcending neutralizing antibody responses against diverse strains of *Plasmodium falciparum* than AMA1 or AMA1-RON2L complex vaccination. This indicates that SBD1 directs neutralizing antibody responses to strain-transcending epitopes in AMA1 that are independent of RON2L binding. This work underscores the importance of neutralization mechanisms that are distinct from RON2 blockade. The stable single-component SBD1 immunogen elicits potent strain-transcending protection that may drive the development of next-generation vaccines for improved malaria and apicomplexan parasite control.

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NOVEL MALARIA VACCINE COMPRISING AMA1 AND RON2 ANTIGENS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 63/524,522, filed June 30, 2023, which is incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This work was made with government support from the National Institutes of Health. The government has certain rights in the invention.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0003] The instant application contains an electronic Sequence Listing that has been submitted electronically and is hereby incorporated by reference in its entirety. The sequence listing was created on June 18, 2024, is named "23-0784-WO_Sequence-Listing.xml" and is 151,552 bytes in size.

BACKGROUND

Field of the Disclosure

[0004] This disclosure generally relates to recombinant proteins, and methods and compositions for inducing an immune response in a subject.

Description of Related Art

[0005] Malaria is a life-threatening disease caused by *Plasmodium* parasite infection initiated by the bite of infected female Anopheles mosquitoes. *Plasmodium falciparum* malaria remains one of the most deadly and prevalent infectious diseases globally (14). The risk of contracting malaria and developing severe illness is considerably higher for infants, children, and pregnant women (14). In addition to the increased risk for these populations, the emergence of antimalarial drug resistance undermines malaria control efforts around the world (14). This emphasizes the need for an effective vaccine that prevents parasites from establishing infection and progressing to the erythrocytic stage characterized by the invasion of red blood cells leading to protection from clinical malaria.

[0006] Adults living in malaria-endemic areas develop robust immunity against clinical disease over the course of multiple natural infections (15-17). A vaccine that induces a similar immune response could successfully prevent malaria pathogenesis. Further, transfer of purified immunoglobulin G (IgG) from malaria-immune adults to non-immune individuals with

acute blood stage malaria greatly reduced parasitemia and clinical symptoms (18-20). This indicates that merozoite surface antigens are prime targets of protective antibody responses in blood-stage malaria immunity. The malaria merozoite protein apical membrane antigen 1 (AMA1) is critical for RBC invasion and is one of the most promising blood-stage vaccine candidates (3, 5). AMA1 has been extensively studied for its role in red cell invasion (6, 8, 21-24) and a role for AMA1 in sporozoite infection of the liver and for transmission to mosquitoes have recently been reported (25, 26). The AMA1-RON2L complex and its role in invasion is conserved among apicomplexan parasites (9-11). This suggests that AMA1 based vaccines have the potential to elicit multi-stage protection against natural malaria parasite infection and clinical malaria, and against diverse apicomplexan parasites.

[0007] Malaria merozoites invade target host cells actively by gliding using the moving junction (MJ) formed between the apex of the parasite and the host cell membrane (9-11, 27-30). The MJ is initiated by the export of the rhoptry neck proteins RONs (RON2, RON4, and RON5) into the host cell. RON2 spans the host cell membrane and serves as a receptor for AMA1 located on the surface of the parasite (6, 27-30). AMA1 binds to RON2 to anchor the parasite to the host cell membrane prior to internalization into a parasitophorous vacuole (PV) (27-30). AMA1 is essential for host cell invasion by *Plasmodium falciparum* and *Toxoplasma gondi* (9-11).

[0008] The presence of AMA1 on the merozoite surface and the ability of AMA1-specific antibodies to neutralize parasites *in vitro* and *in vivo* indicate AMA1 is a potential vaccine candidate (1-4). An AMA1-based vaccine FMP2.1/AS02$_A$ (42) elicited strong and sustained antibody responses in naïve individuals (43, 44) and in malaria-exposed adults and children (45-47). However, AMA1 alleles in endemic areas are highly polymorphic. This suggests that parasites may use polymorphisms as an immune evasion strategy to circumvent strain-transcending protection posing a serious challenge to the development of effective strain-transcending vaccine candidates based on AMA1 (48-51). Antibody responses elicited by single AMA1 alleles show significantly lower efficacy against heterologous strains (48). In order to address this problem and achieve strain-transcending protection, combinations of up to seven AMA1 alleles or the design of three diversity covering (DiCo) variants to elicit strain-transcending antibody responses were evaluated with limited success (37, 49, 52-56).

[0009] AMA1-based vaccines induced strong antibody responses but do not provide significant protection against clinical malaria in controlled infection studies and their efficacy in field studies are lower than expected (44, 47, 57, 58). Variations in the dose, adjuvant, and formulation of AMA1-based vaccines showed only moderate improvements (49,59-61). In contrast, rats immunized with the two-component AMA1-RON2L complex elicited higher levels of anti-AMA1 neutralizing antibodies than AMA1 alone likely because the AMA1-RON2L

complex better mimics the true AMA1 structure on invading merozoites (13). Additionally, mice immunized with a *Plasmodium yoelii* AMA1–RON2L complex show complete antibody-dependent protection against a lethal *Plasmodium yoelii* challenge (13). Further, immunizing Aotus monkeys with the AMA1-RON2L complex protects against a virulent Plasmodium falciparum infection and shows higher neutralizing activity in *in vitro* growth inhibitory activity (GIA) than AMA1 alone (62). These studies suggest that enhancement of the quality of the antibody response towards greater neutralizing antibodies may be required over simply improving the quantity of the antibody response.

[0010] Here, single-component immunogens that mimic the AMA1 complex structure on the invading merozoite were created. Three independent designs were evaluated: one structure-based design (SBD1) of AMA1 to reconfigure the sequence permitting attachment of RON2L to the C-terminus, and two insertion fusions placing RON2L within the sequence of AMA1. These single component AMA1-RON2L immunogens possess improved characteristics over AMA1 and replicate the structure of the two-component AMA1/RON2L complex to varying extents. The RON2L in all designed immunogens occupies the binding site in an irreversible manner, making the designed immunogens incapable of binding exogenous RON2 peptides and immunoglobulin new antigen receptor (IgNAR) 14I-1, which both engage the open binding site in AMA1. The antibody quantity and quality elicited by these immunogens was examined in rats. The designed immunogens do not elicit antibodies that block RON2L binding to AMA1, consistent with a locked RON2 bound in the fused immunogens. Despite the lack of RON2L blocking activity, the antibodies raised against the single component immunogens provided protective GIA with *Plasmodium falciparum 3D7* similar to AMA1 DI-DII, and AMA1 DI-DII-RON2L complex.

[0011] Strikingly, the SBD1 immunogen showed significantly more potent strain-transcending GIA with heterologous *Plasmodium falciparum FVO* and *Dd2* parasites as compared to either the AMA1 DI-DII and AMA1 DI-DII-RON2L complexes. These results demonstrate that antibodies targeting regions of AMA1 DI-DII outside of the RON2 binding site and DII loop contribute substantially to strain-transcending and cross-neutralizing activity. These single-component immunogens form the basis for the next generation of AMA1-based antigens for protection against malaria and other apicomplexan parasites.

SUMMARY

[0012] It is against the above background that the present disclosure provides certain advantages over the prior art.

[0013] Although this disclosure as provided herein is not limited to specific advantages or functionalities, the disclosure provides

[0014] In one aspect, the disclosure provides a recombinant protein, comprising:

(a) a first peptide comprising a C-teriminal portion of an apical membrane antigen 1 (AMA1) protein;

(b) a second peptide comprising an N-terminal portion of the AMA1 protein; and

(c) a third peptide comprising at least a portion of a rhoptry neck protein 2 (RON2) protein.

[0015] In some embodiments of the recombinant protein, the recombinant protein comprises from the N-terminus to the C-terminus the first peptide, then the second peptide, and then the third peptide. In certain embodiments, wherein the recombinant protein comprises from the N-terminus to the C-terminus the third peptide, then the first peptide, and then the second peptide.

[0016] In some embodiments of the recombinant protein, the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein. In some embodiments, the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01. In some embodiments, the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:02. In some embodiments, the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-438 of SEQ ID NO:01. In some embodiments, the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-541 of SEQ ID NO:01. In some embodiments of the recombinant protein, the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of an amino acid sequences selected from the group consisting of SEQ ID NO:46, 47, 48, 53, 54, 57, 58, 62, 63, 69, and 70.

[0017] In some embodiments of the recombinant protein, the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein. In some embodiments, the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01. In some embodiments, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:03. In some embodiments, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 94-358 of SEQ ID NO:01. In some embodiments, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 25-358 of SEQ ID NO:01. In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 104-131 of SEQ ID NO:01. In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 117-131 of SEQ ID NO:01. In some embodiments of the recombinant protein, the second peptide comprises about 10 amino acids to about 520 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of an amino acid sequence selected from the group consisting of SEQ ID NOs:46, 47, 48, 53, 54, 57, 58, 62, 63, 69, and 70.

[0018] In some embodiments of the recombinant protein, the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein. In some embodiments, the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:04. In some embodiments, the third peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or

higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:05-33.

[0019] In some embodiments, the recombinant protein further comprises a linker domain between the first peptide and the second peptide. In some embodiments, the linker domain comprises a flexible linker, for example, a G_4S linker.

[0020] In some embodiments, the recombinant protein comprises at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83. In some embodiments of the recombinant protein, the recombinant protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83. In some embodiments of the recombinant protein, the recombinant protein consists of an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83.

[0021] In some embodiments of the recombinant protein, the AMA1 protein is from the *Plasmodium* genus, the *Babesia* genus, or *Toxoplasma gondii*.

[0022] In some embodiments of the recombinant protein, the RON2 protein is from the *Plasmodium* genus, the *Babesia* genus, or *Toxoplasma gondii*.

[0023] In some embodiments of the recombinant protein, the AMA1 protein and the RON2 protein are from the same species.

[0024] In some embodiments, the recombinant protein further comprises fusion to one or more additional antigens.

[0025] In another aspect, this disclosure provides a nucleic acid composition, comprising a nucleic acid sequence encoding the recombinant protein(s) as disclosed herein.

[0026] In another aspect, this disclosure provides a vector, comprising the nucleic acid sequence(s) as disclosed herein.

[0027] In another aspect, this disclosure provides an immunotherapy composition, comprising the recombinant protein(s) as disclosed herein, the nucleic acid sequence(s) as disclosed herein, or the vector(s) as disclosed herein, and at least one adjuvant and/or a carrier. In some embodiments, the adjuvant is selected from the group consisting of AddaS03™, aluminum hydroxide, aluminum phosphate, aluminum sulfate, monophosphoryl lipid A (MPL), QS-21, TQL1055, QS-18, QS-17, QS-7, Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), oil in water emulsions, CpG, polyglutamic acid, polylysine, AddaVax™, MF59®, and/or combinations thereof. In some embodiments, the

carrier is selected from the group consisting of albumin, diphtheria toxoid (DT), a genetically modified cross-reacting material (CRM) of diphtheria toxin, CRM197, flagellin, *H. influenzae* protein D (HiD), immunoglobulin molecules, KLH (keyhole limpet hemocyanin), mannose-6-phosphate, meningococcal outer membrane protein complex (OMPC), ovalbumin, poly-L-lysine, poly-L-glutamine, rEPA (Pseudomonas aeruginosa exotoxin A), thyroglobulin, and tetanus toxoid (TT). In some embodiments, the immunotherapy composition further comprises a lipid nanoparticle or a nanoparticle.

[0028] In another aspect, this disclosure provides a method of vaccinating a subject, comprising administrating to the subject the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein.

[0029] In another aspect, this disclosure provides a method of treating a subject with malaria or protecting a subject from malaria infection, comprising administrating to the subject the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein.

[0030] In another aspect, this disclosure provides a method of treating a subject with toxoplasmosis or protecting a subject from toxoplasmosis infection, comprising administrating to the subject the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein.

[0031] In another aspect, this disclosure provides a method of treating a subject with babesiosis or protecting a subject from babesiosis infection, comprising administrating to the subject the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein.

[0032] In some embodiments of the methods of treating or protecting a subject, the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein are administered with one or more additional active agents. In some embodiments, the method comprises repeating the administering at least a second time, at least a third time, at least a fourth time, at least a fifth time, or at least a sixth time.

[0033] In another aspect, this disclosure provides an immunoglobulin that binds to the recombinant protein(s) as disclosed herein. In some embodiments, the immunoglobulin is

isolated from a subject using the recombinant protein(s) as disclosed herein, and wherein the subject has naturally acquired immunity to malaria, toxoplasmosis, or babesiosis.

[0034] In another aspect, this disclosure provides an immunoglobulin that binds the recombinant protein(s) as disclosed herein, wherein the immunoglobulin is obtained by immunization with the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein.

[0035] In some embodiments, the immunoglobulin is a monoclonal antibody or a plurality of polyclonal antibodies. In certain embodiments, the immunoglobulin cross-reacts with different strains and/or subtypes of malaria. In some embodiments, the immunoglobulin cross-reacts with different strains and/or subtypes of toxoplasmosis. In some embodiments, the immunoglobulin cross-reacts with different strains and/or subtypes of babesiosis.

[0036] In another aspect, this disclosure provides a method of generating antibodies cross-protective against malaria comprising: (a) immunizing a subject with the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein: (b) isolating antibodies that cross-react with different strains and/or subtypes of malaria.

[0037] In another aspect, this disclosure provides a method of generating antibodies cross-protective against toxoplasmosis comprising: (a) immunizing a subject with the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein: (b) isolating antibodies that cross-react with different strains and/or subtypes of toxoplasmosis.

[0038] In another aspect, this disclosure provides a method of generating antibodies cross-protective against babesiosis comprising: (a) immunizing a subject with the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein: (b) isolating antibodies that cross-react with different strains and/or subtypes of babesiosis.

[0039] The term “cross-protective” as used herein refers to antibodies that inhibit or reduce the severity of infection by multiple different pathogen strains or subtypes.

[0040] These and other features and advantages of the present disclosure will be more fully understood from the following detailed description taken together with the accompanying claims. It is noted that the scope of the claims is defined by the recitations therein and not by the specific discussion of features and advantages set forth in the present description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0041] The following detailed description of the embodiments of the present disclosure can be best understood when read in conjunction with the following drawings, where like structure is indicated with like reference numerals and in which:

[0042] **FIG. 1A – 1F** show an overview of design of single-component immunogens. **FIG.1A** Schematic illustrating the domain organization of the full-length AMA1 (SEQ ID NO:01). **FIG. 1B** Structure of apo AMA1 DI-DII showing the Domain II loop (DII loop) and DIf loop, and the location of RON2L in the bound complex. **FIG. 1C** A circularly permutated immunogen 1 (SBD1 Immunogen) was created by introducing a Gly/Ser linker between the original termini (not shown) and by removing the DII loop, which produced a novel N- and C-termini at residues at Lys386 and Thr357, respectively. Then, RON2L was fused to this new C-terminus without a linker. **FIG. 1D** and **FIG. 1E** Insertion fusion Immunogens 2 (Insertion fusion immunogen 2) and 3 (Insertion fusion immunogen 3) were constructed by replacing the DIf loop of AMA1 DI-DII with RON2L and by removing the DII loop. Immunogen 3 retains Cys263 and its disulfide bridge. FIG. 1B-1E were created using structures of apo AMA1 (PDB ID: 4r19) and AMA1-RON2L complex (PDB ID: 3zwz). An arrow indicates the point of fusion. **FIG. 1F** Schematic illustrating all immunogens discussed in this disclosure.

[0043] **FIG. 2A – 2D** show the yield and stability of single-component immunogens are higher than those of AMA1 DI-DII alone and AMA1 DI-DII-RON2L complex. **FIG. 2A** All three immunogens expressed at higher levels than AMA1 DI-DII and eluted as monomers by size exclusion chromatography (SEC). Inset in **FIG. 2A** shows reducing SDS-polyacrylamide gel electrophoresis (PAGE), which confirms the high purity of immunogens. **FIG. 2B** Purification yield from three separate purifications. Bars represent mean yield from three separate purifications. **FIG. 2C** Differential scanning fluorimetry indicated that three immunogens have higher thermostability than AMA1 DI-DII and AMA1 DI-DII-RON2L complex. **FIG. 2D** T_m from five independent measurements. Bars represent mean.

[0044] **FIG. 3A – 3D** show RON2L is bound to AMA1 in the designed immunogens preventing accessibility to the RON2L binding site. **FIG. 3A** Representative biolayer interferometry (BLI) traces used to quantitatively measure the binding of immunogens to IgNAR 14I-1 demonstrating inaccessibility of the epitope located in the RON2L binding pocket in the immunogens. **FIG. 3B** IgNAR 14I-1 shows little or no binding to immunogens by ELISA. **FIG. 3C** Representative BLI traces used to measure the binding of immunogens to exogenous RON2L demonstrating that the binding site for exogenous RON2L is occupied by the fused RON2L in the designed immunogens. **FIG. 3D** Exogenous RON2L does not bind to

immunogens by ELISA. In FIG. 3B and FIG. 3D, bovine serum albumin (BSA) was used as a negative control.

[0045] **FIG. 4A – 4C** show that the single-component immunogens have a very similar structure to the AMA1-RON2L complex. **FIG. 4A** Crystal structures of single component immunogens 1 (SBD1 immunogen), 2 (Insertion fusion immunogen 2), and 3 (Insertion fusion immunogen 3). The fused RON2L portion of the immunogen is shaded darker than the AMA1 portion. **FIG. 4B** Single-component immunogens superimposed on the AMA1-RON2L complex (PDB: 3zwz). **FIG. 4C** A focused view of RON2L and the surrounding loops in single-component immunogens superimposed on the AMA1/RON2L complex (PDB: 3zwz).

[0046] **FIG. 5A – 5D** show neutralizing antibody levels in rats immunized with single component immunogens are similar to those of AMA1 DI-DII alone or AMA1 DI-DII-RON2L complex. **FIG. 5A** Immunization and blood draw scheme for rats. **FIG. 5B** Serum IgG titers against AMA1 DI-DII. Dashed line indicates detection limit of assay and bars represent the geometric mean titers (GMTs). **FIG. 5C** Serum antibody titers blocking AMA1 DIDII/RON2L interaction depicted as described in FIG. 5B. **FIG. 5D** *In vitro* growth inhibitory activity (GIA) of purified IgG from individual rats from each group at day 63 tested at 5.0 mg/ml against the *Plasmodium falciparum 3D7* blood stage. Dashed line indicates detection limit of assay and bars represent median.

[0047] **FIG. 6A – 6F** show Immunogen 1 (SBD1 immunogen) elicits significantly more potent strain-transcending antibodies than AMA1 DI-DII alone or AMA1 DI-DII-RON2L complex. *In vitro* growth inhibitory activity (GIA) dilution series of pooled purified IgG from each group at day 63 against *Plasmodium falciparum* **FIG. 6A** *3D7* **FIG. 6B** *FVO* **FIG. 6C** *Dd2*. Concentration (mg/ml) of pooled purified IgG required to reduce % GIA by 50% (IC_{50}) against *Plasmodium falciparum* **FIG. 6D** *3D7* **FIG. 6E** *FVO* and **FIG. 6F** *Dd2* were determined by interpolation after fitting data to a four-parameter dose-response curve. The data arise from at least two independent biological replicates and plotted as median with 95 % CI. Statistical comparisons were made using a F-test.

[0048] **FIG. 7A – 7C** show the purification and characterization of the AMA1 DI-DII ΔDII-loop. **FIG. 7A** Size exclusion chromatography (SEC) profile of the AMA1 DI-DII ΔDII-loop. The peak for the AMA1 DI-DII ΔDII-loop is shown between two dotted lines. Inset shows Coomassie Brilliant Bluestained SDS-PAGE gel for AMA1 DI-DII ΔDII-loop (35.4-kDa) under reducing condition. **FIG. 7B** Differential scanning fluorimetry indicated that deletion of DII loop does not improve the stability of engineered AMA1 DI-DII. **FIG. 7C** T_m from five independent measurements. Bar represents mean.

[0049] **FIG. 8A– 8C** show the root-mean-square deviation (RMSD) for the Cα atom of each residue in the **FIG. 8A** SBD1 immunogen (Immunogen 1), **FIG. 8B** Insertion fusion immunogen 2 (Immunogen 2), and **FIG. 8C** Insertion fusion immunogen 3 (Immunogen 3), when aligned to the AMA1 DI-DII-RON2L complex. The residues are labeled based on the wildtype AMA1 and RON2 sequence numbering. The domain I loops are indicated by dark lines and shades beneath the corresponding data points.

[0050] **FIG. 9A– 9C** show that RON2L of the single-component immunogens shows a disulfide-anchored U-shaped conformation in the hydrophobic groove of AMA1. Electron density for the RON2L component of immunogens 1 (SBD1 immunogen; **FIG. 9A**), 2 (Insertion fusion immunogen 2; **FIG. 9B**), and 3 (Insertion fusion immunogen 3; **FIG. 9C**) from a Polder map (gray mesh) contoured at 1.0 σ level (1.43 rmsd).

[0051] **FIG. 10A – 10B** show an influential residue, Arg2041, is located at the tip of the β-hairpin and with its guanidyl group is adequately positioned within the preformed pocket of AMA1. As in **FIG. 10A** AMA1 DI-DII-RON2L complex structure (PDB ID: 3zwz), the Arg residue of RON2L of **FIG. 10B** all three single-component immunogens fits snugly into a deep pocket in the surface of AMA1. The complex network of hydrogen bonds stabilizes this structure.

[0052] **FIG. 11A – 11C** show *in vitro* growth inhibitory activity (GIA) dilution series of pooled purified IgG from each group at day 63 against *Plasmodium falciparum* **FIG. 11A** *3D7* **FIG. 11B** *FVO* **FIG. 11C** *Dd2*. IC_{50} values were determined by interpolation after fitting data to a four-parameter dose-response curve. The data arise from at least two independent biological replicates and plotted as mean.

[0053] **FIG. 12A – 12B** show sequence alignment of AMA1 DI-DII from *Plasmodium falciparum* 3D7 (SEQ ID NO:98), FVO (SEQ ID NO:99), and Dd2 (SEQ ID NO:100) strains and polymorphic residues mapped onto the AMA1 domain I loops. **FIG. 12A** Polymorphic residues, which vary between the strains, are marked by arrows. Residues within the AMA1 domain I loops surrounding the RON2L binding site, in the presence of RON2L, are marked in bold and labeled. The multiple sequence alignments were generated using the Clustal Omega/TCoffee. **FIG. 12B** Polymorphic residues mapped onto the AMA1 domain I loops surrounding the RON2L binding site in the presence of RON2L. The polymorphic residues are indicated within the AMA1 domain I loops. The AMA1 loops are shown (labeled DIa, DIb, DIc, DId, DIe, and DIf loops).

[0054] **FIG. 13** shows Coomassie Brilliant Blue-stained SDS-PAGE gel for AMA1 ectodomain (biotinylated, 63.8-kDa), TrxA-RON2L-1 (18.1-kDa), IgNAR 14I-1 (14.2-kDa),

TrxA-RON2L-2 (biotinylated, 20.2-kDa) and IgNAR 14I-1 (biotinylated, 16.7-kDa) under reducing condition.

[0055] **FIG. 14A – 14C** show purification and characterization of the AMA1 DI-DII-RON2L complex. **FIG. 14A** Size exclusion chromatography (SEC) profile of the AMA1 DI-DII-RON2L complex. The peak for the AMA1 DI-DII-RON2L complex is shown between two dotted lines. A second peak corresponds to cleaved TrxA (6x-His tagged). Inset shows silver-stained SDS-PAGE gel for AMA1 DI-DII (39.2-kDa) and RON2L (4.2-kDa) components of complex under reducing condition. **FIG. 14B** Coomassie Brilliant Blue-stained SDS-PAGE gel for AMA1 DI-DII and RON2L components of complex under non-reducing condition. **FIG. 14C** Western blot for the AMA1 DI-DII and RON2L components of complex is depicted, along with the primary probe used. AMA1 DI-DII (39.2-kDa) and TrxA (14.73-kDa) were used as controls under non-reducing condition.

[0056] Skilled artisans will appreciate that elements in the Figures are illustrated for simplicity and clarity and have not necessarily been drawn to scale. For example, the dimensions of some of the elements in the Figures can be exaggerated relative to other elements to help improve understanding of the embodiment(s) of the present disclosure.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0057] All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

[0058] Before describing the present disclosure in detail, a number of terms will be defined. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. For example, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. It should be understood that the terms “a” and “an” as used herein refer to “one or more” of the enumerated components unless otherwise indicated or dictated by its context. The use of the alternative (*e.g.*, “or”) should be understood to mean either one, both, or any combination thereof of the alternatives unless otherwise indicated.

[0059] In the present disclosure, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated.

[0060] As used herein, the term “about” means ±10% of the indicated range, value, sequence, or structure, unless otherwise indicated.

[0061] It is noted that terms like "preferably," "commonly," and "typically" are not utilized herein to limit the scope of the claimed subject matter or to imply that certain features are critical, essential, or even important to the structure or function of the claimed subject matter. Rather, these terms are merely intended to highlight alternative or additional features that can or cannot be utilized in a particular embodiment of the present disclosure.

[0062] For the purposes of describing and defining the present disclosure it is noted that the term "substantially" is utilized herein to represent the inherent degree of uncertainty that can be attributed to any quantitative comparison, value, measurement, or other representation. The term "substantially" is also utilized herein to represent the degree by which a quantitative representation can vary from a stated reference without resulting in a change in the basic function of the subject matter at issue.

[0063] Unless expressly specified otherwise, the term "comprising" is used in the context of the present disclosure to indicate that further members may optionally be present in addition to the members of the list introduced by "comprising". It is, however, contemplated as a specific embodiment of the present disclosure that the term "comprising" encompasses the possibility of no further members being present, *i.e.*, for the purpose of this embodiment "comprising" is to be understood as having the meaning of "consisting of".

[0064] As utilized in accordance with the present disclosure, unless otherwise indicated, all technical and scientific terms shall be understood to have the same meaning as commonly understood by one of ordinary skill in the art.

[0065] Methods well known to those skilled in the art can be used to construct genetic expression constructs and recombinant cells according to this disclosure. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, *in vivo* recombination techniques, and polymerase chain reaction (PCR) techniques. *See, for example*, techniques as described in Green & Sambrook, 2012, MOLECULAR CLONING: A LABORATORY MANUAL, Fourth Edition, Cold Spring Harbor Laboratory, New York; Ausubel *et al.*, 1989, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, New York, and PCR Protocols: A Guide to Methods and Applications (Innis *et al.*, 1990, Academic Press, San Diego, CA).

[0066] As used herein, the terms "polynucleotide," "nucleotide," "oligonucleotide," and "nucleic acid" can be used interchangeably to refer to nucleic acid comprising DNA, RNA, derivatives thereof, or combinations thereof, in either single-stranded or double-stranded embodiments depending on context as understood by the skilled worker. In the present disclosure, a "nucleic acid" molecule can include, DNA, cDNA and genomic DNA sequences, RNA, messenger RNA, and synthetic nucleic acid sequences. In some embodiments, the

nucleic acid molecules are codon-optimized for expression. Thus, "nucleic acid" also encompasses embodiments in which analogs of DNA and RNA are employed. In some embodiments, the nucleic acid component may comprises one or more RNA molecules, such as viral RNA molecules or mRNA molecules that encode the protein of interest.

[0067] As used herein, the "N-terminus" (also known as the amino-terminus, NH2-terminus, N-terminal end or amine-terminus) refers to the start of a protein or polypeptide, referring to the free amine group (-NH2) located at the end of a polypeptide. Within a peptide, the amine group is bonded to the carboxylic group of another amino acid, making it a chain. That leaves a free carboxylic group at one end of the peptide, called the C-terminus, and a free amine group on the other end called the N-terminus. By convention, peptide sequences are written N-terminus to C-terminus, left to right. This correlates the translation direction to the text direction, because when a protein is translated from messenger RNA, it is created from the N-terminus to the C-terminus, as amino acids are added to the carboxyl end of the protein. As used herein, the "C-terminus" (also known as the carboxyl-terminus, carboxy-terminus, C-terminal tail, C-terminal end, or COOH-terminus) refers to the end of an amino acid chain (protein or polypeptide), terminated by a free carboxyl group (-COOH). The convention for writing peptide sequences is to put the C-terminal end on the right and write the sequence from N- to C-terminus.

[0068] AMA1 is a parasite membrane protein and the ectodomain consists of three disulfide constrained domains (domains I-III) preceded by a prosequence at the N-terminus (*see* **Figure 1A**). During the erythrocytic stage, AMA1 is expressed as an 83-kDa precursor that is routed to secretory organelles at the apical end of the merozoite where it is proteolytically processed to a 66-kDa form (3, 32). Both the 83-kDa precursor and 66-kDa form remain membrane-bound (3, 32). The 66-kDa form selectively translocates to the surface of the merozoite prior to erythrocyte invasion (3, 83) whereas the unprocessed 83-kDa form remains apically restricted (3, 33). At the end of invasion, the 66-kDa form is further proteolytically cleaved at a membrane-proximal site following domain III on the surface of merozoites (34, 35). Consequently, the bulk of the AMA1 ectodomain is shed from the parasite surface predominantly as two soluble forms of 44- and 48-kDa and a rarer 52-kDa form (34).

[0069] The AMA1 ectodomain structure has a stacked three-domain architecture (6, 12, 31). Domains I and II form a RON2L binding site that is partially occupied by the DII loop that extends from domain II (7, 8). The DII loop is highly flexible and undergoes conformational changes to expose the binding site for RON2 (6-8, 12). There are several residues within the RON2 binding site that are conserved across *Plasmodium* and Apicomplexan species (7). Antibodies or peptides that prevent the formation of the AMA1-RON2 complex block red cell

invasion by parasites (36-40). Thus, disrupting the AMA1-RON2 complex is therefore an attractive strategy for developing anti-infectives. Antibodies against AMA1 are also believed to block red cell invasion by disrupting its secondary proteolytic processing on the merozoite surface (41).

[0070] This disclosure provides a recombinant protein, comprising:

(a) a first peptide comprising a C-terminal portion of an apical membrane antigen 1 (AMA1) protein;

(b) a second peptide comprising an N-terminal portion of the AMA1 protein; and

(c) a third peptide comprising at least a portion of a rhoptry neck protein 2 (RON2) protein.

[0071] In some embodiments, the recombinant protein comprises from the N-terminus to the C-terminus the first peptide, then the second peptide, and then the third peptide. In other embodiments, the recombinant protein comprises from the N-terminus to the C-terminus the third peptide, then the first peptide, and then the second peptide.

[0072] As disclosed herein, the terms "recombinant protein" or "antigen" can refer to peptides derived from pathogenic organisms (*e.g.*, malaria, toxoplasmosis and babesiosis), in particular bacterial, viral or protozoological (multicellular) pathogenic organisms, which evoke an immunological reaction by a subject, for example, a mammalian subject or human subject. In certain embodiments, a protein of interest is a surface antigen, *e.g.,* proteins (or fragments of proteins, *e.g.*, the exterior portion of a surface antigen) located at the surface of the virus or the bacterial or protozoological organism.

[0073] In some embodiments of the recombinant protein, the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein.

[0074] In some embodiments of the recombinant protein, the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01.

[0075] In some embodiments of the recombinant protein, the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of an amino acid sequences selected from the group consisting of SEQ ID NO:46, 47, 48, 53, 54, 57, 58, 62, 63, 69, and 70.

[0076] In some embodiments of the recombinant protein, the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:02.

[0077] In some embodiments of the recombinant protein, the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-438 of SEQ ID NO:01.

[0078] In some embodiments of the recombinant protein, the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-541 of SEQ ID NO:01.

[0079] In some embodiments of the recombinant protein, the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein.

[0080] In some embodiments of the recombinant protein, the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01.

[0081] In some embodiments of the recombinant protein, the second peptide comprises about 10 amino acids to about 520 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of an amino acid sequence selected from the group consisting of SEQ ID NOs:46, 47, 48, 53, 54, 57, 58, 62, 63, 69, and 70.

[0082] In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:03.

[0083] In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 94-358 of SEQ ID NO:01.

[0084] In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 25-358 of SEQ ID NO:01. In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 104-131 of SEQ ID NO:01. In some embodiments of the recombinant protein, the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 117-131 of SEQ ID NO:01.

[0085] In some embodiments of the recombinant protein, the third peptide comprises about 25 amino acids to about 50 amino acids from the rhoptry neck protein 2 (RON2).

[0086] In some embodiments of the recombinant protein, the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:04.

[0087] In some embodiments of the recombinant protein, the third peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:05-33.

[0088] In some embodiments of the recombinant protein, the recombinant protein comprises at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83. In some embodiments of the recombinant protein, the recombinant protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83. In some embodiments of the recombinant protein, the recombinant protein consists of an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83.

[0089] As used herein, the terms “sequence identity” or percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*e.g.*, about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher

identity over a specified region (a polypeptide sequence comprising conserved elements), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (*see e.g.*, NCBI web site or the like). Such sequences are then said to be "substantially identical." This definition also refers to, or can be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25, 50, 75, 100, 150, 200 amino acids or nucleotides in length, and oftentimes over a region that is 225, 250, 300, 350, 400, 450, 500 amino acids or nucleotides in length or over the full-length of an amino acid or nucleic acid sequences.

[0090] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0091] A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402 (1977) and Altschul *et al.*, J. Mol. Biol. 215:403-410 (1990), respectively. BLAST software is publicly available through the National Center for Biotechnology Information on the worldwide web at ncbi.nlm.nih.gov/. Both default parameters or other non-default parameters can be used. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0092] In some embodiments, the recombinant protein further comprises a linker domain between the first peptide and the second peptide. Linkers can comprise flexible amino acid residues (*e.g.*, glycine or serine) to permit adjacent domains to move freely related to one another. In some embodiments, the amino acid composition of a linker can mimic the composition of linkers commonly found in recombinant proteins, which can generally by

classified as flexible or rigid linkers. For example, flexible linkers found in recombinant proteins are generally composed of small, non-polar (*e.g.*, Gly) or polar (*e.g.*, Ser or Thr) amino acids whose small size provides flexibility and allows for mobility of the connecting functional domains. The incorporation of, *e.g.*, Ser or Thr can maintain the stability of the linker in aqueous solutions by forming hydrogen bonds with the water molecules, and therefore can reduce interactions between the linker and the immunogens. In some embodiments, a linker comprises stretches of Gly and Ser residues ("GS" linker). An example of a widely used flexible linker is (Gly-Gly-Ser)n, (Gly-Gly-Gly-Ser)n (SEQ ID NO:71) or (Gly-Gly-Gly-Gly-Ser)n (SEQ ID NO:72), where n=1-5. Adjusting the copy number "n" can optimize a linker to achieve sufficient separation of the functional immunogen domains to, *e.g.*, maximize an immunogenic response. Many other flexible linkers have been designed for recombinant fusion proteins that can be used herein. In some embodiments, linkers can be rich in small or polar amino acids such as Gly and Ser but also contain additional amino acids such as Thr and Ala to maintain flexibility, as well as polar amino acids such as Lys and Glu to improve solubility. In certain embodiments, when present, the linker can be an amino acid sequence selected from the group consisting of GGGS (SEQ ID NO:71), GGGSGGGS (SEQ ID NO:73), GGGSGGGSGGGS (SEQ ID NO:74), GGGSGGGSGGGSGGGS (SEQ ID NO:75), GGGGS (SEQ ID NO:72), GGGGSGGGGS (SEQ ID NO:76), GGGGSGGGGSGGGGS (SEQ ID NO:77), and GGGGSGGGGSGGGGSGGGGS (SEQ ID NO:78).

Stabilizer for Protein Expression and Epitope Design (SPEEDesign)

[0093] In some embodiments, the recombinant proteins as disclosed herein can be further modified to include stabilizing mutations on top of SBD1 design. In some embodiments, a computational pipeline is used to improve antigens by making mutations to improve stability, focus immune response, stabilize stages computational design and in vitro screening pipeline to improve vaccine candidates of the recombinant proteins as disclosed herein. Stabilizer for Protein Expression and Epitope Design (SPEEDesign) refers to a pipeline that retains neutralizing epitopes while stabilizing protein domains and removing non-neutralizing epitopes (*see* WO 2022/178545, which is incorporated by reference herein in its entirety). For example, in some embodiments, neutralizing epitopes are unchanged, while residues that are exposed are searched during the design process to identify amino acid changes that would stabilize an accessible epitope, and all remaining residues are allowed to sample a limited sequence space defined by energetic and evolutionary restraints.

[0094] At least one of the goals of the Stabilizer for Protein Expression and Epitope Design (SPEEDesign) is to focus the immune response to conformational neutralizing epitopes from an antigen with neutralizing, non-neutralizing and immunodominant epitopes while stabilizing the domain. Within the context of SPEEDesign, four design approaches are contemplated to

improve vaccine efficacy of the recombinant protein. Neutralizing antibody titers can be improved by: (1) focusing the immune response to neutralizing epitopes, (2) eliminating immunogenicity of non neutralizing epitopes, (3) stabilizing the antigen to lengthen half-life and improve immunogenicity, and (4) promoting transient states. In exemplary embodiments, the core is stabilized by evolutionarily allowed residues from multiple sequence alignments, neutralizing epitope residues remained fixed, exposed residues not under evolutionary constraints are allowed to vary extensively, non-neutralizing/immunodominant epitopes are allowed to mutate. Result candidates are fed into Rosetta design procedure with multiple protocols and clustering analysis samples the most diverse representative designs.

[0095] SPEEDesign residue definition: Each amino acid in the target antigen is categorized as fixed, intermediate, or deep search, defining the depth of the computational search at that position. For example, residues that form an interface with neutralizing antibodies can be defined as fixed. The residues that comprise these fixed epitopes are defined as those that have a > 1A change in solvent accessible surface area upon complex formation, and calculations were performed in PyMOL. Those residues that are exposed can be defined as deep search. Residues exposed upon domain extraction from a larger protein require unique handling during the design processes. These residues are buried or interacting with other residues in the larger protein, and they become fully solvent exposed once the domain is extracted. This dramatic change in chemical environment is accommodated by allowing deep search residues to vary greatly during the design process. Since these residues are not exposed in homologous proteins, conservation or evolutionary-based design principles are unlikely to prove sufficient to redesign these new non-natural surfaces. In some embodiments, these residues were therefore classified for deep search during design where all amino acids except cysteine are allowed. In other embodiments, all amino acids are allowed for deep search. All other residues were defined as intermediate. These residues are allowed to vary to a limited extent that is driven by conservation and evolutionary analysis of similar protein sequences to identify potential amino acid changes.

[0096] SPEEDesign ROSETTA strategies: All ROSETTA strategies leave fixed residues unchanged to preserve neutralizing epitopes, and each strategy differs in the amino acid changes allowed for the intermediate and deep search categories of residues. In strategy 1, intermediate residues were unchanged and all amino acids except cysteine were allowed at deep search positions. In strategy 2, all amino acids were allowed at deep search positions, and intermediate positions were allowed to sample amino acids found in proteins with similar sequences (evolutionary constraints). Strategy 2 samples a very large sequence space, which is constrained in strategy 3 by disallowing amino acid changes that are energetically unfavorable when made individually (energetic constraints), an approach adapted from the

PROSS protocol. Strategy 4 places evolutionary and energetic constraints on the intermediate residues but allows the deep search residues to sample all amino acids.

[0097] SPEEDesign clustering: For each computational strategy, decoys with scores in the 95th percentile were clustered by sequence similarity and the top scoring decoy form each cluster was selected as a representative sequence. In some embodiments, high-scoring decoy sequences are clustered based on sequence similarity, wherein clustering high-scoring decoy sequences comprises clustering decoy sequences with scores in a 90th percentile, a 95th percentile, a 96th percentile, a 97th percentile, a 98th percentile, or a 99th percentile based on sequence similarity. Those of skill in the art will recognize that any suitable clustering method and/or program may be used for decoy clustering as disclosed herein (*e.g.*, CD-HIT, phylogenetic tree generation followed by internal node sequence screening, *etc.*). The number of clusters was selected based on the sequence diversity produced in each computational strategy. For example, strategy 1 samples a limited sequence space, while strategy 2 samples a very large sequence space. Therefore, more clusters were created to sample strategy 2 than strategy 1.

Nucleic acid vectors

[0098] In some aspects, this disclosure provides nucleic acid compositions comprising a nucleic acid sequence encoding the recombinant protein as disclosed herein. In some embodiments, the nucleic acid compositions comprise a vector. As used herein, the term "vector" refers to a nucleic acid molecule that when introduced into a mammal, induces the expression of the encoded recombinant protein of interest within the mammals. In some embodiments, the vector causes the mammals' immune system to become reactive against the protein of interest (*e.g.*, the recombinant protein as disclosed herein and/or an antigen thereof). In certain embodiments the vector is a DNA vaccine in the form of a DNA plasmid. A DNA plasmid is one that includes an encoding sequence of a recombinant protein of interest that is capable of being expressed in a mammalian cell, upon the vector entering after administration. In certain embodiments, administration can be by injection. In some embodiments, the administration uses electroporation. In some embodiments, the vector encodes a sequence for the recombinant protein of interest that elicits an immune response in the subject. In some embodiments, the vector is optimized for mammalian expression, which can include one or more of the following: including the addition of a Kozak sequence, codon optimization, and RNA optimization.

[0099] In certain embodiments, the vector of this disclosure can be formulated for pharmaceutical administration. While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this disclosure, the type of

carrier will vary depending on the mode of administration. For parenteral administration, including intranasal, intradermal, subcutaneous or intramuscular injection or electroporation, the carrier preferably comprises water, saline, and optionally an alcohol, a fat, a polymer, a wax, one or more stabilizing amino acids or a buffer. General formulation technologies are known to those of skill in the art (*see, for example*, Remington: The Science and Practice of Pharmacy (20th edition), Gennaro, ed., 2000, Lippincott Williams & Wilkins; Injectable Dispersed Systems: Formulation, Processing And Performance, Burgess, ed., 2005, CRC Press; and Pharmaceutical Formulation Development of Peptides and Proteins, Frkjr *et al.*, eds., 2000, Taylor & Francis).

[0100] DNA vectors can be administered in solution (*e.g.*, a phosphate-buffered saline solution) by injection, usually by an intra-arterial, intravenous, subcutaneous or intramuscular route. In general, the dose of a naked nucleic acid composition is from about 10 μg to 10 mg for a typical 70 kilogram patient. Subcutaneous or intramuscular doses for naked nucleic acid (typically DNA encoding a fusion protein) will range from 0.1 mg to 50 mg for a 70 kg patient in generally good health. In certain embodiments, about 1 mg to about 20 mg of DNA is administered (for example, about 1 mg, about 2.5 mg, about 4 mg, about 5 mg, about 6 mg, about 7 mg, about 8 mg, about 9 mg, about 10 mg, about 15 mg, or about 20 mg).

[0101] Compositions comprising a DNA vector can be administered once or multiple times. For vaccination with a vector, administration can be performed more than once, for example, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20 or more times as needed to induce the desired response (*e.g.*, specific antigenic response or proliferation of immune cells). Multiple administrations can be administered, for example, bi-weekly, weekly, bi-monthly, monthly, or more or less often, as needed, for a time period sufficient to achieve the desired response.

[0102] The vectors of this disclosure are administered to a mammalian host. The mammalian host usually is a human or a primate. In some embodiments, the mammalian host can be a domestic animal, for example, canine, feline, lagomorpha, rodentia, rattus, hamster, murine. In other embodiment, the mammalian host is an agricultural animal, for example, bovine, ovine, porcine, equine, *etc.*

[0103] The vectors encoding the recombinant proteins as disclosed herein can be formulated in accordance with standard techniques well known to those skilled in the pharmaceutical art. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration.

[0104] The vectors encoding the recombinant proteins as disclosed herein can be administered alone, or can be co-administered or sequentially administered with other immunological, antigenic, vaccine, or therapeutic compositions.

[0105] The vectors encoding the recombinant proteins as disclosed herein can additionally be complexed with other components such as peptides, polypeptides and carbohydrates for delivery. For example, expression vectors, nucleic acid vectors that are not contained within a viral particle, can be complexed to particles or beads that can be administered to an individual.

[0106] DNA vectors and DNA vaccines can be administered by methods well known in the art as described in Donnelly *et al.* (Ann. Rep. Immunol. 15:617-648 (1997)); Feigner *et al.* (U.S. Pat. No. 5,580,859, issued Dec. 3, 1996); Feigner (U.S. Pat. No. 5,703,055, issued Dec. 30, 1997); and Carson *et al.* (U.S. Pat. No. 5,679,647, issued Oct. 21, 1997), each of which is incorporated herein by reference. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector.

[0107] In some embodiments, the vector comprises an RNA construct comprises an mRNA sequence encoding the recombinant protein of interest (*e.g.*, the recombinant protein as disclosed herein and/or an antigen thereof). In an embodiment, the mRNA sequence is a natural and non-modified mRNA. Within the context of the present disclosure, natural and non-modified mRNA encompasses mRNA generated *in vitro*, without chemical modifications or changes in the sequence. In certain embodiments, the mRNA can be an artificial mRNA. In the context of the present disclosure, artificial mRNA encompasses mRNA with chemical modifications, sequence modifications or non-natural sequences.

[0108] An antigen-providing mRNA may be an mRNA, having at least one open reading frame that can be translated by a cell or an organism provided with that mRNA. The product of this translation is a peptide or protein that may act as an antigen, preferably as an immunogen. The product may also be a fusion protein composed of more than one immunogen, *e.g.*, a fusion protein that consist of two or more epitopes, peptides or proteins derived from the same or different virus-proteins, wherein the epitopes, peptides or proteins may be linked by linker sequences.

[0109] An artificial mRNA (sequence) may be understood to be an mRNA molecule that does not occur naturally. In other words, an artificial mRNA molecule may be understood as a non-natural mRNA molecule. Such mRNA molecule may be non-natural due to its individual sequence (which does not occur naturally) and/or due to other modifications, *e.g.*, structural modifications of nucleotides which do not occur naturally. Typically, artificial mRNA molecules

may be designed and/or generated by genetic engineering methods to correspond to a desired artificial sequence of nucleotides (heterologous sequence). In this context an artificial sequence is usually a sequence that may not occur naturally, *i.e.*, it differs from the wild type sequence by at least one nucleotide.

[0110] In certain embodiments, a variant of a nucleic acid sequence refers to variant of nucleic acid sequences, which form the basis of a nucleic acid sequence. For example, a variant nucleic acid sequence may exhibit one or more nucleotide deletions, insertions, additions and/or substitutions compared to the nucleic acid sequence from which the variant is derived. Preferably, a variant of a nucleic acid sequence is at least 40%, preferably at least 50%, more preferably at least 60%, more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, most preferably at least 95% identical to the nucleic acid sequence the variant is derived from. Preferably, the variant is a functional variant. A "variant" of a nucleic acid sequence may have at least 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% nucleotide identity over a stretch of 10, 20, 30, 50, 75 or 100 nucleotide of such nucleic acid sequence.

[0111] A stabilized nucleic acid, preferably mRNA typically, exhibits a modification increasing resistance to *in vivo* degradation (*e.g.* degradation by an exo- or endo-nuclease) and/or *ex vivo* degradation (*e.g.*, by the manufacturing process prior to vaccine administration, *e.g.*, in the course of the preparation of the vaccine solution to be administered). Stabilization of RNA can, *e.g.*, be achieved by providing a 5'-CAP-Structure, a Poly-A-Tail, or any other UTR-modification. It can also be achieved by chemical modification or modification of the G/C-content of the nucleic acid. Various other methods are known in the art and conceivable.

[0112] Suitable quantities of the vector comprising an RNA construct (mRNA) can be about 1 µg to about 100 µg, or about 25 µg to 100 µg, but lower levels such as 1-25 µg can be employed. For example, about 1 µg, about 2.5 µg, about 4 µg, about 5 µg, about 6 µg, about 7 µg, about 8 µg, about 9 µg, about 10 µg, about 15 µg, about 20 µg, about 25 µg, about 30 µg, about 40 µg, about 50 µg, about 60 µg, about 70 µg, about 80 µg, about 90 µg, or about 100 µg. In certain embodiments, an RNA construct as part of a lipid nanoparticle, can be injected into tissue, *e.g.*, intramuscularly or intradermally, in amounts of from 10 µl per site to about 1 mL per site.

[0113] The vector comprising an RNA construct of this disclosure can be administered to a mammalian host. The mammalian host usually is a human or a primate. In some embodiments, the mammalian host can be a domestic animal, for example, canine, feline, lagomorpha, rodentia, rattus, hamster, murine. In other embodiment, the mammalian host is an agricultural animal, for example, bovine, ovine, porcine, equine, *etc.*

[0114] In certain embodiments, this disclosure relates to a vector comprising mRNA formulated with lipid nanoparticles (LNP). In some embodiments, the lipid nanoparticles comprise at least (i) a cationic lipid and/or a PEG-lipid as defined herein; and the RNA construct comprising an mRNA sequence encoding the protein of interest.

Immunotherapy compositions

[0115] In some embodiments, the recombinant protein, the nucleic acid composition, the vector, or the immunotherapy composition as disclosed herein can also comprise suitable pharmaceutically acceptable adjuvants, carriers, and/or excipients. In some embodiments, the disclosure is directed to an immunotherapy composition including the recombinant proteins of the disclosure, wherein the recombinant proteins may be linked to a carrier. Carrier proteins can be effective in increasing vaccine immunogenicity, resulting in enhanced immunogenicity and converting a T-cell independent to a T-cell dependent antigen. In some embodiments, the carrier is selected from the group consisting of albumin, diphtheria toxoid (DT), a genetically modified cross-reacting material (CRM) of diphtheria toxin, CRM197, flagellin, *H. influenzae* protein D (HiD), immunoglobulin molecules, KLH (keyhole limpet hemocyanin), mannose-6-phosphate, meningococcal outer membrane protein complex (OMPC), ovalbumin, poly-L-lysine, poly-L-glutamine, rEPA (*Pseudomonas aeruginosa* exotoxin A), thyroglobulin, and tetanus toxoid (TT).

[0116] As used herein, "a pharmaceutical-acceptable" includes any and all solvents, dispersion media, coatings, stabilizing agents, diluents, preservatives, antibacterial and antifungal agents, isotonic agents, adsorption delaying agents, and the like. The compositions of the present disclosure can also comprise the addition of any stabilizing agent, such as for example saccharides, trehalose, mannitol, saccharose and the like, to increase and/or maintain product shelf-life and/or to enhance stability. In some embodiments, the composition may also include additional components known to those of skill in the art (*see also*, Remington's Pharmaceutical Sciences, 1990, 18th ed. Mack Publ., Easton). Those of skill in the art will understand that the composition herein may incorporate known injectable, physiologically acceptable, sterile solutions. For preparing a ready-to-use solution for parenteral injection or infusion, aqueous isotonic solutions, such as *e.g.*, saline or corresponding plasma protein solutions are readily available. In addition, the immunogenic and vaccine compositions of the present disclosure can include diluents, isotonic agents, stabilizers, or adjuvants. Diluents can include water, saline, dextrose, ethanol, glycerol, and the like. Isotonic agents can include sodium chloride, dextrose, mannitol, sorbitol, and lactose, among others. Stabilizers include albumin and alkali salts of ethylendiamintetracetic acid, among others. Suitable adjuvants are those additional components known to those of skill in the art. When administered as a liquid, a vaccine composition of the present disclosure may

be prepared in the form of an aqueous solution, syrup, an elixir, a tincture and the like. Such formulations are known in the art and are typically prepared by dissolution of the antigen and other typical additives in the appropriate carrier or solvent systems. Suitable carriers or solvents include, but are not limited to, water, saline, ethanol, ethylene glycol, glycerol, *etc.* Typical additives are, for example, certified dyes, flavors, sweeteners and antimicrobial preservatives such as thimerosal (sodium ethylmercurithiosalicylate). Such solutions may be stabilized, for example, by addition of partially hydrolyzed gelatin, sorbitol or cell culture medium, and may be buffered by conventional methods using reagents known in the art, such as sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium hydrogen phosphate, potassium dihydrogen phosphate, a mixture thereof, and the like. In some embodiments, the immunotherapy composition of the present disclosure further comprises a pharmaceutical acceptable salt, preferably a phosphate salt in physiologically acceptable concentrations. Preferably, the pH of said immunotherapy composition is adjusted to a physiological pH, meaning between about 6.5 and 7.5. The immunotherapy compositions described herein can further include one or more other immunomodulatory agents such as, *e.g.*, interleukins, interferons, or other cytokines. The immunotherapy compositions can also include antibiotics or anti-microbiological active agents. It will be found that the immunotherapy compositions comprising the recombinant protein(s) as provided herein are effective in reducing the severity of or incidence of clinical signs associated with malaria and/or toxomplasmosis infections up to and including the prevention of such signs.

[0117] As used herein, the term “adjuvant” can refer to any compound, which is suitable to support administration and delivery of the recombinant protein, the nucleic acid composition, the vector, or the immunotherapy composition as disclosed herein. Furthermore, such an adjuvant may, without being bound thereto, initiate or increase an immune response of the innate immune system, *i.e.*, a non-specific immune response. Put another way, when administered, the recombinant protein, the nucleic acid composition, the vector, or the immunotherapy composition as disclosed herein typically initiates an adaptive immune response due to the recombinant protein and/or an antigen thereof as defined herein or a fragment or variant thereof. In certain embodiments, the term “adjuvant” can be understood not to comprise agents which confer immunity by themselves. An adjuvant assists the immune system unspecifically to enhance the antigen-specific immune response by *e.g.*, promoting presentation of an antigen to the immune system or induction of an unspecific innate immune response. Furthermore, an adjuvant may preferably *e.g.*, modulate the antigen-specific immune response by, *e.g.*, shifting the dominating Th2-based antigen specific response to a more Th1-based antigen specific response or vice versa. Accordingly, an adjuvant may

favorably modulate cytokine expression/secretion, antigen presentation, or type of immune response.

[0118] In some embodiments, an adjuvant may be selected from any adjuvant known to a skilled person and suitable for the present case, *i.e.*, supporting the induction of an immune response in a mammal. For example, an adjuvant may be selected from the group consisting of, without being limited thereto, AS03 (comprising α-tocopherol, squalene and polysorbate 80 in an oil-in-water emulsion), AddaS03™, TDM, MDP, muramyl dipeptide, pluronics, alum solution, aluminum hydroxide, ADJUMER™ (polyphosphazene); aluminum phosphate gel; glucans from algae; algammulin; aluminum hydroxide gel (alum); highly protein-adsorbing aluminum hydroxide gel; low viscosity aluminum hydroxide gel; AF or SPT (emulsion of squalane (5%), Tween 80 (0.2%), Pluronic L121 (1.25%), phosphate-buffered saline, pH 7.4); AVRIDINE™ (propanediamine); BAY R1005TM ((N-(2-deoxy-2-L-leucylamino-b-D-glucopyranosyl)-N-octadecyl-dodecanoyl-amide hydroacetate); CALCITRIOL™ (1-alpha,25-dihydroxy-vitamin D3); calcium phosphate gel; CAP™ (calcium phosphate nanoparticles); cholera holotoxin, cholera-toxin-A1-protein-A-D-fragment fusion protein, subunit B of the cholera toxin; CRL 1005 (block copolymer P1205); cytokine-containing liposomes; DDA (dimethyldioctadecylammonium bromide); DHEA (dehydroepiandrosterone); DMPC (dimyristoylphosphatidylcholine); DMPG (dimyristoylphosphatidylglycerol); DOC/alum complex (deoxycholic acid sodium salt); Freund's complete adjuvant; Freund's incomplete adjuvant; gamma inulin; Gerbu adjuvant (mixture of: i) N-acetylglucosaminyl-(P1-4)-N-acetylmuramyl-L-alanyl-D-glutamine (GMDP), ii) dimethyldioctadecylammonium chloride (DDA), iii) zinc-L-proline salt complex (ZnPro-8); GM-CSF); GMDP (N-acetylglucosaminyl-(b1-4)-N-acetylmuramyl-L-alanyl-D-isoglutamine); imiquimod (1-(2-methypropyl)-1H-imidazo[4,5-c]quinoline-4-amine); ImmTher™ (N-acetylglucosaminyl-N-acetylmuramyl-L-Ala-D-isoGlu-L-Ala-glycerol dipalmitate); DRVs (immunoliposomes prepared from dehydration-rehydration vesicles); interferon-gamma; interleukin-1beta; interleukin-2; interleukin-7; interleukin-12; ISCOMS™; ISCOPREP 7.0.3™; liposomes; LOXORIBINE™ (7-allyl-8-oxoguanosine); LT oral adjuvant (E. coli labile enterotoxin-protoxin); microspheres and microparticles of any composition; Matrix-M™, MF59™; (squalene-water emulsion); MONTANIDE ISA 51™ (purified incomplete Freund's adjuvant); MONTANIDE ISA 720™ (metabolisable oil adjuvant); MPL™ (3-Q-desacyl-4'-monophosphoryl lipid A); MTP-PE and MTP-PE liposomes ((N-acetyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1,2-dipalmitoyl-sn-glycero-3-(hydroxyphosphoryloxy))-ethylamide, monosodium salt); MURAMETIDE™ (Nac-Mur-L-Ala-D-Gln-OCH3); MURAPALMITINE™ and D-MURAPALMITINE™ (Nac-Mur-L-Thr-D-isoGln-sn-glyceroldipalmitoyl); NAGO (neuraminidase-galactose oxidase); nanospheres or nanoparticles of any composition; NISVs (non-ionic surfactant vesicles); PLEURAN™ (P3-

glucan); PLGA, PGA and PLA (homo- and co-polymers of lactic acid and glycolic acid; microspheres/nanospheres); PLURONIC L121™; PMMA (polymethyl methacrylate); PODDS™ (proteinoid microspheres); polyethylene carbamate derivatives; poly-rA: poly-rU (polyadenylic acid-polyuridylic acid complex); polysorbate 80 (Tween 80); protein cochleates (Avanti Polar Lipids, Inc., Alabaster, Ala.); STIMULON™ (QS-21); Quil-A (Quil-A saponin); S-28463 (4-amino-otec-dimethyl-2-ethoxymethyl-1H-imidazo[4,5 c]quinoline-1-ethanol); SAF-1™ ("Syntex adjuvant formulation"); Sendai proteoliposomes and Sendai-containing lipid matrices; Span-85 (sorbitan trioleate); Specol (emulsion of Marcol 52, Span 85 and Tween 85); squalene or Robane® (2,6,10,15,19,23-hexamethyltetracosan and 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexane); stearyltyrosine (octadecyltyrosine hydrochloride); Theramid® (N-acetylglucosaminyl-N-acetylmuramyl-L-Ala-D-isoGlu-L-Ala-dipalmitoxypro- pylamide); Theronyl-MDP (Termurtide™ or [thr 1]-MDP; N-acetylmuramyl-L-threonyl-D-isoglutamine); Ty particles (Ty-VLPs or virus-like particles); Walter-Reed liposomes (liposomes containing lipid A adsorbed on aluminum hydroxide), and lipopeptides, including Pam3Cys, in particular aluminum salts, such as Adju-phos, Alhydrogel, Rehydragel; emulsions, including CFA, SAF, IFA, MF59, Provax, TiterMax, Montanide, Vaxfectin; copolymers, including Optivax (CRL1005), L121, Poloaxmer4010), *etc.*; liposomes, including Stealth, cochleates, including BIORAL; plant derived adjuvants, including QS21, Quil A, Iscomatrix, ISCOM; adjuvants suitable for costimulation including Tomatine, biopolymers, including PLG, PMM, Inulin; microbe derived adjuvants, including Romurtide, DETOX, MPL, CWS, Mannose, CpG nucleic acid sequences, CpG7909, ligands of human TLR 1-10, ligands of murine TLR 1-13, ISS-1018, IC31, Imidazoquinolines, Ampligen, Ribi529, IMOxine, IRIVs, VLPs, cholera toxin, heat-labile toxin, Pam3Cys, Flagellin, GPI anchor, LNFPIII/Lewis X, antimicrobial peptides, UC-1V150, RSV fusion protein, cdiGMP; and adjuvants suitable as antagonists including CGRP neuropeptide.

[0119] In certain embodiments, an adjuvant may be selected from adjuvants, which support induction of a Th1-immune response or maturation of naive T-cells, such as GM-CSF, IL-12, IFN-gamma, any immunostimulatory nucleic acid as defined above, preferably an immunostimulatory RNA and/or CpG DNA. In some embodiments, it is also possible that the compositions disclosed herein contain, besides the antigen-providing RNA, further components which are selected from the group consisting of: further antigens (*e.g.*, in the form of a peptide or protein) or further antigen-encoding nucleic acids; a further immunotherapeutic agent; one or more auxiliary substances; or any further compound, which is known to be immunostimulating due to its binding affinity (as ligands) to human Toll-like receptors; and/or an adjuvant nucleic acid, preferably an immunostimulatory RNA (isRNA).

[0120] In certain embodiments, the recombinant protein as disclosed herein further comprises fusion to one or more additional antigens. For example, one or more additional antigens associated with malaria, toxoplasmosis, and babesiosis can be fused to the recombinant proteins as disclosed herein. Additionally, one or more additional antigens can comprise peptides derived from antigens originating from viruses, bacteria, or protozoa. These peptides may encompass short transmembrane and cytoplasmic domains derived from surface proteins of viral, bacterial, or protozoological origin. Furthermore, antigens well known for their ability to enhance immune responses can also be considered as the one or more additional antigens that can be fused to the recombinant proteins as disclosed herein. For example, immunomodulatory cytokines such as interferons (*e.g.*, IFNα, IFNβ and IFNγ), interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 and IL-20), tumor necrosis factors (*e.g.*, TNFα and TNFβ), erythropoietin (EPO), FLT-3 ligand, gIp10, TCA-3, MCP-1, MIF, MIP-1α, MIP-1β, Rantes, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF), or chemokines including, but not limited to, Mip1α, Mip-1β, Mip-3α (Larc), Mip-3β, Rantes, Hcc-1, Mpif-1, Mpif-2, Mcp-1, Mcp-2, Mcp-3, Mcp-4, Mcp-5, Eotaxin, Tarc, Elc, I309, IL-8, Gcp-2 Gro-α, Gro-β, Gro-γ, Nap-2, Ena-78, Gcp-2, Ip-10, Mig, I-Tac, Sdf-1, and Bca-1 (Blc).

[0121] The immunotherapy compositions as described herein can be applied intramuscularly, intravenously, or intranasally. The amount of a composition that is effective depends on the ingredients of the vaccine and the schedule of administration. A immunotherapy composition of the present disclosure can be administered in a single dose or in repeated doses, with a single dose being preferred. Depending on the desired duration and effectiveness of the treatment, repeated doses of immunotherapy compositions according to the disclosure may be administered once or several times, also intermittently, for instance on a daily, weekly, or monthly basis for several days, weeks or months, and in different dosages.

[0122] As used herein, the terms to "treat" and "treatment" refer to the alleviation or amelioration of one or more symptoms or effects associated with the disease, prevention, inhibition or delay of the onset of one or more symptoms or effects of the disease, lessening of the severity or frequency of one or more symptoms or effects of the disease, and/or increasing or trending toward desired outcomes as described herein.

[0123] The terms "prevention", "prevent", or "preventing" as used herein refer to contacting (for example, administering) the recombinant protein(s) or immunotherapy compositions of the present disclosure with a subject before the onset of a disease (*e.g.*, malaria or toxoplasmosis or babesiosis), thereby delaying the onset of clinical symptoms and/or alleviating symptoms of the disease after the onset of the disease, compared to when the subject is not contacted

with the recombinant protein or immunotherapy compositions, and does not refer to completely suppressing the onset of the disease. In some cases, prevention may occur for limited time after administration of the recombinant protein or immunotherapy compositions of the present disclosure. In other cases, prevention may occur for the duration of a treatment regimen comprising administering the recombinant protein or immunotherapy compositions of the present disclosure.

Lipid nanoparticle (LNP) Formulations

[0124] In some embodiments, the immunotherapy compositions as disclosed herein further comprise lipid nanoparticles or nanoparticles. The term "lipid nanoparticle", also referred to as LNP, refers to a particle having at least one dimension on the order of nanometers (*e.g.*, 1-1,000 nm) which includes one or more lipids. In some embodiments, such lipid nanoparticles comprise a cationic lipid and one or more excipient selected from neutral lipids, charged lipids, steroids and polymer conjugated lipids (*e.g.*, a pegylated lipid such as a pegylated lipid). In some embodiments, vector (*e.g.*, a DNA vaccine, an RNA vaccine or mRNA, is encapsulated in the lipid portion of the lipid nanoparticle or an aqueous space enveloped by some or all of the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other undesirable effects induced by the mechanisms of the host organism or cells, *e.g.*, an adverse immune response. In some embodiments, the mRNA or a portion thereof is associated with the lipid nanoparticles.

[0125] Lipid nanoparticles, cationic lipids and polymer conjugated lipids (PEG-lipid) were prepared and tested according to the general procedures described in WO 2015/199952, WO 2017/004143, WO 2017/075531 and WO 2018/078053, the full disclosures of which are incorporated herein by reference in their entirety. Lipid nanoparticle (LNP)-formulated mRNA can be prepared using an ionizable amino lipid (cationic lipid), phospholipid, cholesterol and a PEGylated lipid. LNPs can be prepared as follows: cationic lipid, DSPC, cholesterol and PEG-lipid can be solubilized in ethanol at a molar ratio of approximately 50:10:38.5:1.5 or 47.5:10:40.8:1.7. Lipid nanoparticles (LNP) can be prepared at a ratio of mRNA to Total Lipid of 0.03-0.04 w/w.

[0126] Lipid nanoparticles are not restricted to any particular morphology, and should be interpreted as to include any morphology generated when a cationic lipid and optionally one or more further lipids are combined, *e.g.*, in an aqueous environment and/or in the presence of a nucleic acid compound. For example, a liposome, a lipid complex, a lipoplex and the like are within the scope of a lipid nanoparticle.

[0127] In some embodiments, the lipid nanoparticles have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150

nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In certain embodiments, the DNA vector, RNA vector or mRNA, when present in the lipid nanoparticles, is resistant in aqueous solution to degradation with a nuclease. As used herein, the mean diameter may be represented by the z-average as determined by dynamic light scattering.

[0128] In some embodiments, a LNP may comprise any lipid capable of forming a particle to which the one or more nucleic acid molecules are attached and/or in which the one or more nucleic acid molecules are encapsulated. The term "lipid" refers to a group of organic compounds that are derivatives of fatty acids (*e.g.*, esters) and are generally characterized by being insoluble in water but soluble in many organic solvents. Lipids are usually divided in at least three classes: (1) "simple lipids" which include fats and oils as well as waxes; (2) "compound lipids" which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0129] In certain embodiments, the mRNA-comprising LNP comprises one or more cationic lipids as defined herein, and one or more stabilizing lipids. Stabilizing lipids include neutral lipids and pegylated lipids.

[0130] In some embodiments, the LNP comprises a cationic lipid. The cationic lipid is preferably cationisable, *i.e.*, it becomes protonated as the pH is lowered below the pKa of the ionizable group of the lipid, but is progressively more neutral at higher pH values. When positively charged, the lipid is then able to associate with negatively charged nucleic acids. In certain embodiments, the cationic lipid comprises a zwitterionic lipid that assumes a positive charge on pH decrease. The LNP may comprise any lipid capable of forming a particle to which the one or more nucleic acid molecules are attached, or in which the one or more nucleic acid molecules are encapsulated.

[0131] In certain embodiments, the LNP may comprise any further cationic or cationisable lipid, *i.e.* any of a number of lipid species which carry a net positive charge at a selective pH, such as physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA); N,N-distearyl-N,N-dimethylammonium bromide (DDAB); N-(2,3dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP); 3-(N--(N',N'dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1-(2,3-

dioleoyloxy)propyl)N-2-(sperminecarboxamido)ethyl)-N,N-dimethyl- ammonium trifluoracetate (DOSPA), dioctadecylamidoglycyl carboxyspermine (DOGS), 1,2-dioleoyl-3-dimethylammonium propane (DODAP), N,N-dimethyl-2,3-dioleoyloxy)propylamine (DODMA), and N-(1,2dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE).

[0132] In some embodiments, a number of commercial preparations of cationic lipids are available which can be used in the LNPs disclosed herein. These can include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and 1,2-dioleoyl-sn-3phosphoethanolamine (DOPE), from GIBCO/BRL, Grand Island, N.Y.); LIPOFECTAMINE® (commercially available cationic liposomes comprising N-(1-(2,3dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethyl- ammonium trifluoroacetate (DOSPA) and (DOPE), from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising dioctadecylamidoglycyl carboxyspermine (DOGS) in ethanol from Promega Corp., Madison, Wis.). The following lipids are cationic and have a positive charge at below physiological pH: DODAP, DODMA, DMDMA, 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

[0133] In an embodiment, the further cationic lipid is an amino lipid. Suitable amino lipids useful in the disclosure include those described in WO 2012/016184, incorporated herein by reference in its entirety. Representative amino lipids include, but are not limited to, 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,Ndilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanediol (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), and 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA).

[0134] In some embodiments, the amount of the permanently cationic lipid or lipidoid should also be selected taking the amount of the nucleic acid cargo into account. In certain embodiments, these amounts are selected such as to result in an N/P ratio of the nanoparticle(s) or of the composition in the range from about 0.1 to about 20. In this context, the N/P ratio is defined as the mole ratio of the nitrogen atoms ("N") of the basic nitrogen-containing groups of the lipid or lipidoid to the phosphate groups ("P") of the nucleic acid which is used as cargo. The N/P ratio may be calculated on the basis that, for example, 1 pg RNA

typically contains about 3 nmol phosphate residues, provided that the RNA exhibits a statistical distribution of bases. The "N"-value of the lipid or lipidoid may be calculated on the basis of its molecular weight and the relative content of permanently cationic and--if present--cationisable groups. Such low N/P ratios are commonly believed to be detrimental to the performance and *in vivo* efficacy of such carrier-cargo complexes, or nucleic-acid loaded nanoparticles. However, such N/P ratios are indeed useful in the context of the present disclosure, in particular when the local or extravascular administration of the nanoparticles is intended. Here, the respectively nanoparticles have been found to be efficacious and at the same time well-tolerated.

[0135] In certain embodiments, the LNP comprises one or more additional lipids which stabilize the formation of particles during their formation. Suitable stabilizing lipids can include neutral lipids and anionic lipids. The term "neutral lipid" refers to any one of a number of lipid species that exist in either an uncharged or neutral zwitterionic form at physiological pH. Representative neutral lipids include diacylphosphatidylcholines, diacylphosphatidylethanolamines, ceramides, sphingomyelins, dihydro sphingomyelins, cephalins, and cerebrosides. Exemplary neutral lipids can include, but are not limited to, for example, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-lcarboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearioyl-2-oleoylphosphatidyethanol amine (SOPE), and 1,2-dielaidoyl-sn-glycero-3-phophoethanolamine (transDOPE). In one embodiment, the neutral lipid is 1,2-distearoyl-sn-glycero-3phosphocholine (DSPC).

[0136] In some embodiments, the LNPs comprise a neutral lipid selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In various embodiments, the molar ratio of the cationic lipid to the neutral lipid ranges from about 2:1 to about 8:1.

[0137] In some embodiments, the LNPs comprise a polymer conjugated lipid. The term "polymer conjugated lipid" refers to a molecule comprising both a lipid portion and a polymer portion. An example of a polymer conjugated lipid is a pegylated lipid. The term "pegylated lipid" refers to a molecule comprising both a lipid portion and a polyethylene glycol portion. Pegylated lipids are known in the art and include 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-s-DMG) and the like.

[0138] In certain embodiments, the LNP can comprise an additional, stabilizing-lipid which is a polyethylene glycol-lipid (pegylated lipid). Suitable polyethylene glycollipids include PEG-modified phosphatidylethanolamine, PEG-modified phosphatidic acid, PEG-modified ceramides (*e.g.*, PEG-CerC14 or PEG-CerC20), PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols. Representative polyethylene glycol-lipids include PEG-c-DOMG, PEG-c-DMA, and PEG-s-DMG. In one embodiment, the polyethylene glycol-lipid is N-[(methoxy poly(ethylene glycol)2000)carbamyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-c-DMA). In one embodiment, the polyethylene glycol-lipid is PEG-c-DOMG). In other embodiments, the LNPs comprise a pegylated diacylglycerol (PEG-DAG) such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a pegylated phosphatidylethanoloamine (PEG-PE), a PEG succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl-1-O-(omega-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a pegylated ceramide (PEG-cer), or a PEG dialkoxypropylcarbamate such as omega-methoxy(polyethoxy)ethyl-N-(2,3di(tetradecanoxy)propyl)carbamate or 2,3-di(tetradecanoxy)propyl-N-(omega-methoxy(polyethoxy)ethyl)carbamate. In various embodiments, the molar ratio of the cationic lipid to the pegylated lipid ranges from about 100:1 to about 25:1.

[0139] In certain embodiments, the PEG lipid is present in the LNP in an amount from about 1 to about 10 mole percent, relative to the total lipid content of the nanoparticle. In an embodiment, the PEG lipid is present in the LNP in an amount from about 1 to about 5 mole percent. In another embodiment, the PEG lipid is present in the LNP in about 1 mole percent or about 1.5 mole percent.

[0140] In certain embodiments, the LNP comprises one or more targeting moieties which are capable of targeting the LNP to a cell or cell population. For example, in an embodiment, the targeting moiety is a ligand which directs the LNP to a receptor found on a cell surface.

[0141] In certain embodiments, the LNP comprises one or more internalization domains. For example, in an embodiment, the LNP comprises one or more domains which bind to a cell to induce the internalization of the LNP. For example, in one embodiment, the one or more internalization domains bind to a receptor found on a cell surface to induce receptor-mediated uptake of the LNP. In certain embodiments, the LNP is capable of binding a biomolecule *in vivo*, where the LNP-bound biomolecule can then be recognized by a cell-surface receptor to induce internalization. For example, in one embodiment, the LNP binds systemic ApoE, which leads to the uptake of the LNP and associated cargo.

[0142] Additional exemplary LNPs and their manufacture are described in the art, for example in U.S. Patent Application Publication No. US 2012/0276209, WO 2019/077053,

Semple *et al.*, 2010, Nat Biotechnol., 28(2):172-176; Akinc *et al.*, 2010, Mol Ther., 18(7): 1357-1364; Basha *et al.*, 2011, Mol Ther, 19(12): 2186-2200; Leung *et al.*, 2012, J Phys Chem C Nanomater Interfaces, 116(34): 18440-18450; Lee *et al.*, 2012, Int J Cancer., 131(5): E781-90; Belliveau *et al.*, 2012, Mol Ther nucleic Acids, 1: e37; Jayaraman *et al.*, 2012, Angew Chem Int Ed Engl., 51(34): 8529-8533; Mui *et al.*, 2013, Mol Ther Nucleic Acids. 2, e139; Maier *et al.*, 2013, Mol Ther., 21(8): 1570-1578; and Tam *et al.*, 2013, Nanomedicine, 9(5): 665-74, each of which are incorporated by reference in their entirety.

[0143] In certain embodiments, the lipid nanoparticles have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. As mentioned, the mean diameter may correspond to the z-average as determined by dynamic light scattering.

[0144] In certain embodiments, a nanoparticle composition further comprises a self-assembling monomeric subunit protein, monomeric subunit protein, self-assembly(SA) protein, self-assembling subunit protein, and the like, which, is capable of directing self-assembly of monomeric self-assembling subunit proteins into a nanoparticle. Such proteins are known to those skilled in the art. Examples of self-assembly proteins useful for producing nanoparticles of the present invention include, but are not limited to, ferritin, encapsulin, sulfur oxygenase reductase (SOR), lumazine synthase (LS), pyruvate dehydrogenase complex (PDC) dihydrolipoamide acetyltransferase (E2) and the envelope (Env) proteins of alphaviruses such as Chikungunya virus.

[0145] In certain embodiments, this disclosure further relates to immunotherapy compositions comprising at least one lipid nanoparticle comprising a vector (*e.g.*, a DNA vaccine, an RNA construct comprising an mRNA sequence encoding the recombinant protein as disclosed herein. In an embodiment, the mRNA sequence encodes the recombinant protein as disclosed herein or antigenic fragement thereof. In an alternative embodiment, the mRNA sequence encodes more than one peptide of interest or antigenic protein.

[0146] In some embodiments, the immunotherapy compositions can comprise a lipid nanoparticle as disclosed herein, wherein the lipid nanoparticle comprises more than one RNA construct, which each RNA construct comprises a different mRNA sequence encoding a peptide of interest or antigenic protein.

[0147] In some embodiments, the immunotherapy compositions are provided as a vaccine. As used herein, a vaccine is typically understood to be a prophylactic or therapeutic material providing at least one antigen or antigenic function. The antigen or antigenic function may stimulate the body's adaptive immune system to provide an adaptive immune response.

Immunization/Vaccination Protocols

[0148] The immunization/vaccination protocol for the recombinant protein, the nucleic acid composition, the vector, or the immunotherapy composition as disclosed herein for the immunization of a subject against the recombinant protein as disclosed herein can comprise a series of single doses or dosages of the recombinant protein, the nucleic acid composition, the vector (DNA or RNA), or the immunotherapy composition as disclosed herein. For example, the recombinant protein as disclosed herein may be expressed on the membrane of a cell, another recombinant protein, or fused to another protein, a domain of a protein or a peptide of a protein. The immunization protocol may include the use of an adjuvant during the primary and/or booster immunizations.

[0149] In some embodiments, a therapeutically effective immunization/vaccination protocol achieves the desired immunological or clinical effect. Regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered at set intervals (*e.g.*, weekly, monthly) or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. In some embodiments, the immunization of a subject against the recombinant protein as disclosed herein comprises a series of single doses. In certain embodiments, the immunization of a subject against the recombinant protein comprises doses separated by at least about 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, or more. In certain embodiments, an immunization regimen comprises an immunization followed by booster dosage(s) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months later.

[0150] In some embodiments, a therapeutically effective immunization/vaccination protocol achieves the desired immunological or clinical effect, for example, production of a monoclonal antibody or a plurality of polyclonal antibodies. In certain embodiments, the monoclonal antibody or plurality of polyclonal antibodies cross-react with different strains and/or subtypes of malaria. In some embodiments, the immunoglobulin cross-reacts with different strains and/or subtypes of toxoplasmosis. In some embodiments, the immunoglobulin cross-reacts with different strains and/or subtypes of babesiosis.

[0151] In another aspect, this disclosure provides a method of generating antibodies cross-protective against malaria comprising: (a) immunizing a subject with the recombinant

protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein: (b) isolating antibodies that cross-react with different strains and/or subtypes of malaria.

[0152] In another aspect, this disclosure provides a method of generating antibodies cross-protective against toxoplasmosis comprising: (a) immunizing a subject with the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein: (b) isolating antibodies that cross-react with different strains and/or subtypes of toxoplasmosis.

[0153] In another aspect, this disclosure provides a method of generating antibodies cross-protective against babesiosis comprising: (a) immunizing a subject with the recombinant protein(s) as disclosed herein, the nucleic acid composition(s) as disclosed herein, the vector(s) as disclosed herein, or the immunotherapy composition(s) as disclosed herein: (b) isolating antibodies that cross-react with different strains and/or subtypes of babesiosis.

[0154] The term “cross-protective” as used herein refers to immunoglobulins or antibodies that inhibit or reduce the severity of infection by multiple different pathogen strains or subtypes.

[0155] Without limiting the disclosure, a number of embodiments of the disclosure are described below for purpose of illustration.

[0156] Embodiment 1. A recombinant protein, comprising:

(a) a first peptide comprising a C-teriminal portion of an apical membrane antigen 1 (AMA1) protein;

(b) a second peptide comprising an N-terminal portion of the AMA1 protein; and

(c) a third peptide comprising at least a portion of a rhoptry neck protein 2 (RON2) protein.

[0157] Embodiment 2. The recombinant protein of embodiment 1, wherein the recombinant protein comprises from the N-terminus to the C-terminus the first peptide, then the second peptide, and then the third peptide.

[0158] Embodiment 3. The recombinant protein of embodiment 1, wherein the recombinant protein comprises from the N-terminus to the C-terminus the third peptide, then the first peptide, and then the second peptide.

[0159] Embodiment 4. The recombinant protein of any one of embodiments 1 to 3, wherein the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein.

[0160] Embodiment 5. The recombinant protein of any one of embodiments 1 to 4, wherein the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01.

[0161] Embodiment 6. The recombinant protein of any one of embodiments 1 to 4, wherein the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:02.

[0162] Embodiment 7. The recombinant protein of any one of embodiments 1 to 4, wherein the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-438 of SEQ ID NO:01.

[0163] Embodiment 8. The recombinant protein of any one of embodiments 1 to 4, wherein the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-541 of SEQ ID NO:01.

[0164] Embodiment 9. The recombinant protein of any one of embodiments 1 to 8, wherein the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein.

[0165] Embodiment 10. The recombinant protein of any one of embodiments 1 to 9, wherein the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01.

[0166] Embodiment 11. The recombinant protein of any one of embodiments 1 to 9, wherein the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:03.

[0167] Embodiment 12. The recombinant protein of any one of embodiments 1 to 9, wherein the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 94-358 of SEQ ID NO:01.

[0168] Embodiment 13. The recombinant protein of any one of embodiments 1 to 9, wherein the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% , 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 25-358 of SEQ ID NO:01.

[0169] Embodiment 14. The recombinant protein of any one of embodiments 1 to 13, wherein the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein.

[0170] Embodiment 15. The recombinant protein of any one of embodiments 1 to 14, wherein the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:04.

[0171] Embodiment 16. The recombinant protein of any one of embodiments 1 to 14, wherein the third peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:05-33.

[0172] Embodiment The recombinant protein of any one of embodiments 1 to 16 further comprising a linker domain between the first peptide and the second peptide.

[0173] Embodiment 18. The recombinant protein of embodiment 17, wherein the linker domain comprises a flexible linker, for example, a G_4S linker.

[0174] Embodiment 19. The recombinant protein of any one of embodiments 1 to 18, wherein the recombinant protein comprises at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83.

[0175] Embodiment 20. The recombinant protein of any one of embodiments 1 to 19, wherein the AMA1 protein is from the *Plasmodium* genus, the *Babesia* genus, or *Toxoplasma gondii*.

[0176] Embodiment 21. The recombinant protein of any one of embodiments 1 to 20, wherein the RON2 protein is from the *Plasmodium* genus, the *Babesia* genus, or *Toxoplasma gondii*.

[0177] Embodiment 22. The recombinant protein of any one of embodiments 1 to 21, wherein the AMA1 protein and the RON2 protein are from the same species.

[0178] Embodiment 23. The recombinant protein of any one of embodiments 1 to 22 further comprising fusion to one or more additional antigens.

[0179] Embodiment 24. A nucleic acid composition, comprising a nucleic acid sequence encoding the recombinant protein of any one of embodiments 1 to 23.

[0180] Embodiment 25. A vector, comprising the nucleic acid sequence of embodiment 24.

[0181] Embodiment 26. An immunotherapy composition, comprising the recombinant protein of any one of embodiments 1 to 23, the nucleic acid sequence of embodiment 24, or the vector of embodiment 25, and at least one adjuvant and/or a carrier.

[0182] Embodiment 27. The immunotherapy composition of embodiment 26, wherein the adjuvant is selected from the group consisting of AddaS03™, aluminum hydroxide, aluminum phosphate, aluminum sulfate, monophosphoryl lipid A (MPL), QS-21, TQL1055, QS-18, QS-17, QS-7, Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), oil in water emulsions, CpG, polyglutamic acid, polylysine, AddaVax™, MF59®, and/or combinations thereof.

[0183] Embodiment 28. The immunotherapy composition of either embodiment 26 or embodiment 27, wherein the carrier is selected from the group consisting of albumin, diphtheria toxoid (DT), a genetically modified cross-reacting material (CRM) of diphtheria toxin, CRM197, flagellin, *HOUR. influenzae* protein D (HiD), immunoglobulin molecules, KLH (keyhole limpet hemocyanin), mannose-6-phosphate, meningococcal outer membrane protein complex (OMPC), ovalbumin, poly-L-lysine, poly-L-glutamine, rEPA (Pseudomonas aeruginosa exotoxin A), thyroglobulin, and tetanus toxoid (TT).

[0184] Embodiment 29. The immunotherapy composition of any one of embodiments 26 to 28, further comprising a lipid nanoparticle or a nanoparticle.

[0185] Embodiment 30. A method of vaccinating a subject, comprising administrating to the subject the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29.

[0186] Embodiment 31. A method of treating a subject with malaria or protecting a subject from malaria infection, comprising administrating to the subject the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29.

[0187] Embodiment 32. A method of treating a subject with toxoplasmosis or protecting a subject from toxoplasmosis infection, comprising administrating to the subject the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 24, or the immunotherapy composition of any one of embodiments 26 to 29.

[0188] Embodiment 33. A method of treating a subject with babesiosis or protecting a subject from babesiosis infection, comprising administrating to the subject the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29.

[0189] Embodiment 34. The method of any one of embodiments 30 to 33, wherein the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29 is administered with one or more additional active agents.

[0190] Embodiment 35. The method of any one of embodiments 30 to 33, further comprising repeating the administering at least a second time, at least a third time, at least a fourth time, at least a fifth time, or at least a sixth time.

[0191] Embodiment 36. An immunoglobulin that binds to the recombinant protein of any one of embodiments 1-23.

[0192] Embodiment 37. The immunoglobulin of embodiment 36, wherein the immunoglobulin is isolated from a subject using the recombinant protein of any one of embodiments 1-23, and wherein the subject has naturally acquired immunity to malaria, toxoplasmosis, or babesiosis.

[0193] Embodiment 38. An immunoglobulin that binds the recombinant protein of any one of embodiments 1-23, wherein the immunoglobulin is obtained by immunization with the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29.

[0194] Embodiment 39. The immunoglobulin of any one of embodiments 36 to 38, wherein the immunoglobulin is a monoclonal antibody or a plurality of polyclonal antibodies.

[0195] Embodiment 40. The immunoglobulin of any one of embodiments 36 to 39, wherein the immunoglobulin cross-reacts with different strains and/or subtypes of malaria.

[0196] Embodiment 41. The immunoglobulin of any one of embodiments 36 to 39, wherein the immunoglobulin cross-reacts with different strains and/or subtypes of toxoplasmosis.

[0197] Embodiment 42. The immunoglobulin of any one of embodiments 36 to 39, wherein the immunoglobulin cross-reacts with different strains and/or subtypes of babesiosis.

[0198] Embodiment 43. A method of generating antibodies cross-protective against malaria comprising:

(a) immunizing a subject with the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29:

(b) isolating antibodies that cross-react with different strains and/or subtypes of malaria.

[0199] Embodiment 44. A method of generating antibodies cross-protective against toxoplasmosis comprising:

(a) immunizing a subject with the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of embodiments 26 to 29:

(b) isolating antibodies that cross-react with different strains and/or subtypes of toxoplasmosis.

[0200] Embodiment 45. A method of generating antibodies cross-protective against babesiosis comprising:

(a) immunizing a subject with the recombinant protein of any one of embodiments 1-23, the nucleic acid composition of embodiment 24, the vector of embodiment 25, or the immunotherapy composition of any one of claims 26 to 29:

(b) isolating antibodies that cross-react with different strains and/or subtypes of babesiosis.

[0201] The subject matter will be further described in the following examples, which do not limit the scope of the subject matter described in the claims.

EXAMPLES

[0202] The Examples that follow are illustrative of specific embodiments of the disclosure, and various uses thereof. They are set forth for explanatory purposes only, and should not be construed as limiting the scope of the claimed subject matter in any way.

Materials and Methods

Expression and purification of AMA1 DI-DII and single-component immunogens.

[0203] All three single-component immunogens, the 3D7 allele of AMA1 DI-DII, AMA1 DI-DII ΔDII-loop and AMA1 ectodomain, TrxA (thioredoxin), TrxA-RON2L fusions, and IgNAR 14I-1 were expressed in HEK293 cells, a system capable of post-translational modifications. The sequences for all constructs were codon optimized for expression in mammalian cells (GenScript) and all N-linked glycosylation sites (NXS/T) were modified by substituting the serine or threonine residue with an alanine residue to prevent glycosylation that is absent in the endogenous *Plasmodium falciparum* proteins.

[0204] These optimized coding sequences for all three single-component immunogens, AMA1 DI-DII, and TrxA were synthesized and cloned into a pHL-sec expression plasmid, which incorporates a 6xHis tag at the C-terminus, and transfected into Expi293F™ cells (Thermo Fisher Scientific, Cat# A14527) and grown according to the manufacturer's instructions. pHL-sec was a gift from Edith Yvonne Jones (Addgene plasmid # 99845; RRID:Addgene_99845) (65). The soluble proteins were purified from cell-free supernatant four days post-transfection using Ni Sepharose™ Excel resin (Cytiva, Cat# 17371203) and size exclusion chromatography (Superdex 200 Increase 10/300 GL; Cytiva) in phosphate buffered saline (pH 7.4) or 20 mM Tris (pH 8.0) containing 100 mM NaCl. Size exclusion chromatography was performed on a ÄKTA pure protein purification system and data was collected using UNICORN 7.3 software.

[0205] Purification yields of single-component immunogens were calculated as described previously (66). Briefly, transfection, expression, and purification were performed in triplicate in order to determine the purification yields for single-component immunogens. A 100-ml culture was used for each replicate, and yields were calculated by integrating the area under the monomeric peak on the Abs280 chromatogram in size exclusion chromatography. These yields were similar to the yields obtained when fractions were pooled. The extinction

coefficients were calculated from protein sequences using the ExPASy ProtParam tool (67) and used to calculate yields.

Expression and purification of AMA1 ectodomain, IgNAR 14I-1, and TrxA-RON2L fusions.

[0206] To produce the biotinylated AMA1 ectodomain and IgNAR 14I-1, the optimized coding sequence was synthesized and cloned into a derivative of the pHL-avitag3 expression plasmid which incorporates an Avi-tag (GLNDIFEAQKIEWHE; SEQ ID NO:79) and a 6xHis tag at the C-terminus (GenScript). pHL-avitag3 was a gift from Edith Yvonne Jones (Addgene plasmid # 99847; RRID:Addgene_99847) (65). Plasmid was co-transfected with the BirA biotin ligase expressing plasmid and 100 µM biotin into Expi293F™ cells and grown according to the manufacturer's instructions. Secreted BirA-Flag was a gift from Gavin Wright (Addgene plasmid # 64395; RRID:Addgene_64395) (68). The soluble biotinylated AMA1 ectodomain and IgNAR 14I-1 were purified from cell-free supernatant four days post-transfection using Ni Sephaose™ Excel resin (Cytiva) and size exclusion chromatography (Superdex 200 Increase 10/300 GL or Superdex 75 Increase 10/300 GL; Cytiva) in a buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl and 3 mM EDTA. Purified biotinylated AMA1 ectodomain and IgNAR 14I-1 were used for BLI experiments and bioassays. The expressed AMA1 ectodomain and IgNAR 14I-1 were biotinylated to at least 90% as evidenced by SDS-PAGE gel-shift (69).

[0207] The TrxA-RON2L-1 fusion protein contains the loop region of RON2 (RON2L; residues Asp2021 to Ser2059) with N-terminal 6xHis and TrxA tags separated from the RON2L sequence by a PreScission Protease cleavage site (LEVLFQ/GP; SEQ ID NO:80). A codon optimized DNA sequence was synthesized and subcloned into a pHL-sec expression plasmid (GenScript). Plasmid was transfected into Expi293F™ cells and grown according to the manufacturer's instructions. Cell-free supernatant was harvested four days after transfection. The soluble TrxA-RON2L-1 fusion was purified using Ni Sepharose™ Excel resin (Cytiva) and size exclusion chromatography (Superdex 75 Increase 10/300 GL; Cytiva) in a phosphate buffered saline (pH 7.4).

[0208] To produce the biotinylated TrxA-RON2L-2 fusion, a codon optimized C-terminal Avi-tag (GLNDIFEAQKIEWHE; SEQ ID NO:79) was appended to the TrxA-RON2L-1 sequence above, synthesized and subcloned into a pHL-sec expression plasmid (GenScript). Plasmid was co-transfected with the BirA biotin ligase expressing plasmid and 100 µM biotin into Expi293F™ cells and grown according to the manufacturer's instructions. The soluble biotinylated TrxA-RON2L-2 fusion was purified from cell-free supernatant four days post-transfection using Ni Sephaose™ Excel resin (Cytiva) and size exclusion chromatography (Superdex 75 Increase 10/300 GL; Cytiva) in a buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl and 3 mM EDTA. Purified biotinylated TrxARON2L-2 fusion was used for BLI

experiments and bioassays. The expressed TrxA-RON2L-2 fusion was biotinylated to at least 90% as evidenced by SDS-PAGE gel-shift (69).

[0209] Purified AMA1 ectodomain, IgNAR 14I-1, and TrxA-RON2L fusions (Table 1) were of high purity and homogeneity (Figure 13).

Table 1: Sequences of immunogens and constructs used in the study after signal peptide cleavage. Cloning scars, tags, and linkers are shown in lowercase.

Name	Sequence
Native AMA1 DI-DII	etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKG IIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKN LDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNK DESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNC EDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTDYEKIKEGFKNKNAAMI KSAFLPTGAFKADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTA LSHPIEVEgtkhhhhhh (SEQ ID NO:81)
AMA1 DI-DII ΔDII-loop	etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKG IIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKN LDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNK DESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNC EDIPHVNEFPAIDLFECNKLVFELSASDQPGGSGYKSHGKGYNWGNYNTETQKCE IFNVKPTCLINNAAYIATTALSHPIEVEgtkhhhhhh(SEQ ID NO:82)
SBD1 immunogen	etgKADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEV EgggggsggggsggggsggggsgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEV AGTQYRLPSGKCPVFGKGIIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSP MTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKC HILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVC PRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHL TTQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALSgtkhhhhhh (SEQ ID NO:83)
Insertion fusion immunogen 2	etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKG IIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKN LDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGggggsg sDITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALSggsgMFCFRPAKDIS FQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECN KLVFELSASDQPggsgYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIAT TALSHPIEVEgtkhhhhhh (SEQ ID NO:84)
Insertion fusion immunogen 3	etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKG IIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKN LDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNG gggsggggsDITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALSggsgMFC FRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFP AIDLFECNKLVFELSASDQPggsgYKSHGKGYNWGNYNTETQKCEIFNVKPTCLI NNAAYIATTALSHPIEVEgtkhhhhhh (SEQ ID NO:85)
AMA1 ectodomain	etgQNYWEHPYQNSDVYRPINEHREHPKEYEYPLHQEHTYQQEDSGEDENTLQHA YPIDHEGAEPAPQEQNLFSSIEIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDL GEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTTFLTPVATGNQYLKDGGFAFPPT EPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVYD DKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYTYLSKNVVD NWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPK

	QYEQHLTDYEKIKEGFKNKNASMIKSAFLPTGAFKADRYKSHGKGYNWGNYNTET QKCEIFNVKPTCLINNSSYIATTALSHPIEVENNFPCSLYKDEIMKEIERESKRI KLNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSTCRFFVCKCVERRAEV TSNNEVVVKEEYKDEYADIPEHKPTgtggsggsglndifeaqkiewhegrtkhhh hhh (SEQ ID NO:86)
TrxA-RON2L-1	etghhhhhhMSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEI ADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLK EFLDANLAgsgslevlfqgpDITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVN TALS (SEQ ID NO:87)
TrxA-RON2L-2	etghhhhhhMSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEI ADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLK EFLDANLAGSGSLEVLFQGPDITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVN TALSGSGSglndifeaqkiewhe (SEQ ID NO:88)
TrxA	etghhhhhhMSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEI ADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLK EFLDANLAGSGSglndifeaqkiewhe (SEQ ID NO:89)
14I-1	etgAWVDQTPRTATKETGESLTINCVLRDASLELKDTGWYRTKLGSTNEQSISIG GRYVETVNKGSKSFSLRISDLRVEDSGTYKCQAFYSLPLRDYNYALLFRGEKGAG TALTVKAAAgtkhhhhhh (SEQ ID NO:90)
14I-1 (biotinylated)	etgAWVDQTPRTATKETGESLTINCVLRDASLELKDTGWYRTKLGSTNEQSISIG GRYVETVNKGSKSFSLRISDLRVEDSGTYKCQAFYSLPLRDYNYALLFRGEKGAG TALTVKAAAgtggsggsglndifeaqkiewhegrtkhhhhhh (SEQ ID NO:91)

Preparation and purification of AMA1 DI-DII-RON2L complex

[0210] To prepare AMA1 DI-DII-RON2L complex, purified AMA1 DI-DII was mixed with purified TrxARON2L-1 fusion in a 1:2 molar ratio and incubated on ice for 30 minutes. The TrxA-RON2L-1 fusion contains a PreScission Protease cleavage site (LEVLFQ/GP; SEQ ID NO:80) between the N-terminal TrxA/6xHis tags and RON2L (residues Asp2021 to Ser2059). A complex formed by mixing AMA1 DI-DII with TrxA-RON2L fusion proteins was proteolytically processed by PreScission Protease.

[0211] Briefly, sample was buffer exchanged and concentrated to 2 mg/ml (in 1 ml total volume) at 4 °C using an Amicon centrifugal filter (MilliporeSigma) equilibrated in cleavage buffer containing 50 mM Tris (pH 7.0), 150 mM NaCl, and 1 mM EDTA. The cleavage buffer did not contain reducing agents to avoid the reduction of intact disulfide bonds in AMA1 DI-DII and RON2L. Then, approximately, 60 units of GST-tagged PreScission Protease was added and incubated at 4 °C for 5 hours on a tube revolver (Thermo Fisher Scientific). One unit of PreScission Protease cleaves 100 µg of a test fusion protein in 16 hours to 90% completion at 5 °C in cleavage buffer with 1 mM DTT. Following cleavage, sample was applied to a column with 1.5 ml bed volume of washed and equilibrated glutathione agarose resin (Gold Biotechnology, Cat# G-250) in cleavage buffer for removal of PreScission Protease. A flow-through fraction of the cleaved sample was collected and concentrated to 1 ml using an

Amicon centrifugal filter (MilliporeSigma). The cleaved sample included AMA1 DI-DII-RON2L complex, free uncomplexed RON2L and TrxA. The AMA1 DI-DII-RON2L complex from cleaved sample was purified by size exclusion chromatography using a Superdex 75 Increase 10/300 GL column (Cytiva) equilibrated in PBS (pH 7.4) (Figure 14A). A peak containing AMA1 D-DII and RON2L confirms the formation of a stable complex and high purity (Figure 14B and 14C). Further, the detection of RON2L in western blot with biotinylated AMA1 ectodomain as a probe confirms that the disulfide bond in RON2L is intact, which is crucial for its binding to AMA1 (Figure 14C).

Western Blotting

[0212] For Western blotting, 5 µg of AMA1 DI-DII-RON2L complex was diluted in Tricine SDS sample buffer (Thermo Fisher Scientific, Catalog# LC1676) without a reducing agent and left at room temperature for 5 minutes. Similarly, 2 µg of purified 6xHis-tagged AMA1 DI-DII and TrxA were diluted in 2x Tricine SDS sample buffer without reducing agent and used as controls. 10 µl of samples were loaded on a 16% Tricine gel (Thermo Fisher Scientific, Cat# EC66955BOX) and separated for 60 min at 150 volts. Proteins were transferred to a nitrocellulose (NC) membrane (Thermo Fisher Scientific, Cat# IB23002) using the iBlot™ Gel Transfer Device (Thermo Fisher Scientific) according to the manufacturer's instructions. The membrane was then washed three times with Tris buffered saline (20 mM Tris (pH 8.0), 150 mM NaCl) containing 0.1 % Tween 20 (TBS/T) and blocked with 25 ml of 3 % bovine serum albumin in TBS/T (blocking buffer) for 1 hour at room temperature with gentle shaking and washed three times with TBS/T. The 6x-His Tag Monoclonal Antibody (Thermo Fisher Scientific, Cat# 37-2900) was diluted 1:10000 in 25 ml of blocking buffer and added to the membrane. The membrane was then incubated for 1 hour at room temperature with gentle shaking, and washed three times with TBS/T. The biotinylated AMA1 ectodomain was then diluted to 2 µg/ml in 25 ml of blocking buffer and added to the membrane, and incubated for 1 hour at room temperature with gentle shaking, followed by three washes with TBS/T. Then, goat anti-mouse antibody conjugated to HRP (Jackson ImmunoResearch Laboratories Inc., Cat# 115-035-164) and streptavidin HRP conjugate (Thermo Fisher Scientific, Cat# 21127) were diluted 1:10000 and 1:5000, respectively, in 25 ml of blocking buffer and added to the membrane, incubated for 1 hour at room temperature with gentle shaking, and washed three times with TBS/T. Following this, chemiluminescent substrate (Thermo Fisher Scientific, Cat# 34579) was applied to the membrane according to the manufacturer's instructions and captured the chemiluminescence image using Amersham™ Imager 600 (GE Healthcare).

Binding kinetics of AMA1 DI-DII and single component immunogens with IgNAR 14I-1 or RON2L using biolayer interferometry

[0213] Binding of the AMA1 DI-DII and single component immunogens to the IgNAR 14I-1 and RON2L were measured by kinetic experiments carried out on an Octet RED96e (Sartorius). All constructs were buffer exchanged into 1x HBS-EP+ buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.05% (v/v) P20 surfactant (Cytiva, Cat# BR100826)] using Zeba™ spin desalting columns (Thermo Fisher Scientific) according to the manufacturer's instructions. All measurements were performed at 200 µl/well in 1x HBS-EP+ buffer at 25 °C in 96-well black plates (Greiner Bio-One, Cat# 655209). Streptavidin (SA) biosensors (Sartorius, Cat# 18-5019) were used to immobilize biotinylated IgNAR 14I-1 [~0.6 binding (nm) units] or TrxA-RON2L [~0.3 binding (nm) units] for 300 s. Immunogens were two-fold serially diluted in HBS-EP+ buffer in the range of 200 nM to 3.125 nM. Assay was performed in five sequential steps with Octet® BLI Discovery 12.2.2.20 software (Sartorius): Step 1, biosensor hydration and equilibration (780 s); Step 2, immobilization of biotinylated IgNAR 14I-1 or TrxA-RON2L on a Streptavidin (SA) biosensor (300 s); Step 3, wash and establish baseline (60 s); Step 4, measure IgNAR 14I-1 or TrxA-RON2L-immunogens association kinetics (300 s); and Step 5, measure IgNAR 14I-1 or RON2L-immunogens dissociation kinetics (300 s). The acquired raw data for binding of AMA1 DI-DII with IgNAR 14I-1 or RON2L were processed and globally fit to a 1:1 binding model with Octet® Analysis Studio 12.2.2.26 Software (Sartorius). The binding kinetics measurements were carried out in three replicates. Values reported are the average and SEM among replicates.

Binding analysis of AMA1 DI-DII and single component immunogens with IgNAR 14I-1 or RON2L using ELISA

[0214] Binding of the AMA1 DI-DII and single component immunogens to the IgNAR 14I-1 and RON2L were analyzed by ELISA. Immunogens were diluted in 50 mM Na-carbonate (pH 9.5) and were coated on Nunc MaxiSorp flat-bottom 96-well ELISA plates (Thermo Fisher Scientific, Cat# 44-2404-21) at 10 nM in 100 µl at 4 °C overnight. The plates were then washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) and blocked with 2% bovine serum albumin in PBS/T for 1 hour at room temperature, and then washed three times with PBS/T. Next, 200 µl of biotinylated 14I-1 or TrxA-RON2L-2 diluted to 200 nM and 1000 nM in blocking buffer (PBS/T with 2% bovine serum albumin) was added to each well of the blocked plates and incubated for 1 hour at room temperature, then washed three times with PBS/T. 200 µl of streptavidin HRP conjugate (Thermo Fisher Scientific, Cat# 21127) was then added to each well at a 1:10000 dilution and incubated for 1 hour at room temperature. The plates were then washed three times with PBS/T and developed with 70 µl of TMB substrate (MilliporeSigma) for 20 min at room temperature in the dark. The reaction was then stopped by adding 160 mM sulfuric acid (H_2SO_4) and an absorbance measured at 450 nm on a BioTek™ Synergy H1 microplate reader using Gen5 3.08.01 software.

Differential scanning fluorimetry

[0215] Differential scanning fluorimetry (DSF) was performed to measure the thermal stability of single-component immunogens using the Protein Thermal Shift™ Dye Kit (Thermo Fisher Scientific, Cat# 4461146) according to the manufacturer's instructions. Each 20 µl assay mixture contained 10 µg of purified immunogen in PBS (pH 7.4), 1× Protein Thermal Shift buffer, and 1× Thermal Shift Dye. The melt-curve experiments were performed on a 7500 Fast Real-Time polymerase chain reaction system (Thermo Fisher Scientific). Fluorescent readings were monitored as the temperature was increased from 25 °C to 95 °C at a ramp rate of 1%. Protein melt fluorescent readings were analysed using Protein Thermal Shift™ software v 1.4 (Thermo Fisher Scientific) and the melting temperature (T_m) was calculated as a peak of the derivative melt curve. Protein melt-curve experiments were performed in five technical replicates on each plate and in biological triplicate. T_m for a biological replicate was calculated by averaging technical replicates, and the reported T_m was calculated by averaging three biological replicates.

Protein crystallization, data collection, and structure solution

[0216] 6xHis-tagged immunogens were purified from cell-free supernatant by affinity chromatography using Ni Sepharose™ Excel resin (Cytiva, Cat# GE17371201) according to the manufacturer's instructions followed by size exclusion chromatography using Superdex 200 Increase 10/300 GL column (Cytiva) equilibrated in 20 mM Tris (pH 8.0) and 100 mM NaCl. Purified immunogens were concentrated to 20 mg/ml using an Amicon centrifugal filter (MilliporeSigma).

[0217] Crystallization experiments were carried out using hanging drop vapor diffusion. Crystals were obtained using a mosquito® crystal (SPT Labtech) to mix 0.2 µl of purified immunogen (20.0 mg/ml) with 0.2 µl reservoir solution in 96-well plates that were incubated at 18 °C.

[0218] Immunogen 1 (SBD1 immunogen) was crystallized with 0.2 M Ammonium sulfate and 20% (w/v) PEG 3350 at 18 °C. Immunogen 2 (Insertion fusion immunogen 2) was crystallized with 0.5 M Lithium Chloride, 0.1 M Tris (pH 8.5), and 34% (w/v) PEG 6000 at 18 °C. Immunogen 3 (Insertion fusion immunogen 3) was crystallized with 0.2 M Magnesium chloride, 0.1 M Tris (pH 8.5), and 20 % (w/v) PEG 8000, at 18 °C. All crystals were cryoprotected with the addition of either 30% glycerol or 30% polyethylene glycol and flash-frozen in liquid nitrogen. Diffraction data for all crystals were collected at 1.0 Å at 100 K on the beamline SER-CAT 22-ID at the Advanced Photon Source (APS). All diffraction data were processed using XDS (70). Reflections were indexed and integrated using XDS (70). Data were scaled and merged using XSCALE70 or POINTLESS and AIMLESS (71) and all

structures were solved by molecular replacement (MR) using Phaser (72-74), rebuilt with AutoBuild (74, 75), manually built in Coot (76), and refined with Phenix.refine (74,77). Resolution cutoffs for scaling were evaluated using standard metrics of signal to noise and CC½. Standard settings in Phenix.refine, TLS parameters (78), B-factors, and weight optimization options (X-ray/stereochemistry weight and X-ray/ADP weight) were enabled for the refinement of the immunogens. The crystal structure of all three immunogens were solved by molecular replacement using AMA1-RON2L peptide complex (PDB: 3zwz) as search model. Following final refinement, the R_{work}/R_{free} values for immunogens 1, 2, and 3 were 0.1750/0.2086, 0.1844/0.2082, and 0.1726/0.2127, respectively. MolProbity was used to evaluate the geometry of the final models (79, 80). All three immunogens showed more than 96.0% of the residues as Ramachandran favored and 0% outlier residues. Figures of molecular structures were generated using the PyMOL Molecular Graphics System, Version 2.5 (Schrödinger, Inc.). Software used in this project was curated by SBGrid (81).

Rat immunizations

[0219] Rat immunogenicity studies were performed in an American Association for Accreditation of Laboratory Animal Care-accredited facility under the guidelines and approval of the Institutional Animal Care and Use Committee at the National Institutes of Health. On Day 0, groups of nine 12-14-week-old CD® (Sprague Dawley) IGS rats, Crl:CD(SD) (Charles River Laboratories) were immunized by subcutaneous injection with 20 µg of each antigen in 100 µL formulated as a 1:1 volume ratio in AddaS03™ adjuvant (InvivoGen, Cat# vac-as03-10) and DPBS (pH 7.4). Rats were boosted twice after the initial prime, on days 21 and 42. On days 14, 35, and 63, blood was collected, and serum was separated and stored at -80 °C.

Serum antibody titer ELISA

[0220] The 3D7 allele of AMA1 DI-DII was diluted in 50 mM Na-carbonate (pH 9.5) and was coated on Nunc MaxiSorp flat-bottom 96-well ELISA plates (Thermo Fisher Scientific, Cat# 44-2404-21) at 20 µg/ml in 100 µl at 4°C overnight. The plates were then washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) and blocked with 2% bovine serum albumin in PBS/T for 1 hour at room temperature, and then washed three times with PBS/T. Next, Serum was diluted in blocking buffer (PBS/T with 2% bovine serum albumin), and 100 µl was added to each well and incubated for 1 hour at room temperature, then washed three times with PBS/T. 200 µl of goat anti-rat antibody conjugated to Horseradish Peroxidase (HRP) (secondary, Jackson ImmunoResearch Laboratories Inc., Cat# 112-035-071) was then added to each well at a 1:5000 dilution and incubated for 1 hour at room temperature. The plates were then washed three times with PBS/T and developed with 70 µl of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (MilliporeSigma, Cat# T0440-1L)

for 20 min at room temperature in the dark. The reaction was then stopped by adding 2 M sulfuric acid (H2SO4) and an absorbance measured at 450 nm on a BioTek™ Synergy H1 microplate reader using Gen5 3.08.01 software.

[0221] The reference standard curve was prepared using pooled serum from rats as described previously (66). Pooled serum from rats immunized with AMA1 DI-DII and having relatively high antibody titers was used as a reference standard curve on each plate to determine the antibody titers of individual animals in all groups. The dilution of reference standard serum required to achieve an Abs450 value of 1 was defined as one antibody unit (AU). Three replicates of two-fold serial dilutions of reference standard serum ranging from 20 to 0.01 AU were included in each plate. Serum from each animal was diluted such that the Abs450 value fell within the dynamic range of the reference standard curve. The Abs450 values for the reference standard curve were fitted to a four-parameter logistic curve, in order to convert Abs450 values into AUs for individual animals in all groups. AUs for each individual animal were measured in three replicates on separate plates, and an average was calculated and reported.

AMA1 DI-DII/RON2L-Blocking assay

[0222] The AMA1 DI-DII/RON2L-blocking assay was carried out similarly to that described previously (66). TrxA-RON2L-1 fusion was diluted in 50 mM Na-carbonate (pH 9.5) and was coated on Nunc MaxiSorp flat-bottom 96-well ELISA plates (Thermo Fisher Scientific, Cat# 44-2404-21) at 20 µg/ml in 100 µl at 4 °C overnight. The plates were then washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) and blocked with 2% bovine serum albumin in PBS/T for 1 hour at room temperature, and then washed three times with PBS/T. Next, serum was diluted in blocking buffer (PBS/T with 2% bovine serum albumin) in a twofold dilution series ranging from 1:50 to 1:6400. 110 µl of diluted serum was mixed with 110 µl of 0.2 nM biotinylated AMA1 ectodomain or 100 µl of buffer as a background control and incubated for 1 hour at room temperature. 200 µl of serum mixture was added to each well of the blocked plates and incubated for 1 hour at room temperature, then washed three times with PBS/T. 200 µl of streptavidin HRP conjugate (Thermo Fisher Scientific, Cat# 21127) was then added to each well at a 1:10000 dilution and incubated for 1 hour at room temperature. The plates were then washed three times with PBS/T and developed with 70 µl of TMB substrate (MilliporeSigma) for 20 min at room temperature in the dark. The reaction was then stopped by adding 160 mM sulfuric acid (H_2SO_4) and an absorbance measured at 450 nm on a BioTek™ Synergy H1 microplate reader using Gen5 3.08.01 software.

[0223] AMA1DI-DII/RON2L binding inhibition was determined by subtracting the Abs450 values from background controls lacking the biotinylated AMA1 ectodomain. The average

maximum signal was calculated using three wells without serum. The following formula was used to calculate inhibition.

$$\% \text{ Inhibition} = 100 \times (1 - X/\text{max})$$

[0224] where X is the Abs_{450} value of a well after background subtraction and max is the average value of the three wells without serum after background subtraction.

[0225] The blocking assay was performed in duplicate and percent inhibition values were calculated for each serum dilution, and average values were plotted in GraphPad Prism 8. Data were fitted using a normalized dose response curve with a variable slope.

$$Y = 100/ (1 + (ID_{50}/X) \wedge \text{HillSlope})$$

[0226] where X is the serum dilution, Y is the % inhibition, and HillSlope and ID_{50} are calculated parameters corresponding to the slope of the curve and the dilution at which 50% inhibition occurs, respectively. For each animal, the ID_{50} values were plotted alongside the geometric mean value for each group.

Growth inhibition assay (GIA)

[0227] All assays for GIA were performed as described in the protocol of the International Growth Inhibition Assay Reference Centre at the National Institutes of Health (82). IgG was purified from individual rat serum using Protein G HTC Agarose resin/Protein G Sepharose 4 Fast Flow resin (GoldBio, Cat# P-430-25 or Cytiva, Cat# 17061805) according to the manufacturer's instructions. Purified IgG were buffer exchanged in RPMI 1640, and concentrated with Amicon centrifugal filters (MilliporeSigma) to 10 mg/ml and aliquots were stored at -80 °C. Test IgG was added to triplicate wells at 5.0 mg/ml and incubated with infected red blood cells (0.3 % parasitemia, 1% hematocrit) in a final volume of 40 µl and returned to a culture incubator (5% O_2–5% CO_2–90% N_2) for 40 hours at 37 °C. Growth inhibition (parasitemia) was assessed by the lactate dehydrogenase activity assay. The percent GIA was calculated using as: % GIA = 100-100 (sample A_{650} – uninfected RBC A_{650})/(infected control A_{650} – uninfected RBC A_{650}).

Example 1: Design of single-component immunogens with improved biophysical characteristics by combining RON2L with AMA1 DI-DII

[0228] We created three single component immunogens **(FIG. 1)** containing domains I and II (DI-DII) of AMA1 fused to RON2L. The RON2L binding site in apo AMA1 comprises a domain I hydrophobic groove and a region that is exposed when the DII loop (Lys351 to Ala387) is displaced by RON2L. Upon displacement, the DII loop adopts a disordered state, does not contact RON2L and appears dispensable for binding. In the absence of RON2L the DII loop is stabilized by domain I (63, 64). RON2L contacts discontinuous residues in AMA1 that are located in the middle of the protein sequence.

[0229] A single-component AMA1-RON2L immunogen cannot be created by simple fusion of RON2L to the N- or C-terminus of AMA1 because the AMA1 termini are located far from the RON2L binding site and would require a large linker to facilitate the correct orientation of RON2L in the pocket. We used structure-based design (SBD) to alter the location of the C-terminus, enabling seamless attachment of RON2L. The SBD1 immunogen is a circular permutation of AMA1 that contains a Gly/Ser linker (G4S x 4; GGGGSGGGGSGGGGSGGGGS; SEQ ID NO:78) between the original N- and C- termini. The DII loop (358-TDYEKIKEGFKNKNASMIKSAFLPTGAF-385; SEQ ID NO:92) is removed in SBD1 to produce novel N- and C-termini at residues at Lys386 and Thr357, respectively. This new AMA1 C-terminus is immediately adjacent to the N-terminal helix of bound RON2L. Some of the residues deleted in SBD1 (360-YEKIKEGFK-368; SEQ ID NO:93) comprise a helix in AMA1, which is replaced by the N-terminal helix of RON2L (4-QQAKDIGAG-12; SEQ ID NO:94). This design approach ensures that the RON2L sequence (3-TQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS-39; SEQ ID NO:95) could be appended without a linker to create SBD1 **(FIG. 1B, C, F)** (7).

[0230] Additionally, we created insertion fusion immunogens by inserting RON2L into an AMA1 loop proximal to the RON2L binding site **(FIG. 1B, D, E, F)**. Insertion fusion immunogens 2 and 3 were constructed by replacing several amino acids in the DIf loop of AMA1 with RON2L and a flanking Gly/Ser linker. Insertion fusion Immunogen 2 lacks amino acids 260-PRYCNKDESKRNS-272 (SEQ ID NO:96) of the DIf loop, including Cys263, consequently disrupting a disulfide bridge **(FIG. 1B, D, F)**. Insertion fusion Immunogen 3 lacks only amino acids 265-KDESKRNS-272 (SEQ ID NO:97), retaining Cys263 and the disulfide bridge **(FIG. 1B, E, F)**. The disordered DII loop was replaced with a Gly/Ser linker in both of these insertion fusion immunogens to prevent the potential displacement of the fused RON2L. We also created AMA1 DI-DII design in which the DII loop was replaced by a short Gly-Ser linker (AMA1 DI-DII ΔDII-loop), in order to examine the impact of the removal of the DII loop.

[0231] AMA1 DI-DII, AMA1 DI-DII ΔDII-loop and each of these three immunogens **(Table 1)** were expressed in HEK293 cells and purified to homogeneity. The expressed AMA1 DIDII, AMA1 DI-DII ΔDII-loop and immunogens were folded, monomeric and monodisperse as evidenced by size exclusion chromatography and SDS-PAGE analysis **(FIG. 2A, FIG. 7A)**. All three designed immunogens had a higher mean purification yield than WT AMA1 DI-DII (8.6 mg/l), with SBD1 immunogen and insertion fusion immunogens 2 and 3 demonstrating purification yields of 14.2 mg/l, 21.9 mg/l and 16.9 mg/l, respectively **(FIG. 2B)**. All three immunogens showed marked improvement in their average melting temperature (T_m) by approximately 21 °C; from 52 °C to 74 °C **(FIG. 2C, D)**. The improvement in Tm is primarily a result of the fusion of RON2L and not due to the removal of the DII loop **(FIG. 7B, C)**. In

summary, fusion of RON2L to AMA1 DI-DII produced three distinct single-component immunogens with substantially improved biophysical characteristics.

Example 2: The fused RON2L is bound to AMA1 in the immunogens

[0232] The fusions of RON2L to AMA1 were designed to replicate the bound state of the complex. The bound state is expected to be unable to bind exogenous RON2L and unable to bind antibodies that compete with RON2L binding. The neutralizing immunoglobulin new antigen receptor (IgNAR) 14I-1 (38) binds to an epitope in AMA1 located within the hydrophobic RON2L binding groove and competes with RON2L binding. We determined the accessibility of the RON2L binding site in the designed immunogens by probing with IgNAR 14I-1 and exogenous RON2L using biolayer interferometry (BLI) and enzyme-linked immunosorbent assay (ELISA). AMA1 DI DII, which has an accessible RON2L binding site, was able to effectively bind to 14I-1 with binding clearly observable by BLI at concentrations as low as ~10 nM **(FIG. 3A)**. In contrast, none of the immunogens bound to 14I-1 even at 200 nM, the highest concentration tested **(FIG. 3A)**, demonstrating that the fused RON2 occupied the binding site and prevents accessibility. Similar results were obtained by ELISA where AMA1 DI-DII bound to 14I-1 while all three immunogens showed little or no binding with 200 nM or 1000 nM of 14I-1 **(FIG. 3B)**. This suggests that antibodies with epitopes in the domain I hydrophobic groove are unable to engage the designed immunogens. In a similar manner, AMA1 DI-DII bound to exogenous RON2L by both BLI and ELISA while the immunogens exhibited little to no binding **(FIG. 3C, D)**. These results indicate the designs were successful in replicating the bound state of the complex.

Example 3: Structures of the designed immunogens recapitulate the AMA1-RON2L complex and reveal the molecular basis for enhanced stability

[0233] We investigated whether fused RON2L is correctly bound to AMA1 in the designed immunogens through structural analysis. We determined the X-ray crystal structures of SBD1, and insertion fusion immunogens 2, and 3 to resolutions 1.80 Å, 1.85 Å, and 2.10 Å, respectively **(FIG. 4A, Table 1)**. We superimposed the structures of these immunogens on the previously characterized AMA1 DI-DII-RON2L complex (PDB ID: 3zwz) (8). The overall structures of the designed immunogens were very similar to the native AMA1 DI-DII-RON2L complex. The SBD1 structure was most similar to the AMA1-RON2L complex with no major structural reorganizations observed and a Cα root mean square deviations (RMSDs) of 0.299 over 245 Calpha residues **(FIG. 4B, FIG. 8)**. In contrast, insertion fusion immunogens 2 and 3 retained the RON2L binding mode of the complex, but displayed local distortions in loops near the vicinity of the insertion sites resulting in C_α root mean square deviations (RMSDs) of 0.381 over 232 C-alpha residues, and 0.309 Å over 228 C-alpha residues respectively **(FIG.**

4B, FIG. 8). In all cases, the N-terminal helix of fused RON2L was located at one end of the binding site with the coil extending into a disulfide-closed loop resulting in a U-shaped structure **(FIG. 4C, FIG. 9)**. A vast majority of the interface residues between fused RON2L and AMA1 DI-DII in the diverse designs were identical to those found in the AMA1 DI-DII-RON2L complex indicating that fused RON2L binds correctly to AMA1 DI-DII **(Tables 2, 3, and 4)**. All three structures revealed the two cysteine residues in the RON2L peptide that are necessary for binding to AMA1 are disulfide-linked **(FIG. 4C)**. Additionally, a key interacting Arg residue, corresponding to ARG2041 in RON2, in the fused RON2L of immunogens fits well into a pocket in a manner identical to that in the complex structure (PDB ID: 3zwz) **(FIG. 10)**. The binding of RON2L appears to improve residue packing in domain I and enhance conformational stability of all three structures.

Table 2: Contact residues between RON2L and AMA1 residues for SBD1 immunogen.

Residues in RON2L	Residues in RON2L (Numbered according to PDB ID:3ZWZ)	Interface with AMA1 (PDB ID: 3ZWZ)	Residues in RON2L (SBD1 immunogen)	Interface with AMA1 (SBD1 immunogen)	Interface with AMA1 (SBD1 immunogen, numbered according to PDB ID:3ZWZ)
Thr3	**Thr2023**	Leu131	**Thr329**	Leu101	Leu131
Gln4	**Gln2024**	Val137	**Gln330**	Val107	Val137
Gln5	**Gln2025**	Thr140	**Gln331**	Thr110	Thr140
Ala6	**Ala2026**	Gln141	**Ala332**	Gln111	Gln141
Lys7	**Lys2027**	Tyr142	**Lys333**	Tyr112	Tyr142
Asp8	**Asp2028**	Arg143	**Asp334**	Arg113	Arg143
Ile9	**Ile2029**	Val169	**Ile335**	Val139	Val169
Gly10	**Gly2030**	Ala170	**Gly336**	Ala140	Ala170
Ala11	**Ala2031**	Thr171	**Ala337**	Thr141	Thr171
Gly12	**Gly2032**	Gly172	**Gly338**	Gly142	Gly172
Pro13	**Pro2033**	Gln174	**Pro339**	Gln144	Gln174
Val14	**Val2034**	Leu176	**Val340**	Tyr145**	Tyr175**
Ala15	**Ala2035**	Phe183	**Ala341**	Leu146	Leu176
Ser16	**Ser2036**	Pro184	**Ser342**	Phe153	Phe183
Cys17	**Cys2037**	Pro185	**Cys343**	Pro154	Pro184
Phe18	**Phe2038**	Thr186	**Phe344**	Pro155	Pro185
Thr19	**Thr2039**	Glu187	**Thr345**	Thr156	Thr186
Thr20	**Thr2040**	Pro188	**Thr346**	Glu157	Glu187
Arg21	**Arg2041**	Met190	**Arg347**	Pro158	Pro188
Met22	**Met2042**	Phe201	**Met348**	Met160	Met190
Ser23	**Ser2043**	Tyr202	**Ser349**	Phe171	Phe201
Pro24	**Pro2044**	Asn205	**Pro350**	Tyr172	Tyr202
Pro25	**Pro2045**	Val208	**Pro351**	Asn175	Asn205
Gln26	**Gln2046**	Arg219	**Gln352**	Val178	Val208
Gln27	**Gln2047**	Gly222	**Gln353**	Arg189	Arg219
Ile28	**Ile2048**	Asn223	**Ile354**	Gly192	Gly222
Cys29	**Cys2049**	Met224	**Cys355**	Asn193	Asn223
Leu30	**Leu2050**	Ile225	**Leu356**	Met194	Met224

Asn31	**Asn2051**	Pro226	**Asn357**	Ile195	Ile225
Ser32	**Ser2052**	Asp227	**Ser358**	Pro196	Pro226
Val33	**Val2053**	Asn228	**Val359**	Asp197	Asp227
Val34	**Val2054**	Ser232	**Val360**	Asn198	Asn228
Asn35	**Asn2055**	Tyr234	**Asn361**	Ser202	Ser232
Thr36	**Thr2056**	Lys235	**Thr362**	Tyr204	Tyr234
Ala37	**Ala2057**	Tyr236	**Ala363**	Lys205	Lys235
Leu38	**Leu2058**	Tyr251	**Leu364**	Tyr206	Tyr236
		Ile252		Tyr221	Tyr251
		Ala253		Ile222	Ile252
		Ala254		Ala223	Ala253
		Gln255		Ala224	Ala254
		Glu256		Gln225	Gln255
		Asn257		Glu226	Glu256
		Tyr262		Asn227	Asn257
		Asn271		Tyr232	Tyr262
		Met273		Asn241	Asn271
		Phe274		Met243	Met273
		Phe276		Phe244	Phe274
		Gln349		Phe246	Phe276
		Pro350		Gln319	Gln349
		Arg389		Pro320	Pro350
		Tyr390		Gln325**	Gln355**
				Leu327**	Leu357**
				Thr328**	Thr358**
				Arg4	Arg389
				Tyr5	Tyr390

A box with a * indicates residues present at the interface of the AMA1 DI-DII-RON2L complex (PDB: 3zwz) but not in the SBD1 immunogen, while a box with ** indicates residues present at the interface of the SBD1 immunogen but absent in the complex.

Table 3: Contact residues between RON2L and AMA1 residues for Insertion fusion immunogen 2.

Residues in RON2L	Residues in RON2L (Numbered according to PDB ID:3ZWZ)	Interface with AMA1 (PDB ID: 3ZWZ)	Residues in RON2L (Insertion fusion immunogen 2)	Interface with AMA1 (Insertion fusion immunogen 2)	Interface with AMA1 (Insertion fusion immunogen 2, numbered according to PDB ID:3ZWZ)
Asp8	**Asp2028**	Leu131	**Asp174**	Leu31	Leu131
Ile9	**Ile2029**	Val137	**Ile175**	Val37	Val137
Gly10	**Gly2030**	Tyr142	**Gly176**	Tyr42	Tyr142
Ala11	**Ala2031**	Arg143	**Ala177**	Arg43	Arg143
Gly12	**Gly2032**	Val169	**Gly178**	Val69	Val169
Pro13	**Pro2033**	Ala170	**Pro179**	Ala70	Ala170
Val14	**Val2034**	Thr171	**Val180**	Thr71	Thr171
Ala15	**Ala2035**	Gly172	**Ala181**	Gly72	Gly172
Ser16	**Ser2036**	Gln174*	**Ser182**	Asn73**	Asn173**
Cys17	**Cys2037**	Leu176*	**Cys183**	Lys77**	Lys177**
Phe18	**Phe2038**	Phe183	**Phe184**	Phe83	Phe183
Thr19	**Thr2039**	Pro184	**Thr185**	Pro84	Pro184
Thr20	**Thr2040**	Pro185	**Thr186**	Pro85	Pro185

Arg21	**Arg2041**	Thr186	**Arg187**	Thr86	Thr186
Met22	**Met2042**	Glu187	**Met188**	Glu87	Glu187
Ser23	**Ser2043**	Pro188	**Ser189**	Pro88	Pro188
Pro24	**Pro2044**	Met190	**Pro190**	Met90	Met190
Pro25	**Pro2045**	Phe201	**Pro191**	Phe101	Phe201
Gln26	**Gln2046**	Tyr202	**Gln192**	Tyr102	Tyr202
Gln27	**Gln2047**	Asn205	**Gln193**	Asp104**	Asp204**
Ile28	**Ile2048**	Val208*	**Ile194**	Asn105	Asn205
Cys29	**Cys2049**	Arg219*	**Cys195**	Gly122	Gly222
Leu30	**Leu2050**	Gly222	**Leu196**	Asn123	Asn223
Asn31	**Asn2051**	Asn223	**Asn197**	Met124	Met224
Ser32	**Ser2052**	Met224	**Ser198**	Ile125	Ile225
Val33	**Val2053**	Ile225	**Val199**	Pro126	Pro226
Val34	**Val2054**	Pro226	**Val200**	Asp127	Asp227
Asn35	**Asn2055**	Asp227	**Asn201**	Asn128	Asn228
		Asn228		Lys130**	Lys230**
		Ser232		Ser132	Ser232
		Tyr234		Asn133**	Asn233**
		Lys235		Tyr134	Tyr234
		Tyr236		Lys135	Lys235
		Tyr251		Tyr136	Tyr236
		Ile252		Tyr151	Tyr251
		Ala253		Ile152	Ile252
		Ala254		Ala153	Ala253
		Gln255		Ala154	Ala254
		Glu256		Gln155	Gln255
		Asn257		Glu156	Glu256
		Tyr262*		Asn157	Asn257
		Asn271*		Phe213	Phe276
		Phe276		Gln286	Gln349
		Gln349		Pro287	Pro350
		Pro350			
		Tyr390*			

A box with a * indicates residues present at the interface of the AMA1 DI-DII-RON2L complex (PDB: 3zwz) but not in the Insertion fusion immunogen 2, while a box with ** indicates residues present at the interface of the Insertion fusion immunogen 2 but absent in the complex.

Table 4: Contact residues between RON2L and AMA1 residues for Insertion fusion immunogen 3.

Residues in RON2L	Residues in RON2L (Numbered according to PDB ID:3ZWZ)	Interface with AMA1 (PDB ID: 3ZWZ)	Residues in RON2L (Insertion fusion immunogen 3)	Interface with AMA1 (Insertion fusion immunogen 3)	Interface with AMA1 (Insertion fusion immunogen 3, numbered according to PDB ID:3ZWZ)
Thr3	**Thr2023**	Leu131	**Thr177**	Leu31	Leu131
Gln4	**Gln2024**	Val137	**Gln178**	Val37	Val137
Gln5	**Gln2025**	Thr140	**Gln179**	Thr40	Thr140
Ala6	**Ala2026**	Gln141	**Ala180**	Gln41	Gln141
Lys7	**Lys2027**	Tyr142	**Lys181**	Tyr42	Tyr142
Asp8	**Asp2028**	Arg143	**Asp182**	Arg43	Arg143
Ile9	**Ile2029**	Val169	**Ile183**	Val69	Val169
Gly10	**Gly2030**	Ala170	**Gly184**	Ala70	Ala170

Ala11	**Ala2031**	Thr171	**Ala185**	Thr71	Thr171
Gly12	**Gly2032**	Gly172	**Gly186**	Gly72	Gly172
Pro13	**Pro2033**	Gln174*	**Pro187**	Asn73**	Asn173**
Val14	**Val2034**	Leu176*	**Val188**	Lys77**	Lys177**
Ala15	**Ala2035**	Phe183	**Ala189**	Phe83	Phe183
Ser16	**Ser2036**	Pro184	**Ser190**	Pro84	Pro184
Cys17	**Cys2037**	Pro185	**Cys191**	Pro85	Pro185
Phe18	**Phe2038**	Thr186	**Phe192**	Thr86	Thr186
Thr19	**Thr2039**	Glu187	**Thr193**	Glu87	Glu187
Thr20	**Thr2040**	Pro188	**Thr194**	Pro88	Pro188
Arg21	**Arg2041**	Met190	**Arg195**	Met90	Met190
Met22	**Met2042**	Phe201	**Met196**	Phe101	Phe201
Ser23	**Ser2043**	Tyr202	**Ser197**	Tyr102	Tyr202
Pro24	**Pro2044**	Asn205	**Pro198**	Asp104**	Asp204**
Pro25	**Pro2045**	Val208*	**Pro199**	Asn105	Asn205
Gln26	**Gln2046**	Arg219*	**Gln200**	Gly122	Gly222
Gln27	**Gln2047**	Gly222	**Gln201**	Asn123	Asn223
Ile28	**Ile2048**	Asn223	**Ile202**	Met124	Met224
Cys29	**Cys2049**	Met224	**Cys203**	Ile125	Ile225
Leu30	**Leu2050**	Ile225	**Leu204**	Pro126	Pro226
Asn31	**Asn2051**	Pro226	**Asn205**	Asp127	Asp227
Ser32	**Ser2052**	Asp227	**Ser206**	Asn128	Asn228
Val33	**Val2053**	Asn228	**Val207**	Lys130**	Lys230**
Val34	**Val2054**	Ser232	**Val208**	Ser132	Ser232
Asn35	**Asn2055**	Tyr234	**Asn209**	Asn133**	Asn233**
		Lys235		Tyr134	Tyr234
		Tyr236		Lys135	Lys235
		Tyr251		Tyr136	Tyr236
		Ile252		Tyr151	Tyr251
		Ala253		Ile152	Ile252
		Ala254		Ala153	Ala253
		Gln255		Ala154	Ala254
		Glu256		Gln155	Gln255
		Asn257		Glu156	Glu256
		Tyr262*		Asn157	Asn257
		Asn271*		Phe221	Phe276
		Phe276		Gln294	Gln349
		Gln349		Pro295	Pro350
		Pro350		Tyr300	Tyr390
		Arg389*			
		Tyr390			

A box with a * indicates residues present at the interface of the AMA1 DI-DII-RON2L complex (PDB: 3zwz) but not in the fusion 3, while a box with ** indicates residues present at the interface of the fusion 3 but absent in the complex.

The designed immunogens produce similar antibody titers to control groups indicating the quantity of the antibody response is unchanged

[0234] We examined how these improved biophysical characteristics and altered epitope availability impacted immunogenicity and growth inhibitory activity (GIA). Groups of nine rats were immunized three times at three-week intervals with 20 μg of single-component SBD1 immunogen, insertion fusion immunogen 2 or insertion fusion immunogen 3, apo AMA1 DI-DII, or the AMA1 DI-DII-RON2L two-component complex. All antigens were adjuvanted with

AddaS03^{TM}, which is a research grade mimic of AS03, an adjuvant approved for human use **(FIG. 5A)**. There was no significant difference between the levels of AMA1 DI-DII-specific antibodies induced by the immunogens and the levels induced by AMA1 DI-DII or AMA1 DI-DII-RON2L two-component complex. This similarity in titers is noteworthy because the immunogens do not elicit antibodies to the deleted DII loop nor the blocked hydrophobic pocket (vide infra). These results suggest that the majority of antibodies induced by AMA1 DI-DII target epitopes distinct from the DII loop and RON2L binding site **(FIG. 5B)**.

Antibodies raised by the designed immunogens do not block RON2L binding by AMA1 indicating a drastically different quality of the antibody response.

[0235] We measured inhibition of the direct protein-protein interaction between AMA1 DI-DII and RON2L in a blocking assay that measures RON2L binding to AMA1. Blocking antibody titers were determined by serially diluting sera from individual rats after the third vaccination on day 63 (d63). Rats immunized with either AMA1 DI-DII or the AMA1 DI-DII-RON2L two-component complex elicited high titers of RON2L blocking antibodies. In contrast, all three immunogens elicited significantly lower levels of blocking antibodies, typically at the limit of detection of the assay, than AMA1 DI-DII or AMA1 DI-DII-RON2L two-component complex **(FIG. 5C)**. This result indicates that the immunogens do not elicit antibodies that recognize the domain I hydrophobic groove and DII loop of AMA1 DI-DII and is consistent with the formation of an irreversibly bound RON2L complex.

Functional antibody responses outside of the RON2L binding site contribute substantially to strain-transcending parasite neutralization

[0236] The neutralizing activity of immunogen-induced antibodies was evaluated in the GIA assay using the day 63 sera. Purified total IgG from individual rats was first evaluated in the GIA assay against *Plasmodium falciparum 3D7*, which contains the same AMA1 and RON2 sequences used for design and vaccination. Antibodies from all groups, except the adjuvant only group, showed potent GIA in the range of 60%-81% against *Plasmodium falciparum 3D7* **(FIG. 5D)**. This demonstrates that the immunogens elicit a potent inhibitory antibody response similar to AMA1 DI-DII and AMA1 DI-DII-RON2L complexes despite having drastically different RON2L *in vitro* blocking activity. We measured the IC_{50} of pooled IgG from each group to further quantify the GIA elicited by the immunogens. All groups had potent GIA against *Plasmodium falciparum 3D7*, and there were insignificant differences in IC_{50} of AMA1 DI-DII (p=1.000) and SBD1 immunogen (p=0.815), when compared to AMA1 DI-DII-RON2L complex **(FIG. 6A, D, FIG. 11A)**. However, the median IC_{50} value elicited by SBD1 immunogen was approximately 1.5-2-fold more potent than insertion fusion immunogen 2 and insertion fusion immunogen 3 **(FIG. 6A, D, FIG. 11A)**.

[0237] Strikingly, when the same pooled IgGs were tested for strain-transcending GIA with *Plasmodium falciparum FVO* **(FIG. 6B, E, FIG. 11B)** and *Plasmodium falciparum Dd2* **(FIG. 6C, F, FIG. 11C)**, SBD1 outperformed all groups, including the AMA1 DI-DII-RON2L complex group (p<0.001). The results suggest that either the local structural changes in domain I loops **(FIG. 8B, C and 12A, B)** disrupt strain-transcending epitopes or that the epitopes within the disrupted DIf loop along with other conserved domain I loops are important for broad protection **(FIG. 12A, B)**. These results indicate the presence of functional epitopes on AMA1 DI-DII outside of its hydrophobic groove and DII loop that induced strain-transcending antibody responses that can neutralize diverse strains of malaria parasites.

[0238] Having described the invention in detail and by reference to specific embodiments thereof, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims. More specifically, although some aspects of the present invention are identified herein as particularly advantageous, it is contemplated that the present invention is not necessarily limited to these particular aspects of the invention.

SEQUENCES

>SEQ ID NO:01 Full-length Pf AMA1 sequence
MRKLYCVLLLSAFEFTYMINFGRGQNYWEHPYQNSDVYRPINEHREHPKEYEYPLHQEHTYQQEDSGEDENTL QHAYPIDHEGAEPAPQEQNLFSSIEIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPS GKCPVFGKGIIIENSNTTFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSR HAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYTYLSK NVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTDYEKIKE GFKNKNASMIKSAFLPTGAFKADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNSSYIATTALSHPIEVE NNFPCSLYKDEIMKEIERESKRIKLNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSTCRFFVCKCVE RRAEVTSNNEVVVKEEYKDEYADIPEHKPTYDKMKIIIASSAAVAVLATILMVYLYKRKGNAEKYDKMDEPQD YGKSNSRNDEMLDPEASFWGEEKRASHTTPVLMEKPYY
>SEQ ID NO:02 Pf AMA1_117-541 - possible first peptide range
YDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTTFLTPVATGNQYLKDGGFAFPPTEPL MSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRY CNKDESKRNSMFCFRPAKDISFQNYTYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLF ECNKLVFELSASDQPKQYEQHLTDYEKIKEGFKNKNASMIKSAFLPTGAFKADRYKSHGKGYNWGNYNTETQK CEIFNVKPTCLINNSSYIATTALSHPIEVENNFPCSLYKDEIMKEIERESKRIKLNDNDDEGNKKIIAPRIFI SDDKDSLKCPCDPEMVSNSTCRFFVCKCVERRAEVTSNNEVVVKEEYKDEYADIPEHKPT
>SEQ ID NO:03 Pf AMA1_25-541 - possible second peptide range
QNYWEHPYQNSDVYRPINEHREHPKEYEYPLHQEHTYQQEDSGEDENTLQHAYPIDHEGAEPAPQEQNLFSSI EIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTTFLTPVA TGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDK DKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYTYLSKNVVDNWEKVCPRKNLQNAKFGLWV DGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTDYEKIKEGFKNKNASMIKSAFLPTGAFKADR YKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNSSYIATTALSHPIEVENNFPCSLYKDEIMKEIERESKRIK LNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSTCRFFVCKCVERRAEVTSNNEVVVKEEYKDEYADI PEHKPT
>SEQ ID NO:04 Full-length Pf RON2 sequence
MLKFFIFILHIYLYIDSIYSSELSKHVKHDTDELKYASPTYDPKKGTKVIFYMPGNEQGVIPNNIQNKQHGTS IYPAIEYPTTKYPAIEYPGSEMNNGKTGTTNSGIYNKAHGSSNDYNASNSQDKTINLHIDENNSNNSYQPYDS NNTNNNTNSNNNSNNNSNNNSNNNSNNYSNNNSNTNSNTNSNTNSNNNTTNYDLNSEYEKIRRKEEEAARRIE

RERRADINRKNQDNNSNKYNNEQNGGYESDGNSPNSRVNINITNNGTHGNPHNNNSYGHKNNMHNTTNGNYAN
GNYANGNYSKGDYTNGDYTNGDYTNGDYTNGINNNMHGKNNYNTANGEYVNGAYDGLNNGSYKLIGNLNNNQN
VDNSYNQGNENDKTYTSNYNINLEDNKNSPDNNQNYISTFNKDIGPNKSERSYYDVYGREYDDNKYNPYNKTN
NHNTNQNGSTTYGSNANGTYGPNGTYGSNGTYEPNESYGPNGAYGPNGTYGHNGKYGSKGTYGNNETPYYVEH
PEYDNGKSMSDFHVKDSKDNIGPGGDYPNLYQNIYGNEKNPNIFPGSPRNINVYSVHHIPNNGANGGLNSGAN
GGLNNGANDGLNNGANGGLNNGANGGLNNGANGGLNNGMNNGMNNGMNNGMNNGMNNGMNNGMNNGTNGGLNN
GMNNGMNNGMNNGMNNGIHDDLYNSENSTFNNGLNNSGRTGLNNAYPHNGMLNNGTEYNVHYGNSDSNNTNDS
MLNENYYSDSDYDDHTPGNKKKVYKSVAERNKKSASQDSLGAGFSDSDSDSEYEVVDGENKKYKKKNKENNEK
DKYENNWDSNNYNSDNEIKDGYLSESEREYARNKANEIEDKMKKGEYSRKYKNSKSNESGYASKQTSDSDDSD
IEANAFYVDNGQEMLIKEKEHYSSDSEHHKEESASIGNLNVFFPAENYHFSTYMGFDRRSFLPSNEIELEKMI
GANFSNEVKNYCSRQNVAQKIGDYLNISFEYSRALEELRSEMILDFNKRKHLTNNTDDTILHMIENAEKRKND
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KSLASSTIDFNYEIHMASASNIYLLAHFLVLSLAYLSYNEYFTTGTKSFYSLPTILTANSDNSFFMLNEMCNI
HYNPNKNFKKDITFIPIESRPKRTTTFYGERRLTCDLLELVLNAIMLININEINNVFSNNNVDGYENSLSFSH
NAIRIFSKVCPKINNDNVLKCEFEESSLYNPKIIKNDTSEKSSQKNLKKAFDLLRTYAEIEGHSAEGSTSPYY
VSLILDDMKYNDFYKYTLWYEPRELIYGDIRGMQMKKKKKTKYIYNDFMKKSTQLKKKLIKNDLKYNLKSKGL
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FAEYADNLMAKWFSKGFEEYKREQIENFKMENSIDSELKDSEREDENDSSEESAKKKLQDLQLEEREKMKKEN
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LSQMFYMQAPYFGHFIGKWQKKRQQSRLKEIMSFMTLGSLSAYTLFSAMDITQQAKDIGAGPVASCFTTRMSP
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RON2L sequences
SEQ ID NO:5 PF3D7_1452000 RON2L: 2021-2059
DITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS
SEQ ID NO:6 >Pf7G8-2_000525800
DITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS
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DITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS
SEQ ID NO:8 >PfCD01_140057400
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SEQ ID NO:9 >PfDd2_140056400
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SEQ ID NO:10 >PfGA01_140057500
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SEQ ID NO:11 >PfGB4_140058100
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SEQ ID NO:12 >PfGN01_140057300
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SEQ ID NO:13 >PfHB3_140057700
DITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS
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SEQ ID NO:15 >PfKE01_140056900
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SEQ ID NO:19 >PfNF135_140056100
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SEQ ID NO:21 >PfNF54_140055700
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SEQ ID NO:22 >PfSD01_140055300
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SEQ ID NO:24 >PfTG01_140057300
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SEQ ID NO:25 >PF3D7_1452000_RON2L
DITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS
SEQ ID NO:26 >PvSal1_RON2L
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SEQ ID NO:27 >TgSporo_RON2L
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SEQ ID NO:28 >Tg_RON2L
DIVQHMEDIGGAPPVSCVTNEILGVTCAPQAIAKATT
SEQ ID NO:29 >Bb_RON2L
DIAQHAADVGVGPAESCFIMVKPPALHCVLKPVETLMK
SEQ ID NO:30 >NcLIV_RON2L
DIVQHMEDIGGAPPASCVTNEILGVTCAPQAIAKAT
SEQ ID NO:31 PBANKA_1315700 RON2L: 1913- DITQHATDIGMGPSTSCYTSLVPPPKSICIQQTVKAVLT-1951
SEQ ID NO:32 PYYM_1316500 RON2L: 1738- DITQHATDIGMGPSTSCYTSLLPPPKSICIQQTVKTVLT-1776
SEQ ID NO:33 NcRON2L: 1296-DIVQHMEDIGGAPPASCVTNEILGVTCAPQAIAKATT-1332
SEQ ID NO:26 PvSal1 RON2L: 2034-MDISQHATDIGMGPATSCYTSTIPPPKQVCIQQAVKATLT- 2072 (PDB: 5NQG)

SEQ ID NO:27 TgSporo RON2L: 999-DIAQFLTDSGMKAIEDCSWNPIMQQMACVVVAGSGSL-1035 (PDB: 3ZLD)
SEQ ID NO:28 Tg RON2L: 1297-DIVQHMEDIGGAPPVSCVTNEILGVTCAPQAIAKATT-1333 (PDB: 2Y8T)
SEQ ID NO:29 BbRON2L: 1211-DIAQHAADVGVGPAESCFIMVKPPALHCVLKPVETLMK-1248 (UniProt: A0A3G5AQB4)
SBD-1 immunogen sequences for Plasmodium and Toxoplasma parasites
All N-linked glycosylation sites (NXS/T) were modified by substituting the serine or threonine residue with an alanine residue to prevent the glycosylation that is absent in the endogenous P. falciparum proteins.
Sequences of immunogens and constructs used in the study after signal peptide cleavage. Cloning scars, tags, and linkers are shown in lowercase.
Native AMA1 DI-DII etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTAFLT PVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVY DDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFG LWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTDYEKIKEGFKNKNAAMIKSAFLPTGAFK ADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVEgtkhhhhhh (SEQ ID No:34)
AMA1 DI-DII ΔDII-loop (AMA1 DI-DII with deleted DII loop) etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTAFLT PVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVY DDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFG LWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPGGSGYKSHGKGYNWGNYNTETQKCEIFNVKPTCLIN NAAYIATTALSHPIEVEgtkhhhhhh(SEQ ID No:35)
SBD1 immunogen etgKADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVEggggsggggsg gggsggggsNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTAFLT PVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVY DDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFG LWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTTQQAKDIGAGPVASCFTTRMSPPQQICL NSVVNTALSgtkhhhhhh (SEQ ID No:36)
Insertion fusion immunogen 2 etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTAFLT PVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVY DDKDKKCHILYIAAQENNGggggsgsDITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALSggsgMFCF RPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQ PggsgYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVEgtkhhhhhh (SEQ ID No:37)
Insertion fusion immunogen 3 etgNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTAFLT PVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVY DDKDKKCHILYIAAQENNGPRYCNGggggsggggsDITQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS ggsgMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLV FELSASDQPggsgYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVEgtkhhhhhh (SEQ ID No:38)
AMA1 ectodomain etgQNYWEHPYQNSDVYRPINEHREHPKEYEYPLHQEHTYQQEDSGEDENTLQHAYPIDHEGAEPAP QEQNLFSSIEIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENS

NTTFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNY KYPAVYDDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYTYLSKNVVDNWEKVCPRKNL QNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTDYEKIKEGFKNKNASMIKSAFL PTGAFKADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNSSYIATTALSHPIEVENNFPCSLYKDEIMKE IERESKRIKLNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSTCRFFVCKCVERRAEVTSNNEVVVKE EYKDEYADIPEHKPTgtggsggsglndifeaqkiewhegrtkhhhhhh (SEQ ID No:39)
Parasite and strain: Plasmodium falciparum 3D7
The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4) for the following sequence.
> SBD1-AMA1D1-D3_Pf3D7
KADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVENNFPCSLYKDEIMKEIERES KRIKLNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSACRFFVCKCVERRAEVTSNNEVVVKEEYKDE YGGGGSGGGGSGGGGSGGGGSEIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKC PVFGKGIIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAG NMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVV DNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTTQQAKDIGAG PVASCFTTRMSPPQQICLNSVVNTALS (SEQ ID No:40)
The fusion immunogen consists of RON2L peptide and domains I and II of AMA1. The linker length (G4S) is of 15 amino acid residues (3x G4S) for the following sequence.
>TDAMA1018A
KADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVEGGGGSGGGGSGGGGSNYMGN PWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGKGIIIENSNTAFLTPVATGNQYLKDGGF AFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAA QENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVN EFPAIDLFECNKLVFELSASDQPKQYEQHLTTQQAKDIGAGPVASCFTTRMSPPQQICLNSVVNTALS (SEQ ID No:41)
The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 15 amino acid residues (3x G4S) for the following sequence.
>TDAMA1021A
KADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVENNFPCSLYKDEIMKEIERES KRIKLNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSACRFFVCKCVERRAEVTSNNEVVVKEEYKDE YGGGGSGGGGSGGGGSEIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLPSGKCPVFGK GIIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCSRHAGNMIPD NDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLSKNVVDNWEK VCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTTQQAKDIGAGPVASC FTTRMSPPQQICLNSVVNTALS (SEQ ID No:42)
The fusion immunogen consists of RON2L peptide and full-length ectodomain of AMA1. The linker length (G4S) is of 15 amino acid residues (3x G4S) for the following sequence.
>TDAMA1023A
KADRYKSHGKGYNWGNYNTETQKCEIFNVKPTCLINNAAYIATTALSHPIEVENNFPCSLYKDEIMKEIERES KRIKLNDNDDEGNKKIIAPRIFISDDKDSLKCPCDPEMVSNSACRFFVCKCVERRAEVTSNNEVVVKEEYKDE YADIPEHKPTGGGGSGGGGSGGGGSQNYWEHPYQNSDVYRPINEHREHPKEYEYPLHQEHTYQQEDSGEDENT LQHAYPIDHEGAEPAPQEQNLFSSIEIVERSNYMGNPWTEYMAKYDIEEVHGSGIRVDLGEDAEVAGTQYRLP SGKCPVFGKGIIIENSNTAFLTPVATGNQYLKDGGFAFPPTEPLMSPMTLDEMRHFYKDNKYVKNLDELTLCS RHAGNMIPDNDKNSNYKYPAVYDDKDKKCHILYIAAQENNGPRYCNKDESKRNSMFCFRPAKDISFQNYAYLS KNVVDNWEKVCPRKNLQNAKFGLWVDGNCEDIPHVNEFPAIDLFECNKLVFELSASDQPKQYEQHLTTQQAKD IGAGPVASCFTTRMSPPQQICLNSVVNTALS (SEQ ID No:43)
Parasite and strain: Plasmodium berghei ANKA

The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
> SBD1-AMA1D1-D3_PbANKA
KSDQIKSHGRGYNWGNYDSQNKKCYIFETKPTCLINDRNFIATTALSSTEEFEEQFPCDIYKNKINEEIKVLN KNIANGNNAIEFPRIFISTDKNSLNCPCEPTQLTESSCNFYVCNCVEKRQYIAENNDVEIKEEFRSEYGGGGS GGGGSGGGGSGGGGSENTERSTKLINPWEKYMEKYDIEKMHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKG ITIQNSEVSFLTPVATGDQSVRSGGLALPKTDVHLSPITIDNLKTMYKEHPEIVKLNNMALCAKHTSFYVPGN NANSAYRHPAVYDKSNSACYMLYVAAQENMGPRYCSNNANNDNQPFCFTPEKIEKYKNLAYLTKNLRDDWETS CPNKAIKNAKFGIWVDGYCKDYQKHTVHDSDSLLKCNQIIFNEAASDQPKQYEKHLEDITQHATDIGMGPSTS CYTSLVPPPKSICIQQTVKAVLT (SEQ ID No:44)
The fusion immunogen consists of RON2L peptide and domains I and II of AMA1. The linker length (G4S) is of 20 amino acid residues (3x G4S) for the following sequence.
> SBD1-AMA1D1-D2_PbANKA
KSDQIKSHGRGYNWGNYDSQNKKCYIFETKPTCLINDRNFIATTALSSTEEFEGGGGSGGGGSGGGGSGGGGS TKLINPWEKYMEKYDIEKMHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLTPVATGDQSV RSGGLALPKTDVHLSPITIDNLKTMYKEHPEIVKLNNMALCAKHTSFYVPGNNANSAYRHPAVYDKSNSACYM LYVAAQENMGPRYCSNNANNDNQPFCFTPEKIEKYKNLAYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCKD YQKHTVHDSDSLLKCNQIIFNEAASDQPKQYEKHLEDITQHATDIGMGPSTSCYTSLVPPPKSICIQQTVKAV LT (SEQ ID No:45)
The native AMA1 consists of domains I, II, and III.
>PbAMA1_D1-D3_PbANKA
TKLINPWEKYMEKYDIEKMHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLTPVATGDQSV RSGGLALPKTDVHLSPITIDNLKTMYKEHPEIVKLNNMALCAKHTSFYVPGNNANSAYRHPAVYDKSNSACYM LYVAAQENMGPRYCSNNANNDNQPFCFTPEKIEKYKNLAYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCKD YQKHTVHDSDSLLKCNQIIFNEAASDQPKQYEKHLEDTTKFRQGVAERNGKLIGEALLPIGSYKSDQIKSHGR GYNWGNYDSQNKKCYIFETKPTCLINDRNFIATTALSSTEEFEEQFPCDIYKNKINEEIKVLNKNIANGNNAI EFPRIFISTDKNSLNCPCEPTQLTESSCNFYVCNCVEKRQYIAENNDVEIKEEFRSEYESPS (SEQ ID No:46)
The native AMA1 consists of domains I and II.
>PbAMA1_D1-D2_PbANKA
TKLINPWEKYMEKYDIEKMHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLTPVATGDQSV RSGGLALPKTDVHLSPITIDNLKTMYKEHPEIVKLNNMALCAKHTSFYVPGNNANSAYRHPAVYDKSNSACYM LYVAAQENMGPRYCSNNANNDNQPFCFTPEKIEKYKNLAYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCKD YQKHTVHDSDSLLKCNQIIFNEAASDQPKQYEKHLEDTTKFRQGVAERNGKLIGEALLPIGSYKSDQIKSHGR GYNWGNYDSQNKKCYIFETKPTCLINDRNFIATTALSSTEEFE (SEQ ID No:47)
The native AMA1 consists of full-length ectodomain.
>PbAMA1_ectodomain_PbANKA
SEGPNNVISENGHINYDMIQKENTERSTKLINPWEKYMEKYDIEKMHGSGIRVDLGEDARVENRDYRIPSGKC PVIGKGITIQNSEVSFLTPVATGDQSVRSGGLALPKTDVHLSPITIDNLKTMYKEHPEIVKLNNMALCAKHTS FYVPGNNANSAYRHPAVYDKSNSACYMLYVAAQENMGPRYCSNNANNDNQPFCFTPEKIEKYKNLAYLTKNLR DDWETSCPNKAIKNAKFGIWVDGYCKDYQKHTVHDSDSLLKCNQIIFNEAASDQPKQYEKHLEDTTKFRQGVA ERNGKLIGEALLPIGSYKSDQIKSHGRGYNWGNYDSQNKKCYIFETKPTCLINDRNFIATTALSSTEEFEEQF PCDIYKNKINEEIKVLNKNIANGNNAIEFPRIFISTDKNSLNCPCEPTQLTESSCNFYVCNCVEKRQYIAENN DVEIKEEFRSEYESPS (SEQ ID No:48)
Parasite: Plasmodium yoelii
The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
>SBD1-PyAMA1D1-D3_01

KSGQIKSHGKGYNWGNYDSKNNKCYIFETKPTCLINDKNFIATTALSSTEEFEENFPCEIYKNKIAEEIKVLN LNQNTANGNNAIKFPRIFISTDKNSLNCPCDPTKLTESTCEFYVCSCVEQRQYIAENNDVIIKEEFIGDYENP KQKGGGGSGGGGSGGGGSGGGGSENTERSIKLINPWDKYMEKYDIEKVHGSGIRVDLGEDARVENRDYRIPSG KCPVIGKGITIQNSEVSFLKPVATGDKPVRSGGLAFPETDVHISPITITNLKTMYKDHQDIVNLNDMSLCAKH TSLYVPGKDATSAYRHPVVYDKSNSACYMLYVAAQENMGPRYCSNDANNENQPFCFTPEKIENYKDLSYLTKN LRDDWETSCPNKAIKNAKFGIWVDGYCTDYQKHVVHDSDSLLKCNQIIFNEAASDQPKQYERHLETQHATDIG MGPSTSCYTSLLPPPKSICIQQTVKTVLT (SEQ ID No:49)
The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence. This immunogen has a slightly longer RON2L.
>SBD1-PyAMA1D1-D3_02
KSGQIKSHGKGYNWGNYDSKNNKCYIFETKPTCLINDKNFIATTALSSTEEFEENFPCEIYKNKIAEEIKVLN LNQNTANGNNAIKFPRIFISTDKNSLNCPCDPTKLTESTCEFYVCSCVEQRQYIAENNDVIIKEEFIGDYENP KQKGGGGSGGGGSGGGGSGGGGSENTERSIKLINPWDKYMEKYDIEKVHGSGIRVDLGEDARVENRDYRIPSG KCPVIGKGITIQNSEVSFLKPVATGDKPVRSGGLAFPETDVHISPITITNLKTMYKDHQDIVNLNDMSLCAKH TSLYVPGKDATSAYRHPVVYDKSNSACYMLYVAAQENMGPRYCSNDANNENQPFCFTPEKIENYKDLSYLTKN LRDDWETSCPNKAIKNAKFGIWVDGYCTDYQKHVVHDSDSLLKCNQIIFNEAASDQPKQYERHLEDITQHATD IGMGPSTSCYTSLLPPPKSICIQQTVKTVLTNSTLASMK (SEQ ID No:50)
The fusion immunogen consists of RON2L peptide and domains I and II of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
>SBD1-PyAMA1D1-D2_01
KSGQIKSHGKGYNWGNYDSKNNKCYIFETKPTCLINDKNFIATTALSSTEEFEGGGGSGGGGSGGGGSGGGGS IKLINPWDKYMEKYDIEKVHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLKPVATGDKPV RSGGLAFPETDVHISPITITNLKTMYKDHQDIVNLNDMSLCAKHTSLYVPGKDATSAYRHPVVYDKSNSACYM LYVAAQENMGPRYCSNDANNENQPFCFTPEKIENYKDLSYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCTD YQKHVVHDSDSLLKCNQIIFNEAASDQPKQYERHLETQHATDIGMGPSTSCYTSLLPPPKSICIQQTVKTVLT (SEQ ID No:51)
The fusion immunogen consists of RON2L peptide and domains I and II of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence. This immunogen has a slightly longer RON2L.
>SBD1-PyAMA1D1-D2_02
KSGQIKSHGKGYNWGNYDSKNNKCYIFETKPTCLINDKNFIATTALSSTEEFEGGGGSGGGGSGGGGSGGGGS IKLINPWDKYMEKYDIEKVHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLKPVATGDKPV RSGGLAFPETDVHISPITITNLKTMYKDHQDIVNLNDMSLCAKHTSLYVPGKDATSAYRHPVVYDKSNSACYM LYVAAQENMGPRYCSNDANNENQPFCFTPEKIENYKDLSYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCTD YQKHVVHDSDSLLKCNQIIFNEAASDQPKQYERHLEDITQHATDIGMGPSTSCYTSLLPPPKSICIQQTVKTV LTNSTLASMK (SEQ ID No:52)
The native AMA1 consists of domains I, II, and III.
>PyAMA1_D1-D3
IKLINPWDKYMEKYDIEKVHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLKPVATGDKPV RSGGLAFPETDVHISPITITNLKTMYKDHQDIVNLNDMSLCAKHTSLYVPGKDATSAYRHPVVYDKSNSACYM LYVAAQENMGPRYCSNDANNENQPFCFTPEKIENYKDLSYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCTD YQKHVVHDSDSLLKCNQIIFNEAASDQPKQYERHLEDATKIRQGIVERNGKLIGEALLPIGSYKSGQIKSHGK GYNWGNYDSKNNKCYIFETKPTCLINDKNFIATTALSSTEEFEENFPCEIYKNKIAEEIKVLNLNQNTANGNN AIKFPRIFISTDKNSLNCPCDPTKLTESTCEFYVCSCVEQRQYIAENNDVIIKEEFIGDYENPKQK (SEQ ID No:53)
The native AMA1 consists of domains I and II.
>PyAMA1_D1-D2
IKLINPWDKYMEKYDIEKVHGSGIRVDLGEDARVENRDYRIPSGKCPVIGKGITIQNSEVSFLKPVATGDKPV RSGGLAFPETDVHISPITITNLKTMYKDHQDIVNLNDMSLCAKHTSLYVPGKDATSAYRHPVVYDKSNSACYM LYVAAQENMGPRYCSNDANNENQPFCFTPEKIENYKDLSYLTKNLRDDWETSCPNKAIKNAKFGIWVDGYCTD YQKHVVHDSDSLLKCNQIIFNEAASDQPKQYERHLEDATKIRQGIVERNGKLIGEALLPIGSYKSGQIKSHGK GYNWGNYDSKNNKCYIFETKPTCLINDKNFIATTALSSTEEFE (SEQ ID No:54)

Parasite and strain: Plasmodium vivax Sal1
The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
> SBD1-AMA1D1-D3_PvSal1
NSDNFKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDLEFPCSIYKDEIEREIKKQS RNMNLYSVDGERIVLPRIFISNDKESIKCPCEPERISNSACNFYVCNCVEKRAEIKENNQVVIKEEFRDYYGG GGSGGGGSGGGGSGGGGSPTVERSTRMSNPWKAFMEKYDIERTHSSGVRVDLGEDAEVENAKYRIPAGRCPVF GKGIVIENSDVSFLRPVATGDQKLKDGGFAFPNANDHISPMTLANLKERYKDNVEMMKLNDIALCRTHAASFV MAGDQNSAYRHPAVYDEKEKTCHMLYLSAQENMGPRYCSPDAQNRDAVFCFKPDKNEAFENLVYLSKNVRNDW DKKCPRKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMDISQHATDIGMGPA TSCYTSTIPPPKQVCIQQAVKATLT (SEQ ID No:55)
The fusion immunogen consists of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
> SBD1-AMA1D1-D2_PvSal1
NSDNFKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDGGGGSGGGGSGGGGSGGGGS PTVERSTRMSNPWKAFMEKYDIERTHSSGVRVDLGEDAEVENAKYRIPAGRCPVFGKGIVIENSDVSFLRPVA TGDQKLKDGGFAFPNANDHISPMTLANLKERYKDNVEMMKLNDIALCRTHAASFVMAGDQNSAYRHPAVYDEK EKTCHMLYLSAQENMGPRYCSPDAQNRDAVFCFKPDKNEAFENLVYLSKNVRNDWDKKCPRKNLGNAKFGLWV DGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMDISQHATDIGMGPATSCYTSTIPPPKQVCIQQ AVKATLT (SEQ ID No:56)
The native AMA1 consists of domains I, II, and III.
>PvAMA1_D1-D3_PvSal1
PTVERSTRMSNPWKAFMEKYDIERTHSSGVRVDLGEDAEVENAKYRIPAGRCPVFGKGIVIENSDVSFLRPVA TGDQKLKDGGFAFPNANDHISPMTLANLKERYKDNVEMMKLNDIALCRTHAASFVMAGDQNSAYRHPAVYDEK EKTCHMLYLSAQENMGPRYCSPDAQNRDAVFCFKPDKNEAFENLVYLSKNVRNDWDKKCPRKNLGNAKFGLWV DGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDN FKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDLEFPCSIYKDEIEREIKKQSRNMN LYSVDGERIVLPRIFISNDKESIKCPCEPERISNSACNFYVCNCVEKRAEIKENNQVVIKEEFRDYY (SEQ ID No:57)
The native AMA1 consists of domains I and II.
>PvAMA1_D1-D2_PvSal1
NPWKAFMEKYDIERTHSSGVRVDLGEDAEVENAKYRIPAGRCPVFGKGIVIENSDVSFLRPVATGDQKLKDGG FAFPNANDHISPMTLANLKERYKDNVEMMKLNDIALCRTHAASFVMAGDQNSAYRHPAVYDEKEKTCHMLYLS AQENMGPRYCSPDAQNRDAVFCFKPDKNEAFENLVYLSKNVRNDWDKKCPRKNLGNAKFGLWVDGNCEEIPYV KEVEAEDLRECNRIVFGASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNW ANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVD (SEQ ID No:58)
Parasite and strain: Toxoplasma gondii
The fusion immunogens consist of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
>SBD1-TgAMA1D1-D3_01
QSDQPHSGGVGRNYGFYYVDTTGEGKCALSDQVPDCLVSDSAAVSYTAAGSLSEETPNFIIPSNPAVTPPTPE TALQCTADKFPDSFGACDVQACKRQKTSCVGGQIQSTSVDCTADEQNECGGGGSGGGGSGGGGSGGGGSGNPF QANVEMKTFMERFNLAHHHQSGIYVDLGQDKEVDGTLYREPAGLCPIWGKHIELQQPDRPPYRNNFLEDVPTE KEYKQSGNPLPGGFNLNFVTPSGQRISPFPMELLEKNSNIKASTDLGRCAEFAFKTVAMDKNNKATKYRYPFV YDSKKRLCHILYVSMQLMEGKKYCSVKGEPPDLTWYCFKPRKSVTENHHLIYGSAYVGENPDAFISKCPNQAL RGYRFGVWKKGRCLDYTELTDTVIERVESKAQCWVKTFENDGVASDQPHTYPLDIVQHMEDIGGAPPVSCVTN EILGVTCAPQAIAKATT (SEQ ID No:59)
The fusion immunogens consist of RON2L peptide and domains I, II, and III of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S)

for the following sequence. The fusion immunogen contains five additional residues.
>SBD1-TgAMA1D1-D3_02
QSDQPHSGGVGRNYGFYYVDTTGEGKCALSDQVPDCLVSDSAAVSYTAAGSLSEETPNFIIPSNPAVTPPTPE TALQCTADKFPDSFGACDVQACKRQKTSCVGGQIQSTSVDCTADEQNECGSNTAGGGGSGGGGSGGGGSGGGG SASTSGNPFQANVEMKTFMERFNLAHHHQSGIYVDLGQDKEVDGTLYREPAGLCPIWGKHIELQQPDRPPYRN NFLEDVPTEKEYKQSGNPLPGGFNLNFVTPSGQRISPFPMELLEKNSNIKASTDLGRCAEFAFKTVAMDKNNK ATKYRYPFVYDSKKRLCHILYVSMQLMEGKKYCSVKGEPPDLTWYCFKPRKSVTENHHLIYGSAYVGENPDAF ISKCPNQALRGYRFGVWKKGRCLDYTELTDTVIERVESKAQCWVKTFENDGVASDQPHTYPLDIVQHMEDIGG APPVSCVTNEILGVTCAPQAIAKATT (SEQ ID No:60)
The fusion immunogens consist of RON2L peptide and domains I and II of AMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
>SBD1-TgAMA1D1-D2_01
QSDQPHSGGVGRNYGFYYVDTTGEGKCALSDQVPDCLVSDSAAVSYTAAGSLSEETGGGGSGGGGSGGGGSGG GGSGNPFQANVEMKTFMERFNLAHHHQSGIYVDLGQDKEVDGTLYREPAGLCPIWGKHIELQQPDRPPYRNNF LEDVPTEKEYKQSGNPLPGGFNLNFVTPSGQRISPFPMELLEKNSNIKASTDLGRCAEFAFKTVAMDKNNKAT KYRYPFVYDSKKRLCHILYVSMQLMEGKKYCSVKGEPPDLTWYCFKPRKSVTENHHLIYGSAYVGENPDAFIS KCPNQALRGYRFGVWKKGRCLDYTELTDTVIERVESKAQCWVKTFENDGVASDQPHTYPLDIVQHMEDIGGAP PVSCVTNEILGVTCAPQAIAKATT (SEQ ID No:61)
The native AMA1 consists of domains I and II.
>TgAMA1D1-D2
SGNPFQANVEMKTFMERFNLAHHHQSGIYVDLGQDKEVDGTLYREPAGLCPIWGKHIELQQPDRPPYRNNFLE DVPTEKEYKQSGNPLPGGFNLNFVTPSGQRISPFPMELLEKNSNIKASTDLGRCAEFAFKTVAMDKNNKATKY RYPFVYDSKKRLCHILYVSMQLMEGKKYCSVKGEPPDLTWYCFKPRKSVTENHHLIYGSAYVGENPDAFISKC PNQALRGYRFGVWKKGRCLDYTELTDTVIERVESKAQCWVKTFENDGVASDQPHTYPLTSQASWNDWWPLHQS DQPHSGGVGRNYGFYYVDTTGEGKCALSDQVPDCLVSDSAAVSYTAAGSLSEET (SEQ ID No:62)
The native AMA1 consists of domains I, II, and III.
>TgAMA1D1-D3
SGNPFQANVEMKTFMERFNLAHHHQSGIYVDLGQDKEVDGTLYREPAGLCPIWGKHIELQQPDRPPYRNNFLE DVPTEKEYKQSGNPLPGGFNLNFVTPSGQRISPFPMELLEKNSNIKASTDLGRCAEFAFKTVAMDKNNKATKY RYPFVYDSKKRLCHILYVSMQLMEGKKYCSVKGEPPDLTWYCFKPRKSVTENHHLIYGSAYVGENPDAFISKC PNQALRGYRFGVWKKGRCLDYTELTDTVIERVESKAQCWVKTFENDGVASDQPHTYPLTSQASWNDWWPLHQS DQPHSGGVGRNYGFYYVDTTGEGKCALSDQVPDCLVSDSAAVSYTAAGSLSEETPNFIIPSNPAVTPPTPETA LQCTADKFPDSFGACDVQACKRQKTSCVGGQIQSTSVDCTADEQNECGSNTA (SEQ ID No:63)
The fusion immunogen consists of RON2L peptide and domains I, II and III of SporoAMA1. The linker length (G4S) is of 25 amino acid residues (5x G4S) for the following sequence.
> SBD1-25L-TgSporoAMA1D1-D3
EQSSPKSGGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVNLPQCDSASFIPIEGPC NNCVQVVTECVGNQFDQTSKACCGGGGSGGGGSGGGGSGGGGSGGGGSGQNPWATTTAFADFMKRFNIPQVHG SGIFVDLGRDTEGYREVGGKCPVFGKAIQMHQPAEYSNNFLDDAPTSNDASKKPLPGGFNNPQVYTSGQKFSP IDDSLLQERLGTAGPKTAIGRCALYAYSTIAVNPATNYASTYKYPFVYDAVSRKCYVLSVSAQLLKGEKYCSV NGAPSGLTWACFEPVKEKSSARALVYGSAFVAEGNPDAWQSACPNDAVKDALFGKWEDGQCVPFDTKTSVQSD QATNKEECWKRVFANPLVASDAPTTYPEDIAQFLTDSGMKAIEDCSWNPIMQQMACVVVAGSGSL (SEQ ID No:64)
The fusion immunogen consists of RON2L peptide and domains I, II and III of SporoAMA1. The linker length (G4S) is of 20 amino acid residues (4x G4S) for the following sequence.
> SBD1-20L-TgSporoAMA1D1-D3
EQSSPKSGGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVNLPQCDSASFIPIEGPC NNCVQVVTECVGNQFDQTSKACCGGGGSGGGGSGGGGSGGGGSGGGGSGQNPWATTTAFADFMKRFNIPQVHGSGIFV DLGRDTEGYREVGGKCPVFGKAIQMHQPAEYSNNFLDDAPTSNDASKKPLPGGFNNPQVYTSGQKFSPIDDSL LQERLGTAGPKTAIGRCALYAYSTIAVNPATNYASTYKYPFVYDAVSRKCYVLSVSAQLLKGEKYCSVNGAPS GLTWACFEPVKEKSSARALVYGSAFVAEGNPDAWQSACPNDAVKDALFGKWEDGQCVPFDTKTSVQSDQATNK

EECWKRVFANPLVASDAPTTYPEDIAQFLTDSGMKAIEDCSWNPIMQQMACVVVAGSGSL (SEQ ID No:65)
The fusion immunogen consists of RON2L peptide and domains I, II and III of SporoAMA1. The linker length (G4S) is of 15 amino acid residues (3x G4S) for the following sequence.
> SBD1-15L-TgSporoAMA1D1-D3
EQSSPKSGGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVNLPQCDSASFIPIEGPC NNCVQVVTECVGNQFDQTSKACCGGGGSGGGGSGGGGSGQNPWATTTAFADFMKRFNIPQVHGSGIFVDLGRD TEGYREVGGKCPVFGKAIQMHQPAEYSNNFLDDAPTSNDASKKPLPGGFNNPQVYTSGQKFSPIDDSLLQERL GTAGPKTAIGRCALYAYSTIAVNPATNYASTYKYPFVYDAVSRKCYVLSVSAQLLKGEKYCSVNGAPSGLTWA CFEPVKEKSSARALVYGSAFVAEGNPDAWQSACPNDAVKDALFGKWEDGQCVPFDTKTSVQSDQATNKEECWK RVFANPLVASDAPTTYPEDIAQFLTDSGMKAIEDCSWNPIMQQMACVVVAGSGSL (SEQ ID No:66)
The fusion immunogen consists of RON2L peptide and domains I, II and III of SporoAMA1. The linker length (G4S) is of 10 amino acid residues (2x G4S) for the following sequence.
> SBD1-10L-TgSporoAMA1D1-D3
EQSSPKSGGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVNLPQCDSASFIPIEGPC NNCVQVVTECVGNQFDQTSKACCGGGGSGGGGSGQNPWATTTAFADFMKRFNIPQVHGSGIFVDLGRDTEGYR EVGGKCPVFGKAIQMHQPAEYSNNFLDDAPTSNDASKKPLPGGFNNPQVYTSGQKFSPIDDSLLQERLGTAGP KTAIGRCALYAYSTIAVNPATNYASTYKYPFVYDAVSRKCYVLSVSAQLLKGEKYCSVNGAPSGLTWACFEPV KEKSSARALVYGSAFVAEGNPDAWQSACPNDAVKDALFGKWEDGQCVPFDTKTSVQSDQATNKEECWKRVFAN PLVASDAPTTYPEDIAQFLTDSGMKAIEDCSWNPIMQQMACVVVAGSGSL (SEQ ID No:67)
The fusion immunogen consists of RON2L peptide and domains I, II and III of SporoAMA1. The linker length (G4S) is of 5 amino acid residues (1x G4) for the following sequence.
> SBD1-5L-TgSporoAMA1D1-D3
EQSSPKSGGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVNLPQCDSASFIPIEGPC NNCVQVVTECVGNQFDQTSKACCGGGGSGQNPWATTTAFADFMKRFNIPQVHGSGIFVDLGRDTEGYREVGGK CPVFGKAIQMHQPAEYSNNFLDDAPTSNDASKKPLPGGFNNPQVYTSGQKFSPIDDSLLQERLGTAGPKTAIG RCALYAYSTIAVNPATNYASTYKYPFVYDAVSRKCYVLSVSAQLLKGEKYCSVNGAPSGLTWACFEPVKEKSS ARALVYGSAFVAEGNPDAWQSACPNDAVKDALFGKWEDGQCVPFDTKTSVQSDQATNKEECWKRVFANPLVAS DAPTTYPEDIAQFLTDSGMKAIEDCSWNPIMQQMACVVVAGSGSL (SEQ ID No:68)
The native AMA1 consists of domains I and II.
>TgSporoAMA1D1-D2
GQNPWATTTAFADFMKRFNIPQVHGSGIFVDLGRDTEGYREVGGKCPVFGKAIQMHQPAEYSNNFLDDAPTSN DASKKPLPGGFNNPQVYTSGQKFSPIDDSLLQERLGTAGPKTAIGRCALYAYSTIAVNPATNYASTYKYPFVY DAVSRKCYVLSVSAQLLKGEKYCSVNGAPSGLTWACFEPVKEKSSARALVYGSAFVAEGNPDAWQSACPNDAV KDALFGKWEDGQCVPFDTKTSVQSDQATNKEECWKRVFANPLVASDAPTTYPEAAQKNWNDFWPVHEQSSPKS GGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVN (SEQ ID No:69)
The native AMA1 consists of domains I, II and III.
>TgSporoAMA1D1-D3
GQNPWATTTAFADFMKRFNIPQVHGSGIFVDLGRDTEGYREVGGKCPVFGKAIQMHQPAEYSNNFLDDAPTSN DASKKPLPGGFNNPQVYTSGQKFSPIDDSLLQERLGTAGPKTAIGRCALYAYSTIAVNPATNYASTYKYPFVY DAVSRKCYVLSVSAQLLKGEKYCSVNGAPSGLTWACFEPVKEKSSARALVYGSAFVAEGNPDAWQSACPNDAV KDALFGKWEDGQCVPFDTKTSVQSDQATNKEECWKRVFANPLVASDAPTTYPEAAQKNWNDFWPVHEQSSPKS GGFGANWANFYLEKESGETICAIFDQVPDCFAPITGAVAYTALGSSTEVNLPQCDSASFIPIEGPCNNCVQVV TECVGNQFDQTSKACC (SEQ ID No:70)

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WHAT IS CLAIMED IS:

1. A recombinant protein, comprising:
    (a) a first peptide comprising a C-teriminal portion of an apical membrane antigen 1 (AMA1) protein;
    (b) a second peptide comprising an N-terminal portion of the AMA1 protein; and
    (c) a third peptide comprising at least a portion of a rhoptry neck protein 2 (RON2) protein.

2. The recombinant protein of claim 1, wherein the recombinant protein comprises from the N-terminus to the C-terminus the first peptide, then the second peptide, and then the third peptide.

3. The recombinant protein of claim 1, wherein the recombinant protein comprises from the N-terminus to the C-terminus the third peptide, then the first peptide, and then the second peptide.

4. The recombinant protein of any one of claims 1 to 3, wherein the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein.

5. The recombinant protein of any one of claims 1 to 4, wherein the first peptide comprises about 10 amino acids to about 425 amino acids from the C-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01.

6. The recombinant protein of any one of claims 1 to 4, wherein the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:02.

7. The recombinant protein of any one of claims 1 to 4, wherein the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%,

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-438 of SEQ ID NO:01.

8. The recombinant protein of any one of claims 1 to 4, wherein the first peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 386-541 of SEQ ID NO:01.

9. The recombinant protein of any one of claims 1 to 8, wherein the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein.

10. The recombinant protein of any one of claims 1 to 9, wherein the second peptide comprises about 10 amino acids to about 520 amino acids from the N-terminus of the AMA1 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:01.

11. The recombinant protein of any one of claims 1 to 9, wherein the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to the amino acid sequence of SEQ ID NO:03.

12. The recombinant protein of any one of claims 1 to 9, wherein the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 94-358 of SEQ ID NO:01.

13. The recombinant protein of any one of claims 1 to 9, wherein the second peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to amino acids 25-358 of SEQ ID NO:01.

14. The recombinant protein of any one of claims 1 to 13, wherein the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein.

15. The recombinant protein of any one of claims 1 to 14, wherein the third peptide comprises about 25 amino acids to about 50 amino acids from the RON2 protein and shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to all or a portion of the amino acid sequence of SEQ ID NO:04.

16. The recombinant protein of any one of claims 1 to 14, wherein the third peptide shares at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:05-33.

17. The recombinant protein of any one of claims 1 to 16 further comprising a linker domain between the first peptide and the second peptide.

18. The recombinant protein of claim 17, wherein the linker domain comprises a flexible linker, for example, a G_4S linker.

19. The recombinant protein of any one of claims 1 to 18, wherein the recombinant protein comprises at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOs:36, 40-45, 49-52, 55, 56, 59-61, 64-68 and 83.

20. The recombinant protein of any one of claims 1 to 19, wherein the AMA1 protein is from the *Plasmodium* genus, the *Babesia* genus, or *Toxoplasma gondii*.

21. The recombinant protein of any one of claims 1 to 20, wherein the RON2 protein is from the *Plasmodium* genus, the *Babesia* genus, or *Toxoplasma gondii*.

22. The recombinant protein of any one of claims 1 to 21, wherein the AMA1 protein and the RON2 protein are from the same species.

23. The recombinant protein of any one of claims 1 to 22 further comprising fusion to one or more additional antigens.

24. A nucleic acid composition, comprising a nucleic acid sequence encoding the recombinant protein of any one of claims 1 to 23.

25. A vector, comprising the nucleic acid sequence of claim 24.

26. An immunotherapy composition, comprising the recombinant protein of any one of claims 1 to 23, the nucleic acid sequence of claim 24, or the vector of claim 25, and at least one adjuvant and/or a carrier.

27. The immunotherapy composition of claim 26, wherein the adjuvant is selected from the group consisting of AddaS03™, aluminum hydroxide, aluminum phosphate, aluminum sulfate, monophosphoryl lipid A (MPL), QS-21, TQL1055, QS-18, QS-17, QS-7, Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), oil in water emulsions, CpG, polyglutamic acid, polylysine, AddaVax™, MF59®, and/or combinations thereof.

28. The immunotherapy composition of either claim 26 or claim 27, wherein the carrier is selected from the group consisting of albumin, diphtheria toxoid (DT), a genetically modified cross-reacting material (CRM) of diphtheria toxin, CRM197, flagellin, *H. influenzae* protein D (HiD), immunoglobulin molecules, KLH (keyhole limpet hemocyanin), mannose-6-phosphate, meningococcal outer membrane protein complex (OMPC), ovalbumin, poly-L-lysine, poly-L-glutamine, rEPA (Pseudomonas aeruginosa exotoxin A), thyroglobulin, and tetanus toxoid (TT).

29. The immunotherapy composition of any one of claims 26 to 28, further comprising a lipid nanoparticle or a nanoparticle.

30. A method of vaccinating a subject, comprising administrating to the subject the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29.

31. A method of treating a subject with malaria or protecting a subject from malaria infection, comprising administrating to the subject the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29.

32. A method of treating a subject with toxoplasmosis or protecting a subject from toxoplasmosis infection, comprising administrating to the subject the recombinant

protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 24, or the immunotherapy composition of any one of claims 26 to 29.

33. A method of treating a subject with babesiosis or protecting a subject from babesiosis infection, comprising administrating to the subject the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29.

34. The method of any one of claims 30 to 33, wherein the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29 is administered with one or more additional active agents.

35. The method of any one of claims 30 to 33, further comprising repeating the administering at least a second time, at least a third time, at least a fourth time, at least a fifth time, or at least a sixth time.

36. An immunoglobulin that binds to the recombinant protein of any one of claims 1-23.

37. The immunoglobulin of claim 36, wherein the immunoglobulin is isolated from a subject using the recombinant protein of any one of claims 1-23, and wherein the subject has naturally acquired immunity to malaria, toxoplasmosis, or babesiosis.

38. An immunoglobulin that binds the recombinant protein of any one of claims 1-23, wherein the immunoglobulin is obtained by immunization with the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29.

39. The immunoglobulin of any one of claims 36 to 38, wherein the immunoglobulin is a monoclonal antibody or a plurality of polyclonal antibodies.

40. The immunoglobulin of any one of claims 36 to 39, wherein the immunoglobulin cross-reacts with different strains and/or subtypes of malaria.

41. The immunoglobulin of any one of claims 36 to 39, wherein the immunoglobulin cross-reacts with different strains and/or subtypes of toxoplasmosis.

42. The immunoglobulin of any one of claims 36 to 39, wherein the immunoglobulin cross-reacts with different strains and/or subtypes of babesiosis.

43. A method of generating antibodies cross-protective against malaria comprising:
(a) immunizing a subject with the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29:
(b) isolating antibodies that cross-react with different strains and/or subtypes of malaria.

44. A method of generating antibodies cross-protective against toxoplasmosis comprising:
(a) immunizing a subject with the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29:
(b) isolating antibodies that cross-react with different strains and/or subtypes of toxoplasmosis.

45. A method of generating antibodies cross-protective against babesiosis comprising:
(a) immunizing a subject with the recombinant protein of any one of claims 1-23, the nucleic acid composition of claim 24, the vector of claim 25, or the immunotherapy composition of any one of claims 26 to 29:
(b) isolating antibodies that cross-react with different strains and/or subtypes of babesiosis.

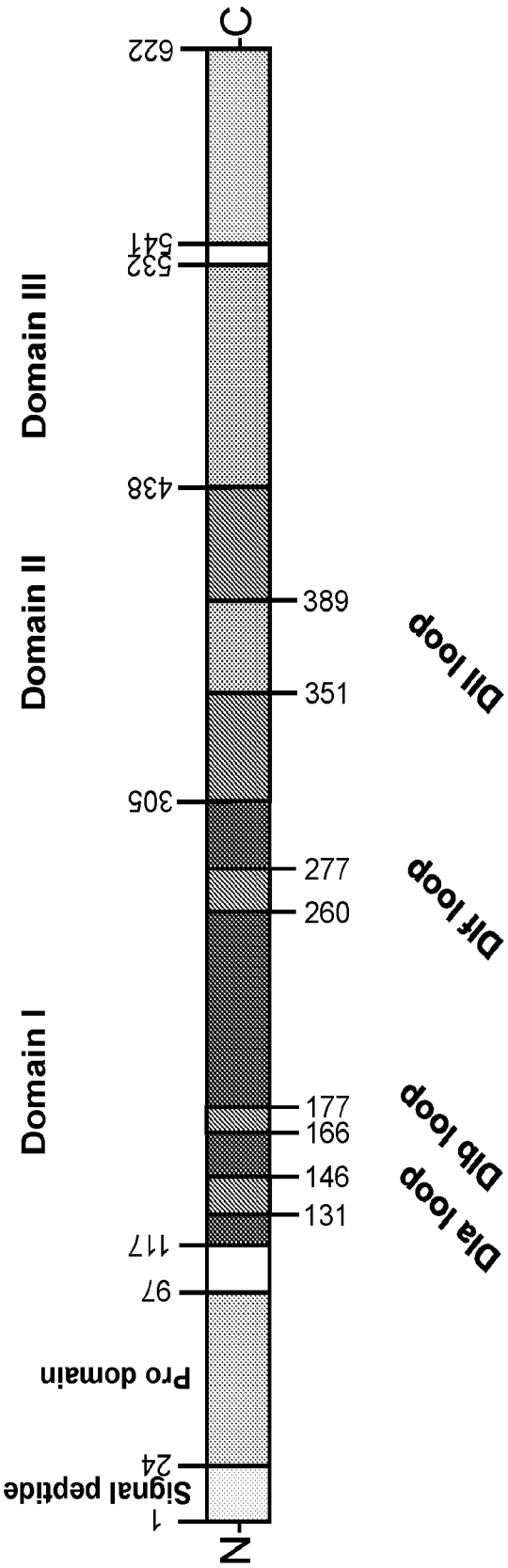
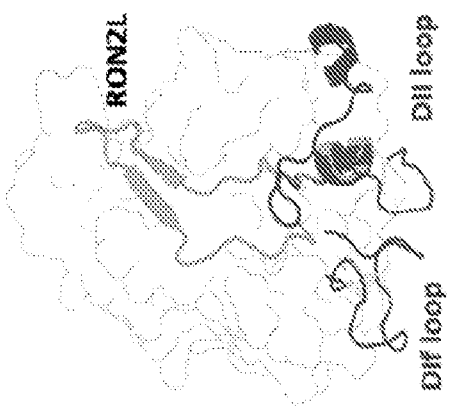

C
622
541
532
438
389
351
305
277
260
177
166
146
131
117
97
1
24
N
Domain III
Domain II
Domain I
Pro domain
Signal peptide
DII loop
DIf loop
DIb loop
DIa loop

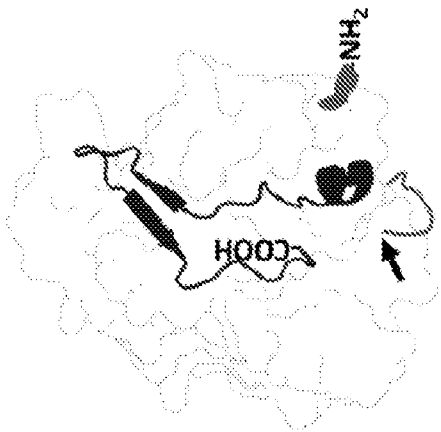
FIG. 1A

FIG. 1B



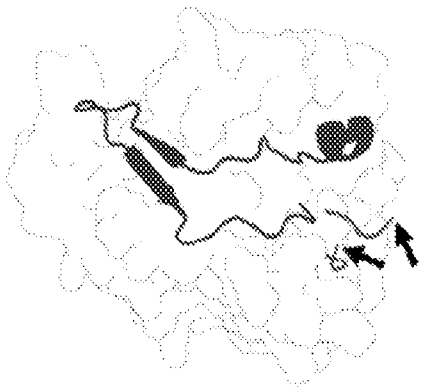
AMA1 DI-DII & RON2L

FIG. 1C



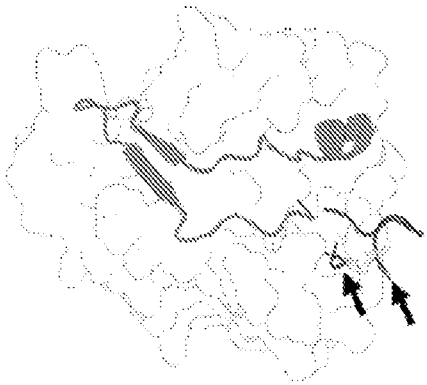
SBD1 Immunogen

FIG. 1D



Insertion fusion immunogen 2

FIG. 1E



Insertion fusion immunogen 3

FIG. 1F

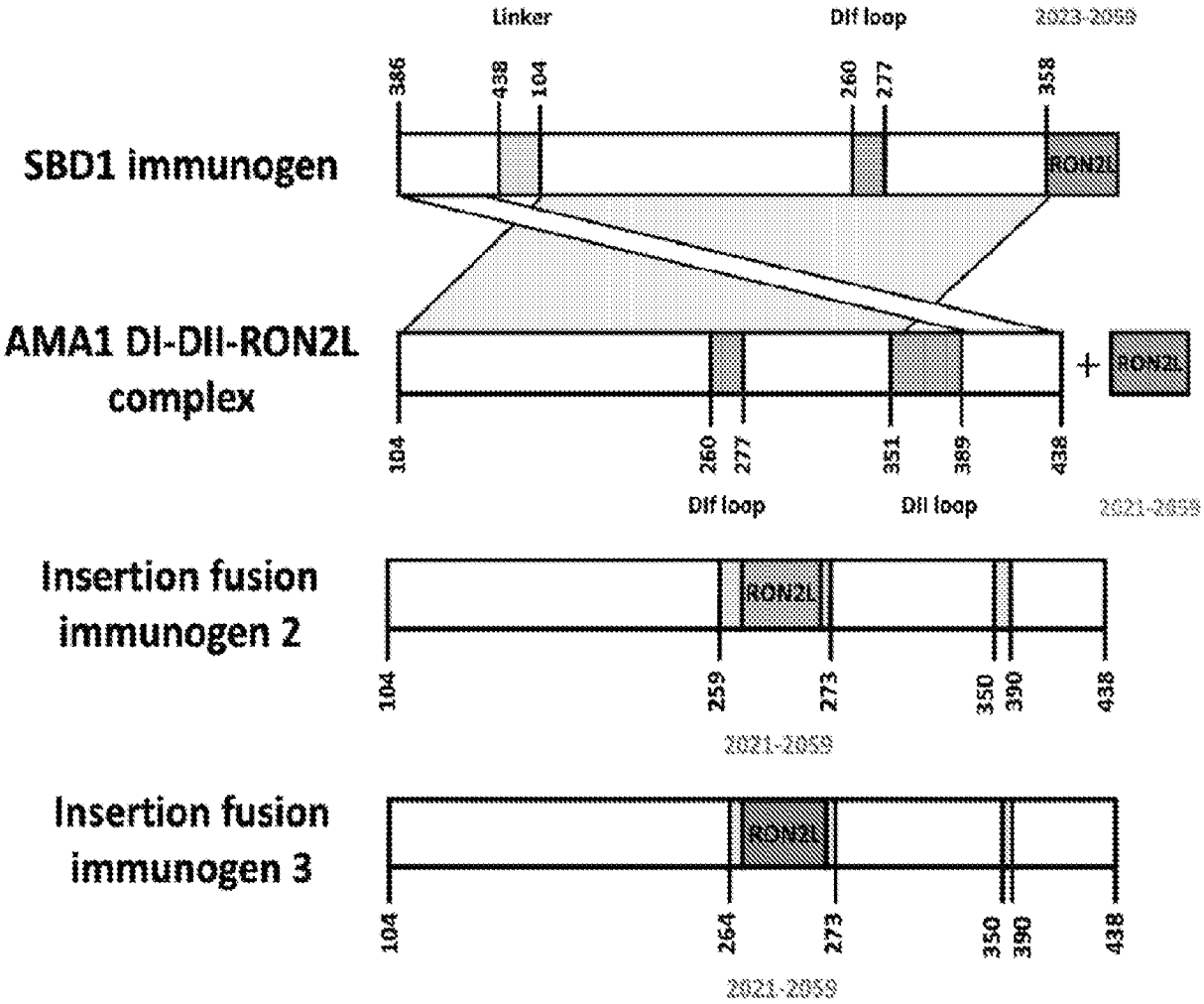

Linker
Dlf loop
2023-2059
386
438
104
260
277
358
SBD1 immunogen
RON2L
AMA1 DI-DII-RON2L complex
+
RON2L
104
260
277
351
389
438
Dlf loop
Dll loop
2021-2059
Insertion fusion immunogen 2
RON2L
104
259
273
350
390
438
2021-2059
Insertion fusion immunogen 3
RON2L
104
264
273
350
390
438
2021-2059

FIG. 2A

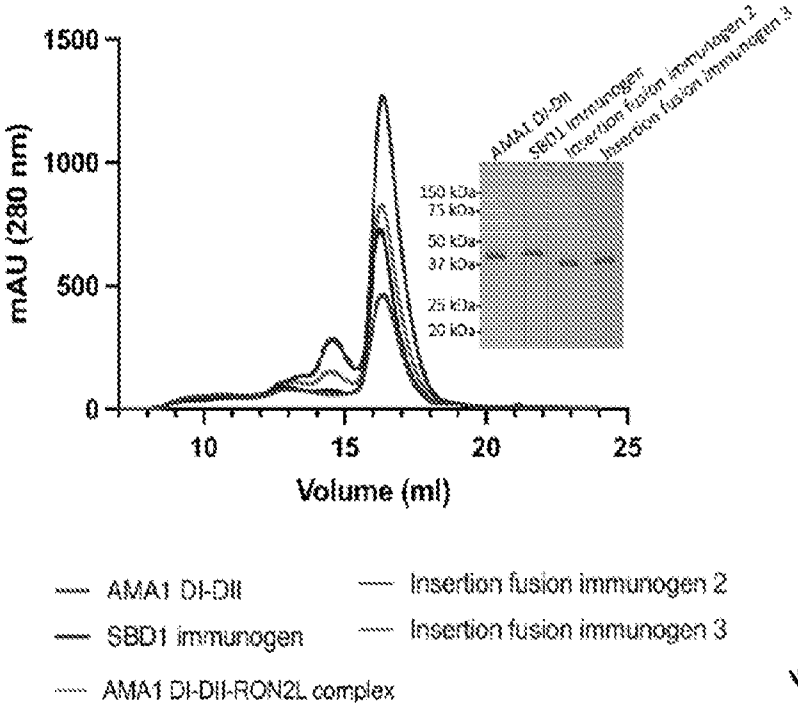


FIG. 2B

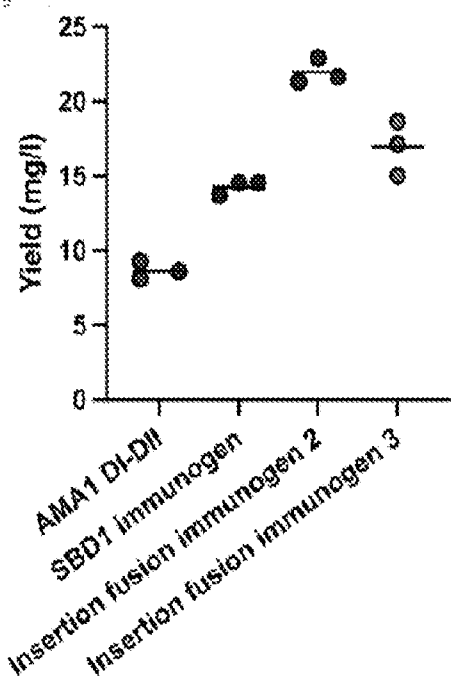


FIG. 2C

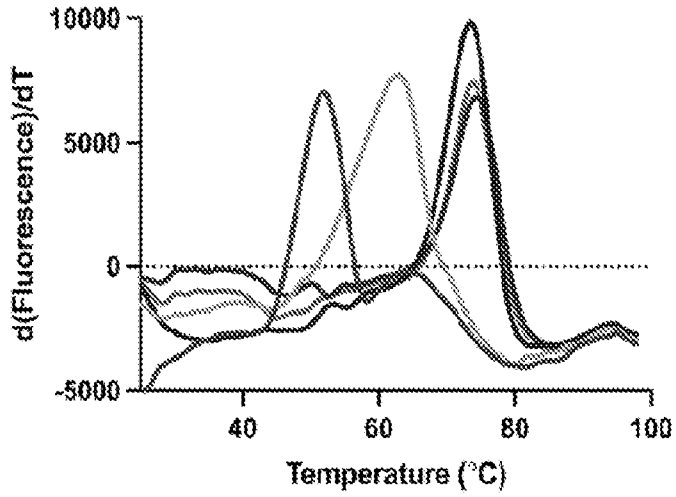


FIG. 2D

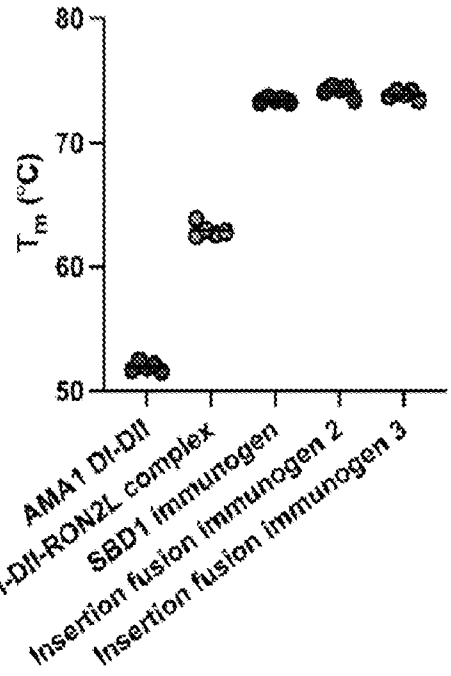


FIG. 3A

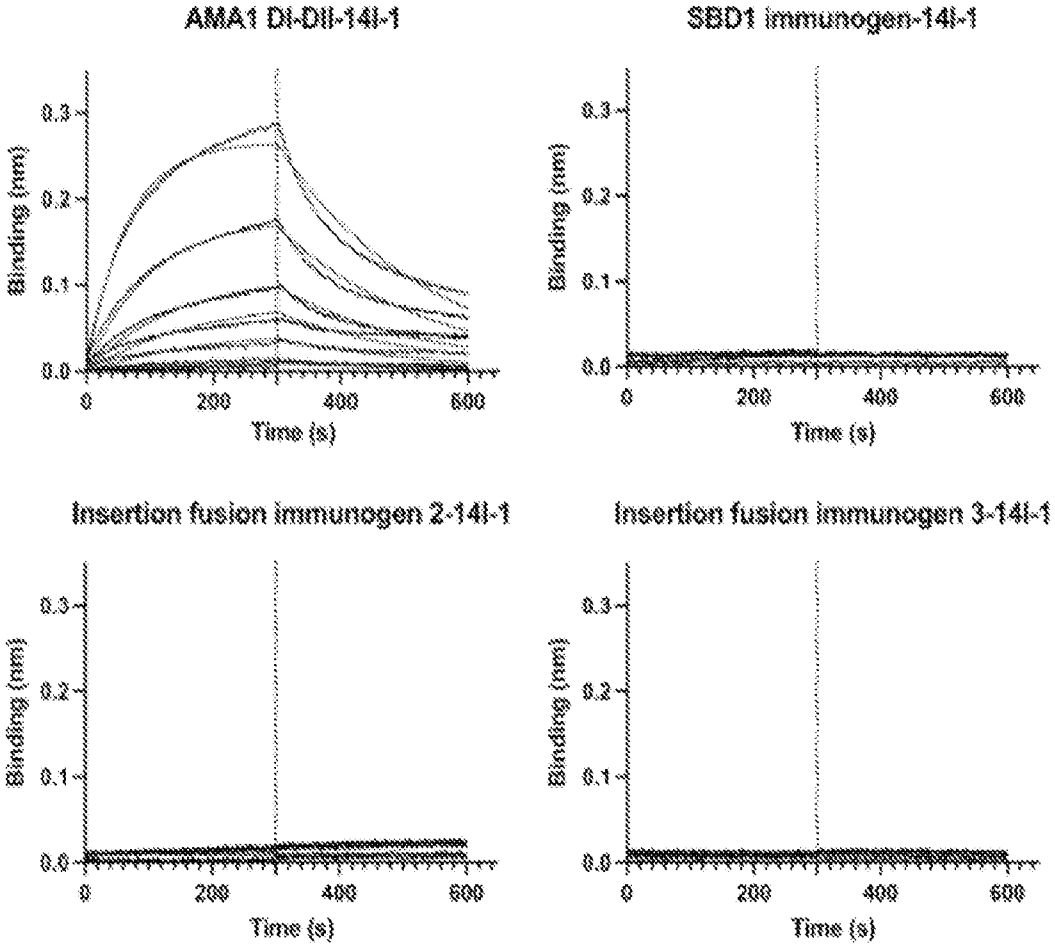

AMA1 DI-DII-14I-1
SBD1 immunogen-14I-1
Insertion fusion immunogen 2-14I-1
Insertion fusion immunogen 3-14I-1
Binding (nm)
Time (s)
0.0
0.1
0.2
0.3
0
200
400
600

FIG. 3B

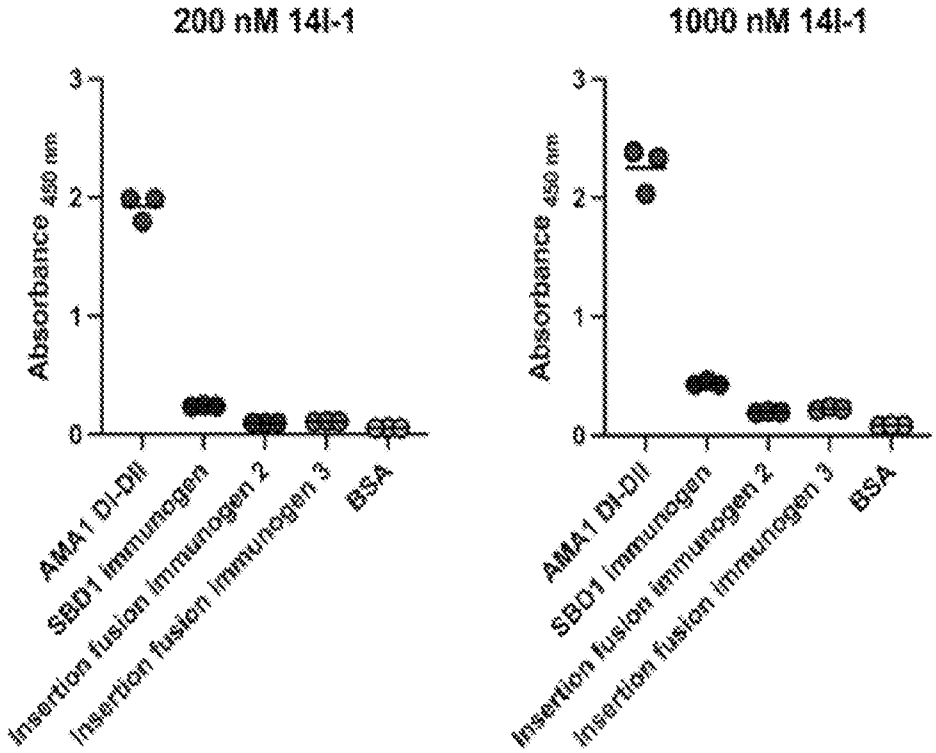

200 nM 14I-1
1000 nM 14I-1
Absorbance 450 nm
0
1
2
3
AMA1 DI-DII
SBD1 immunogen
Insertion fusion immunogen 2
Insertion fusion immunogen 3
BSA

FIG. 3C

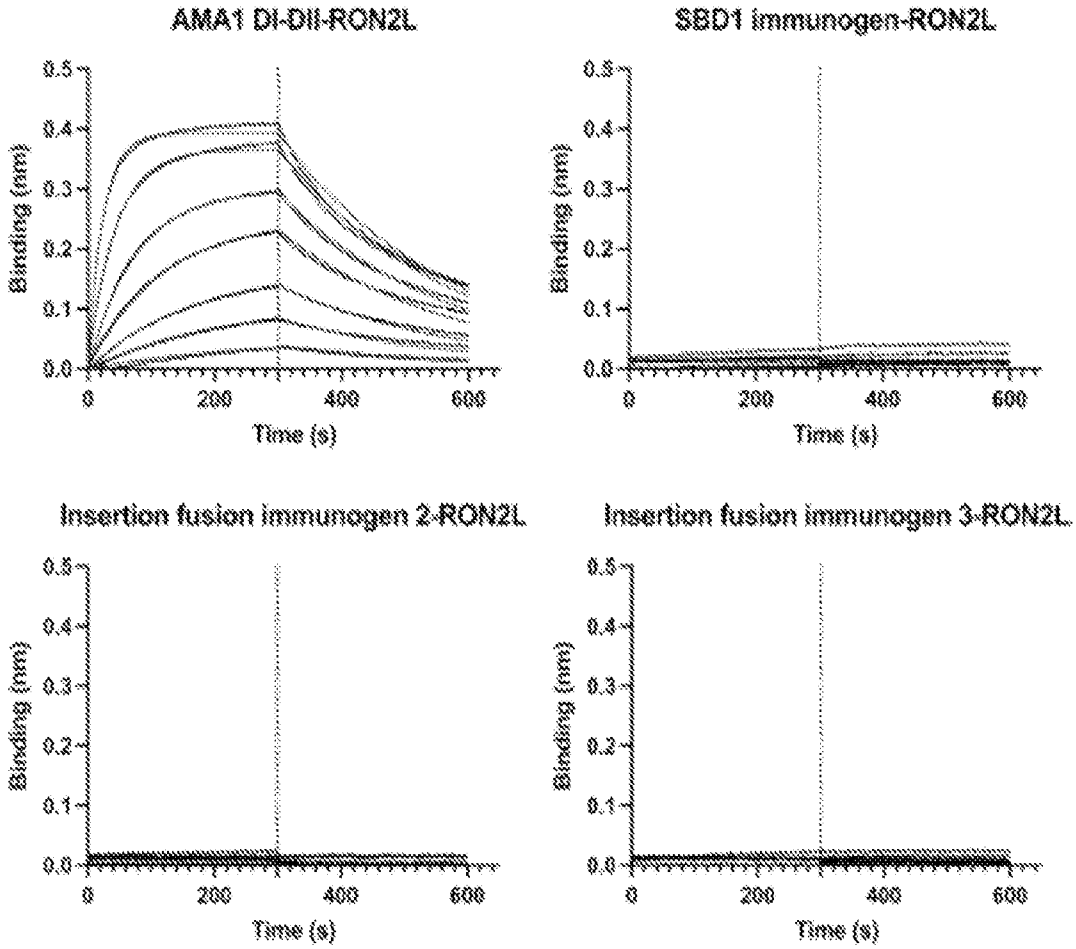

AMA1 DI-DII-RON2L
SBD1 immunogen-RON2L
Insertion fusion immunogen 2-RON2L
Insertion fusion immunogen 3-RON2L
Binding (nm)
Time (s)
0.0
0.1
0.2
0.3
0.4
0.5
0
200
400
600

FIG. 3D

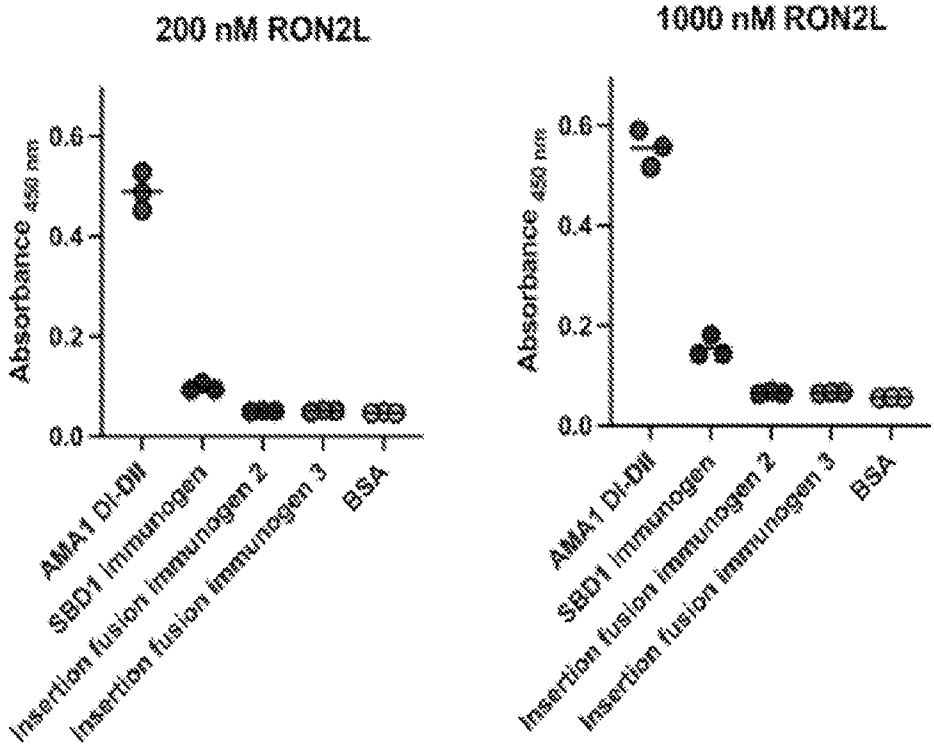

200 nM RON2L
1000 nM RON2L
Absorbance 450 nm
0.0
0.2
0.4
0.6
AMA1 DI-DII
SBD1 immunogen
Insertion fusion immunogen 2
Insertion fusion immunogen 3
BSA

FIG. 4A

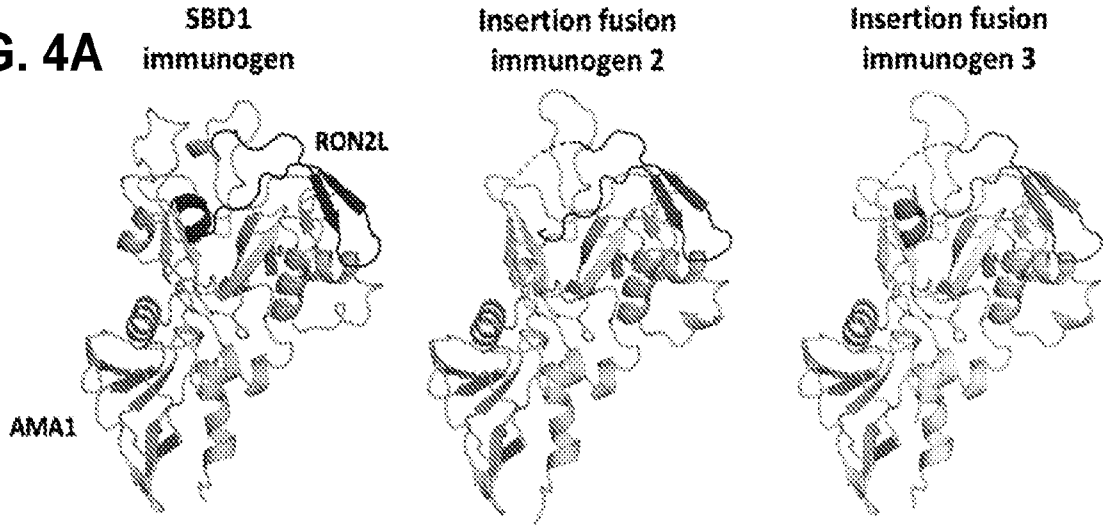


FIG. 4B

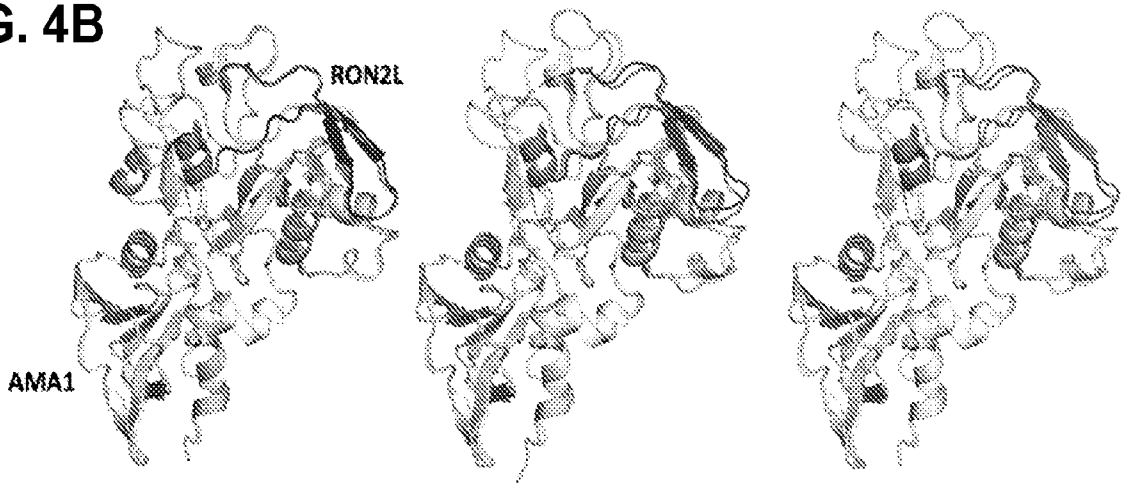


FIG. 4C

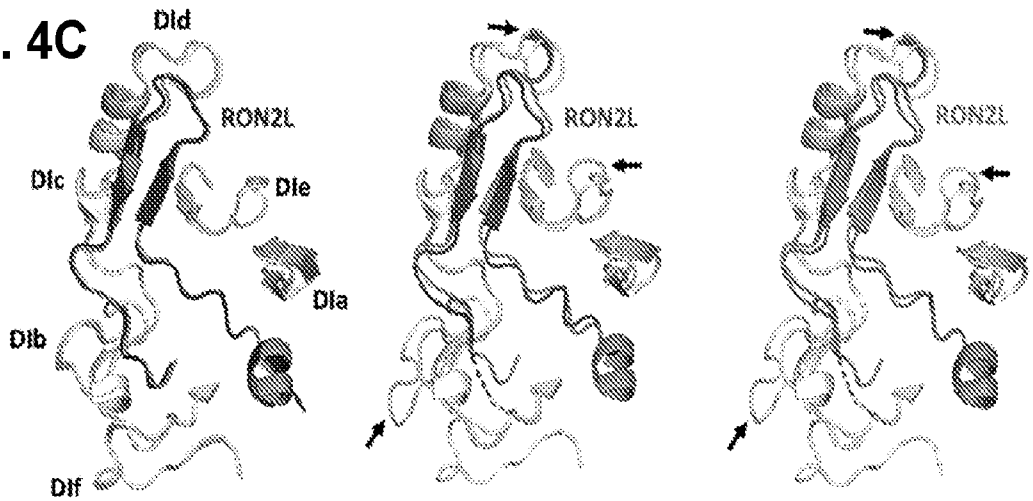


FIG. 5A

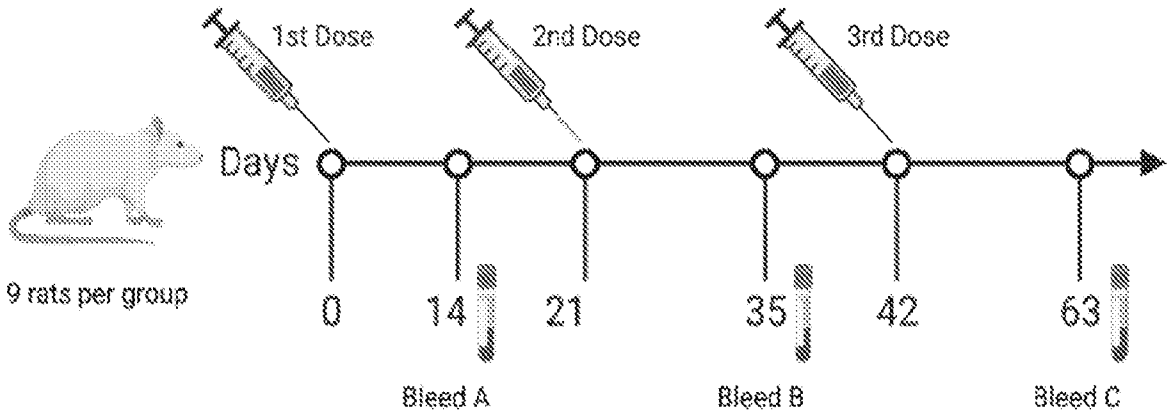

1st Dose
2nd Dose
3rd Dose
Days
9 rats per group
0
14
21
35
42
63
Bleed A
Bleed B
Bleed C

FIG. 5B

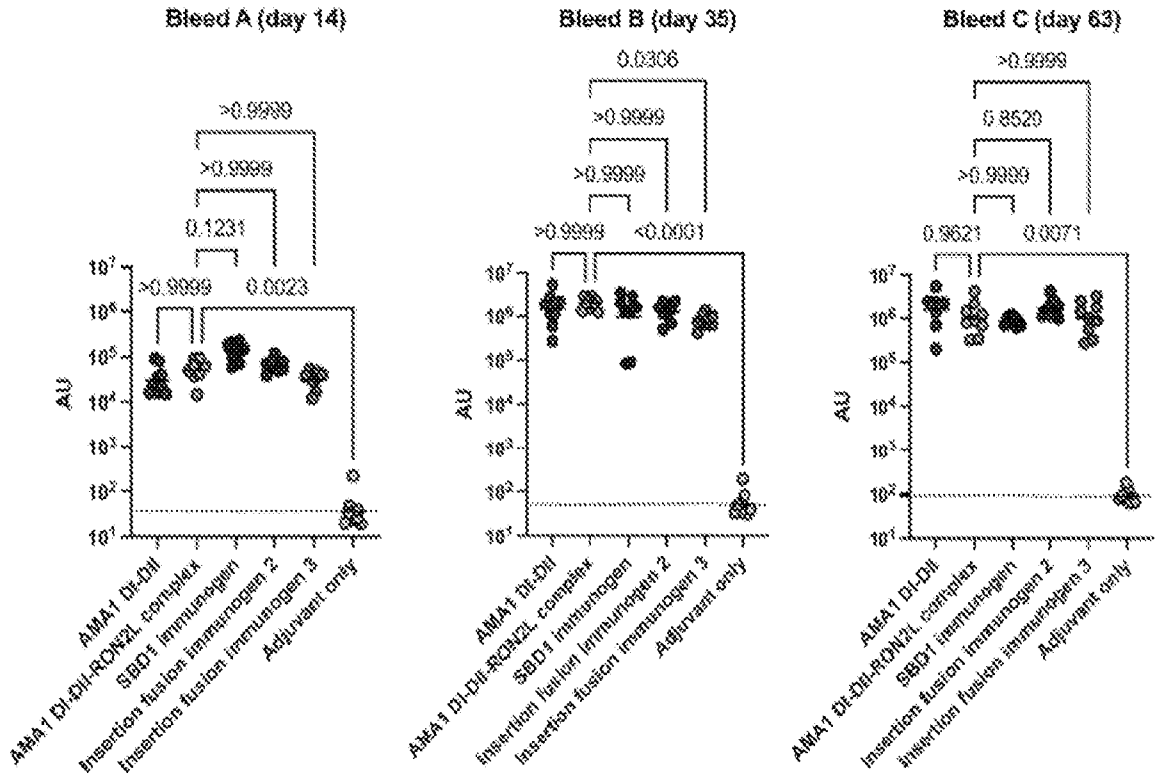

Bleed A (day 14)
Bleed B (day 35)
Bleed C (day 63)
AU
AMA1 DI-DII
AMA1 DI-DII-RON2L complex
SBD1 immunogen
Insertion fusion immunogen 2
Insertion fusion immunogen 3
Adjuvant only

FIG. 5C

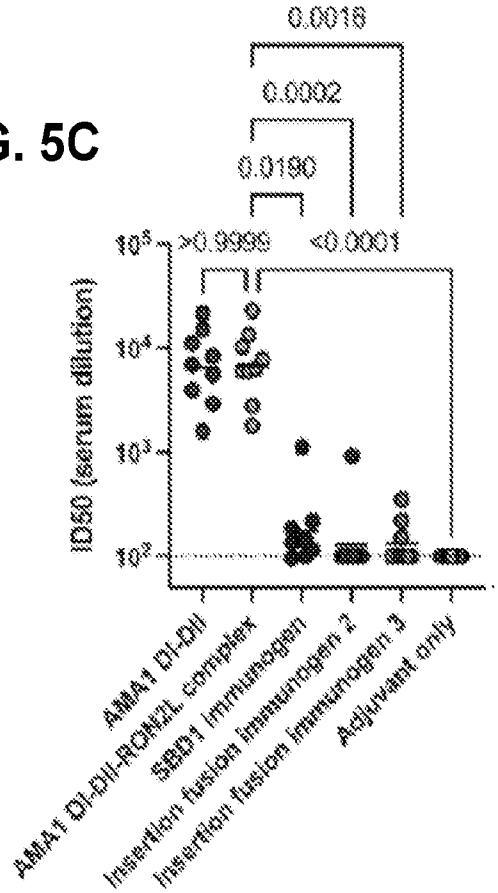

0.0018
0.0002
0.0190
>0.9999
<0.0001
ID50 (serum dilution)
AMA1 DI-DII
AMA1 DI-DII-RON2L complex
SSD1 immunogen
Insertion fusion immunogen 2
Insertion fusion immunogen 3
Adjuvant only

FIG. 5D

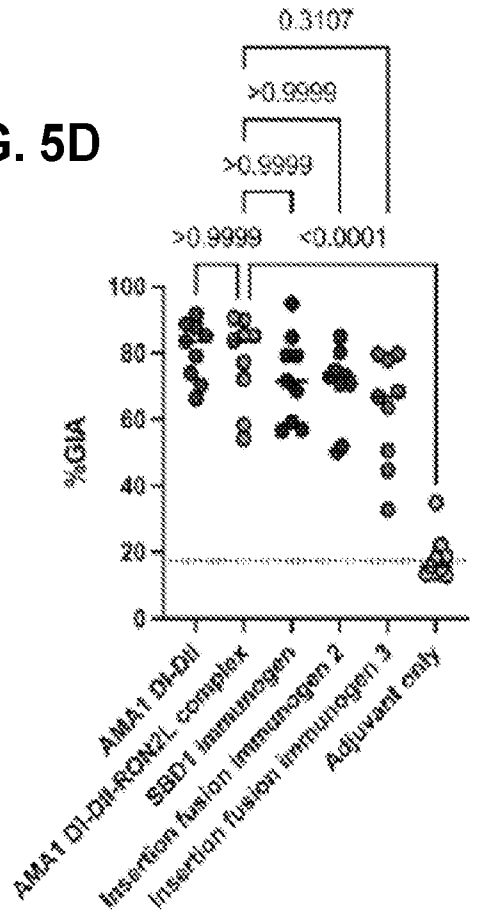

0.3107
>0.9999
>0.9999
>0.9999
<0.0001
%GIA
AMA1 DI-DII
AMA1 DI-DII-RON2L complex
SSD1 immunogen
Insertion fusion immunogen 2
Insertion fusion immunogen 3
Adjuvant only

FIG. 6A

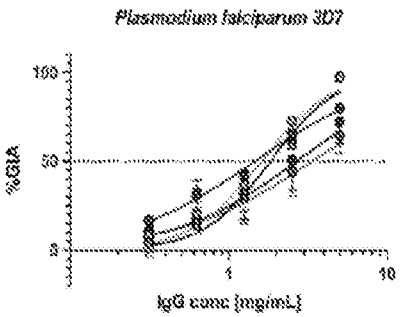


FIG. 6B

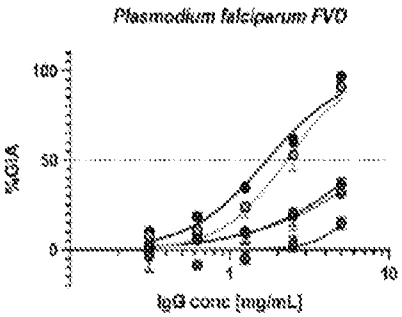
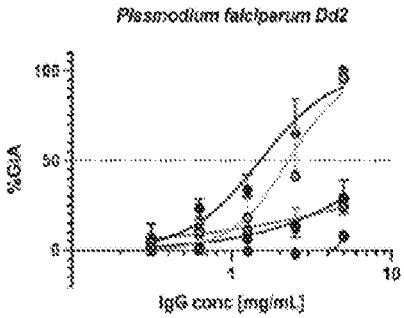


FIG. 6C



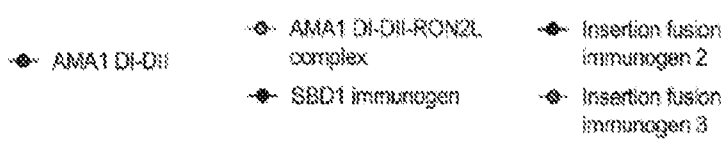


FIG. 6D

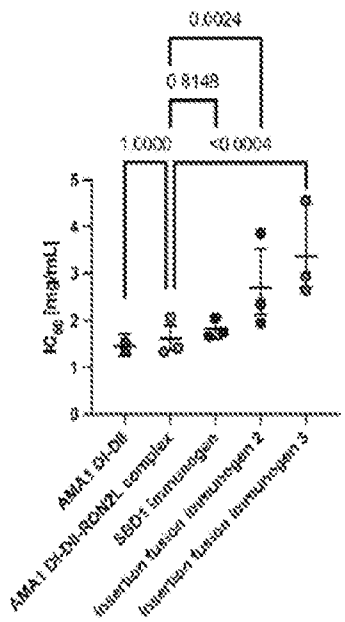


FIG. 6E

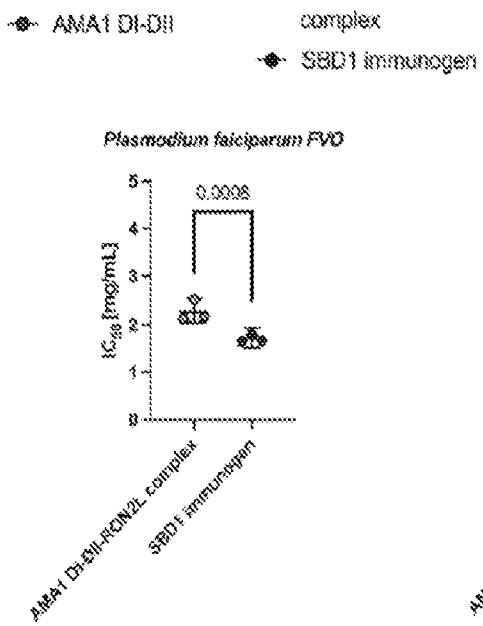


FIG. 6F

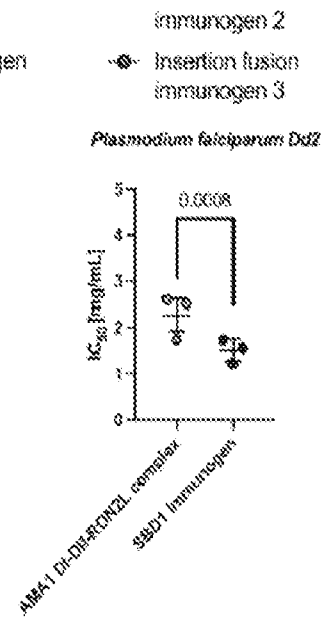


FIG. 7A

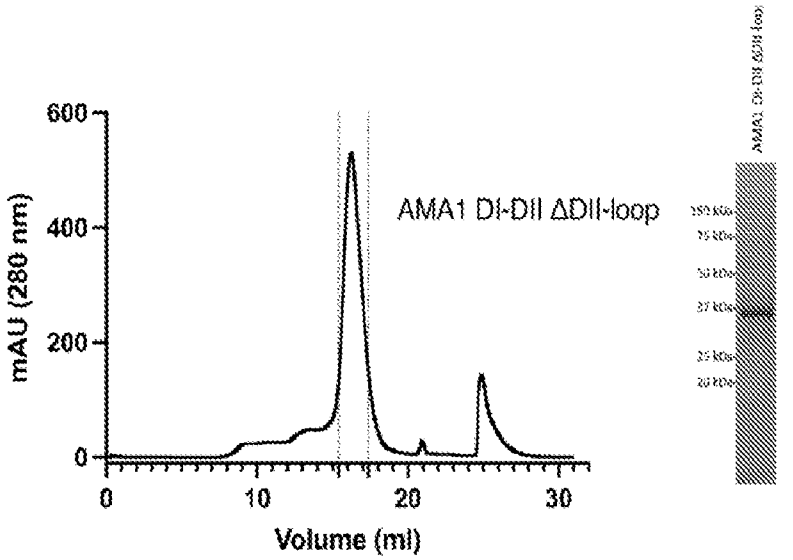


FIG. 7B

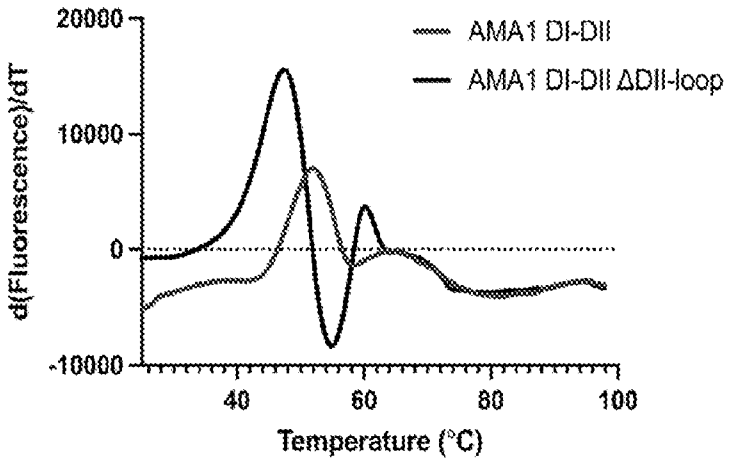


FIG. 7C

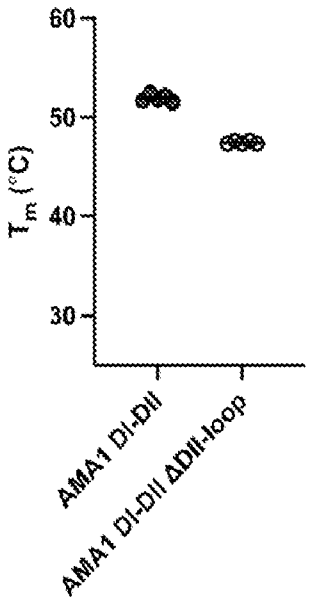


FIG. 8A

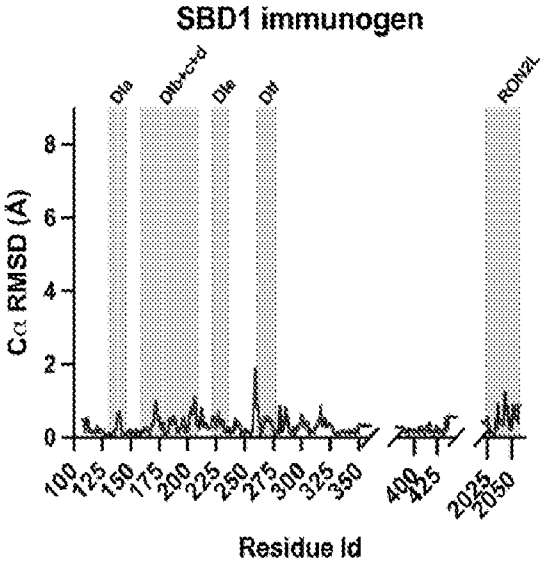


FIG. 8B

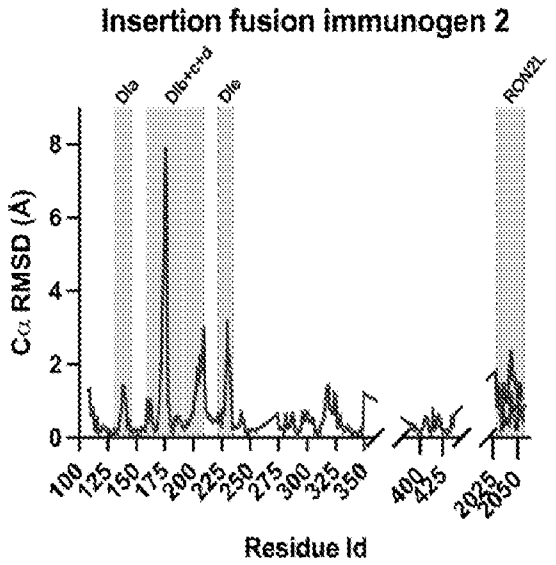


FIG. 8C

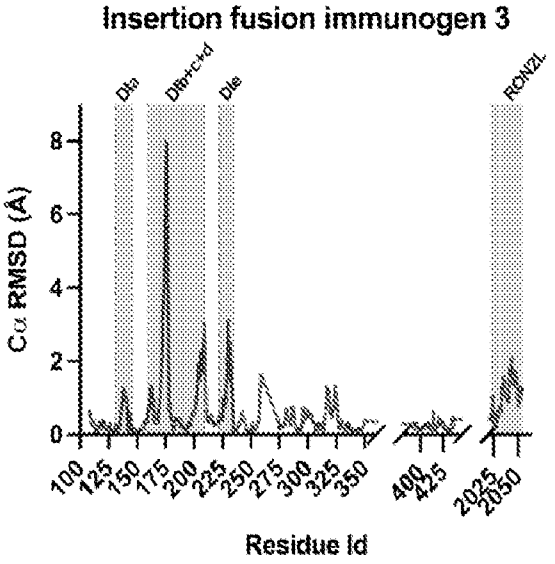
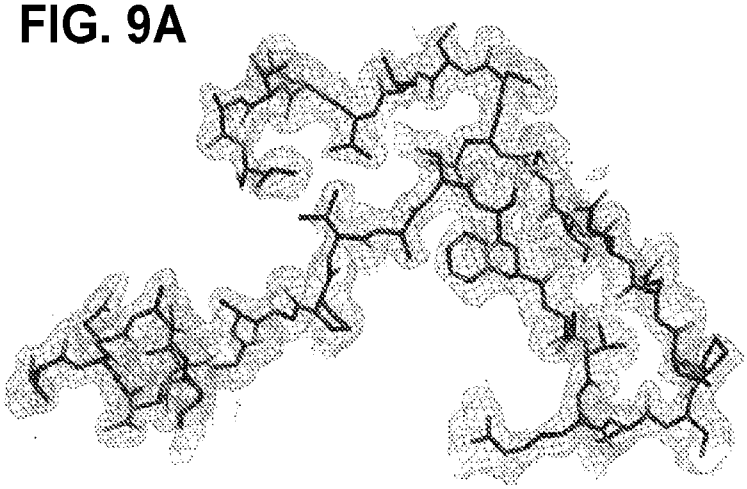
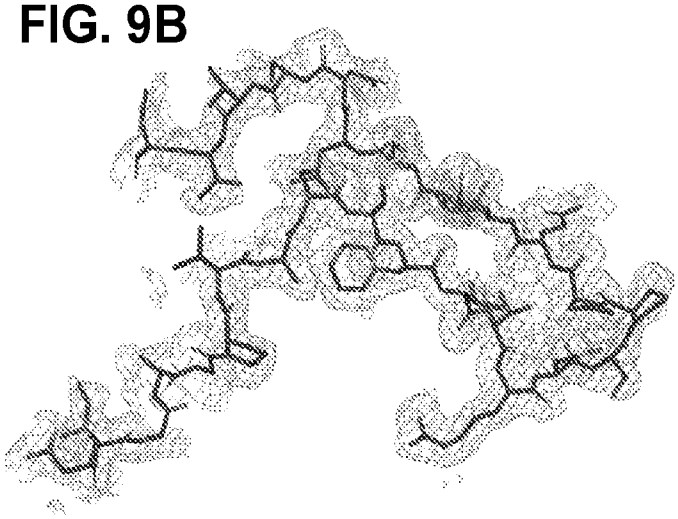


FIG. 9A



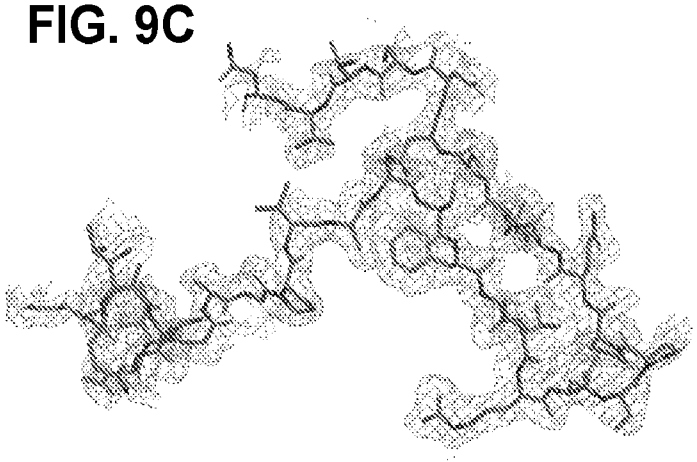
SBD1 immunogen

FIG. 9B



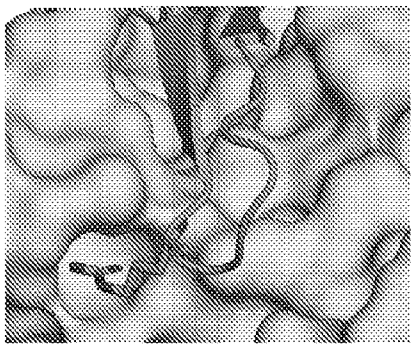
Insertion fusion immunogen 2

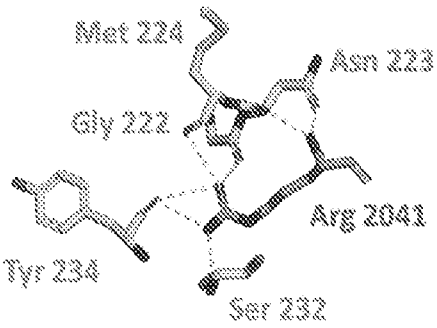
FIG. 9C



Insertion fusion immunogen 3

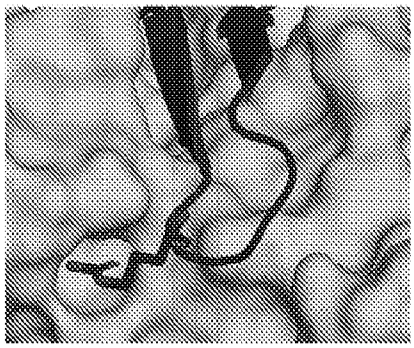
FIG. 10A

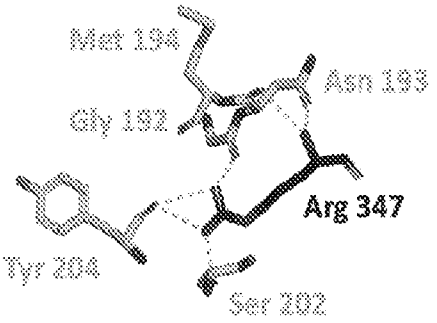




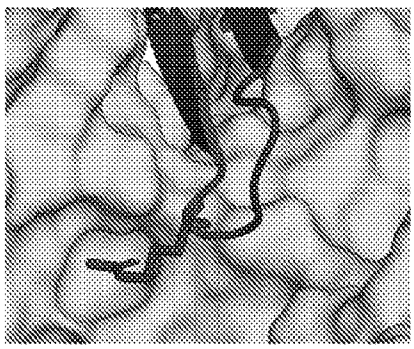
AMA1 DI-DII-RON2L complex

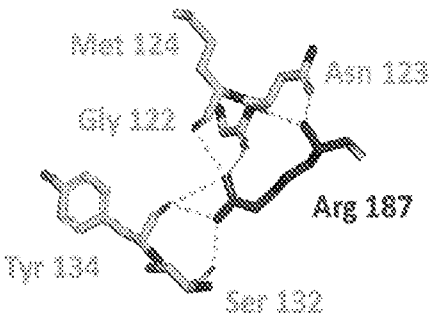
FIG. 10B



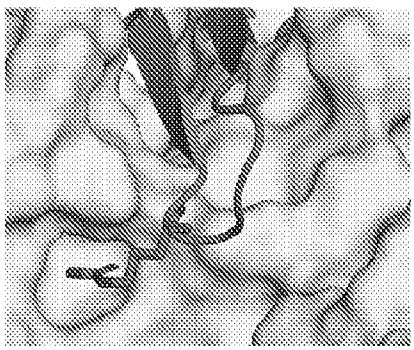


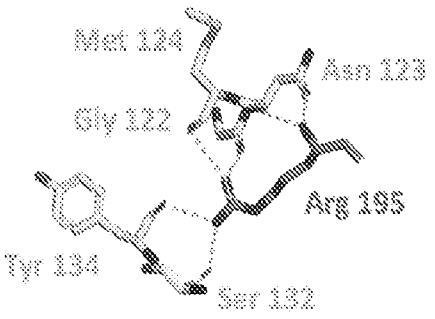
SBD1 immunogen





Insertion fusion immunogen 2





Insertion fusion immunogen 3

FIG. 11A

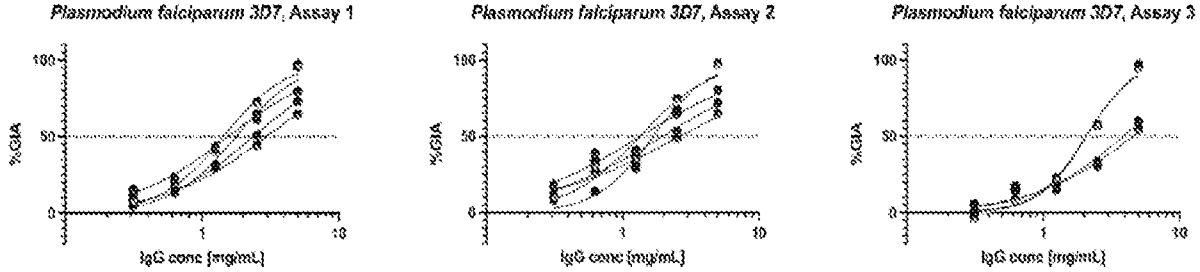


FIG. 11B

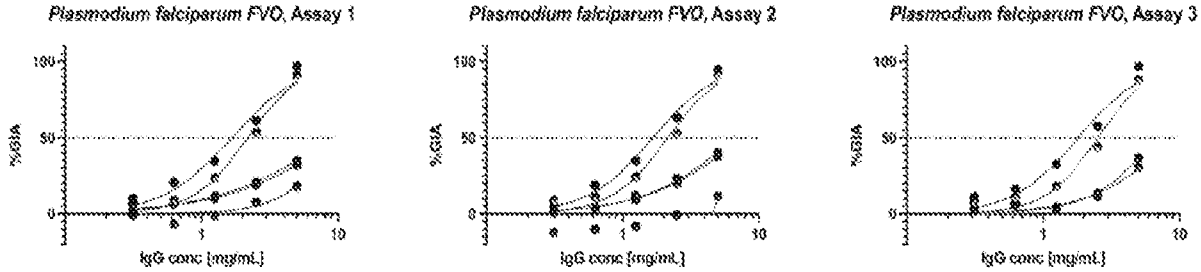


FIG. 11C

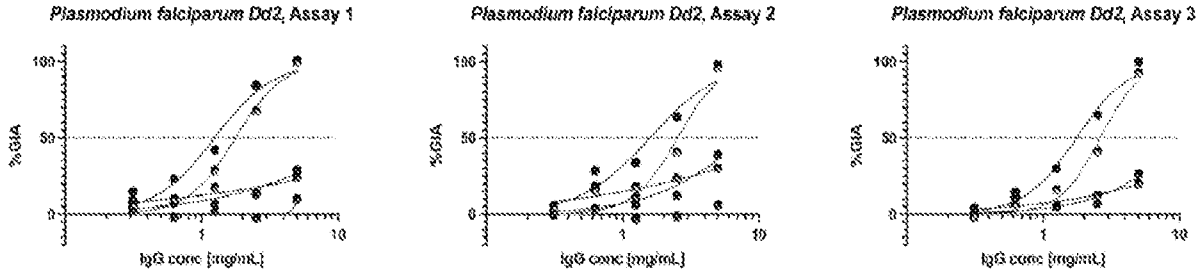


FIG. 12A

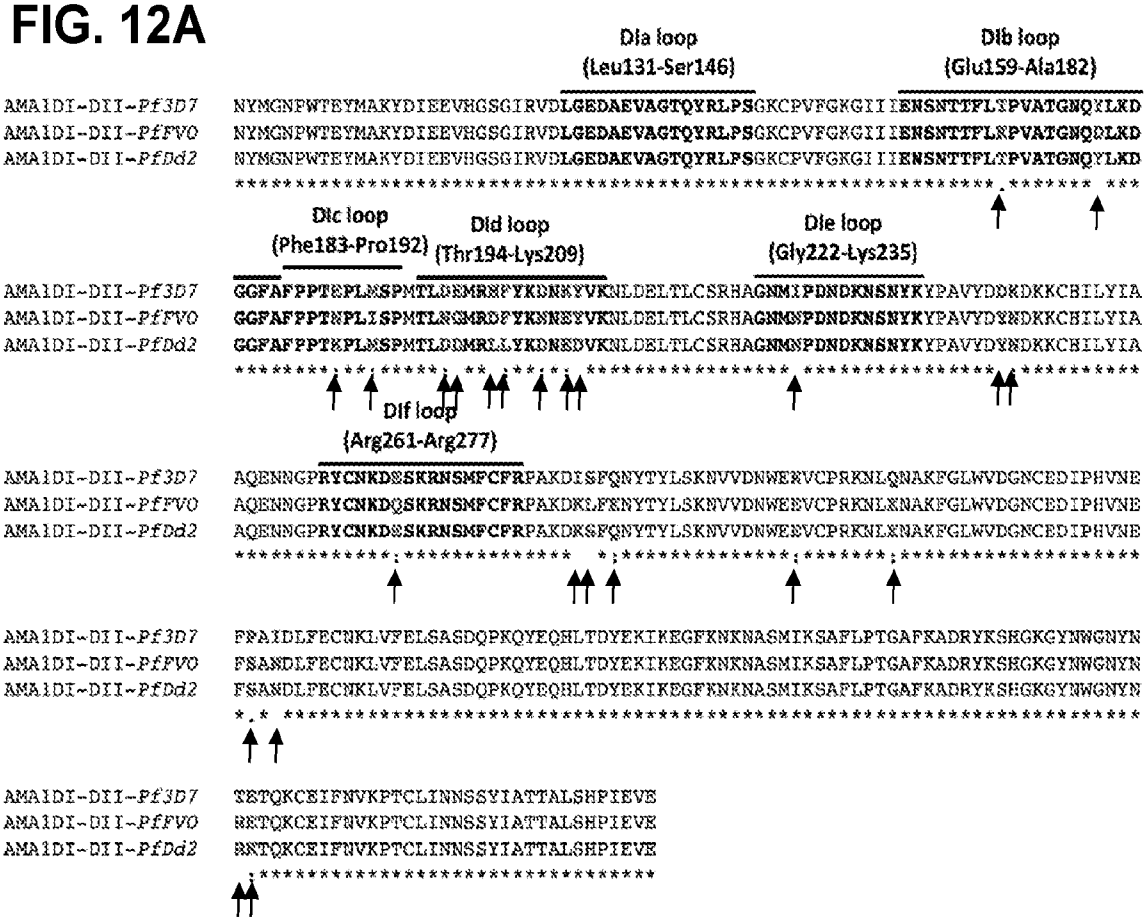


FIG. 12B

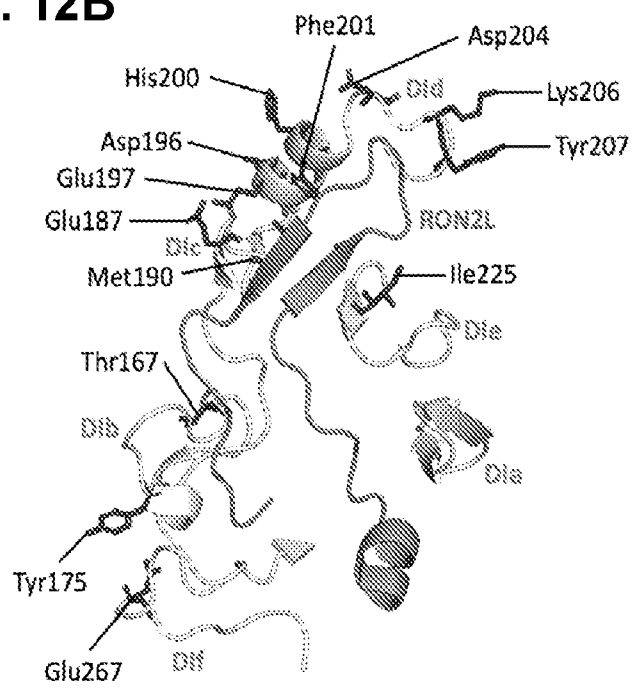


FIG. 13

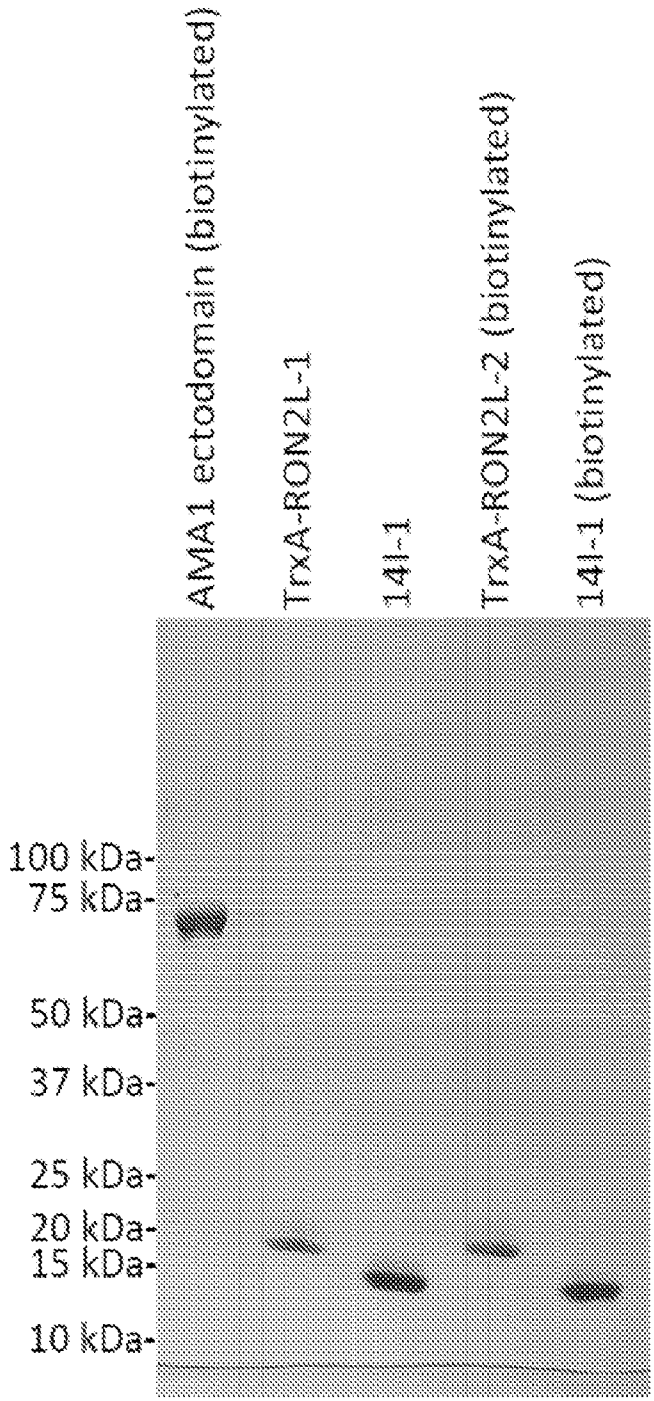

AMA1 ectodomain (biotinylated)
TrxA-RON2L-1
14I-1
TrxA-RON2L-2 (biotinylated)
14I-1 (biotinylated)
100 kDa-
75 kDa-
50 kDa-
37 kDa-
25 kDa-
20 kDa-
15 kDa-
10 kDa-

FIG. 14A

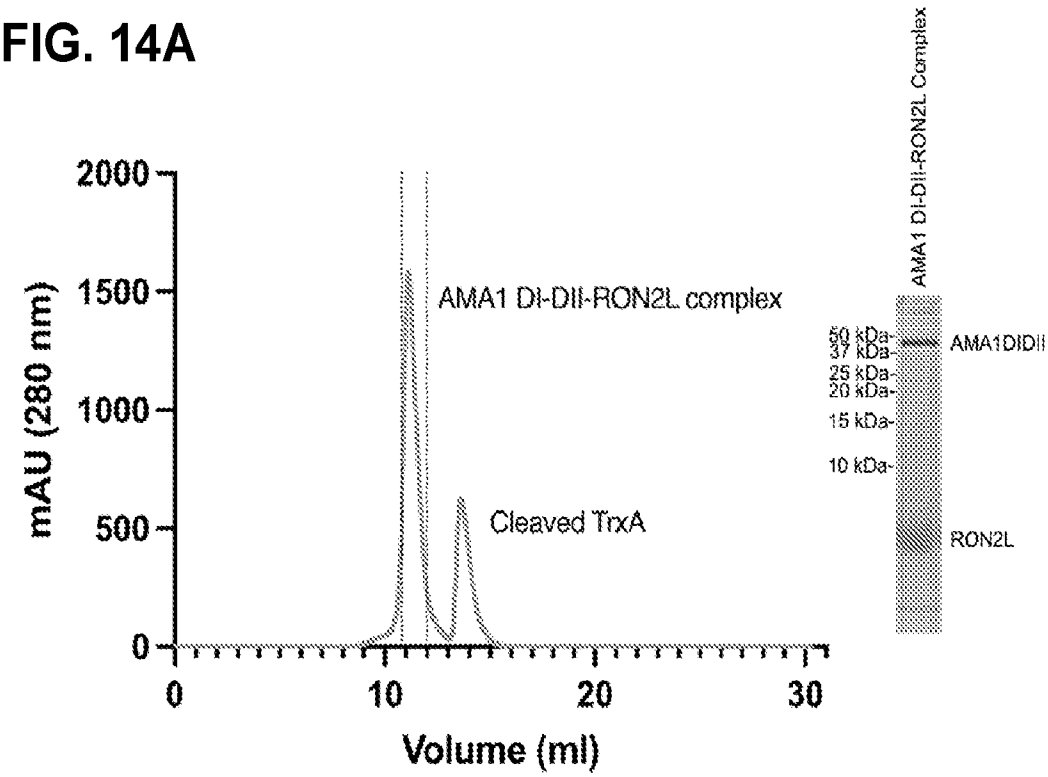


FIG. 14B

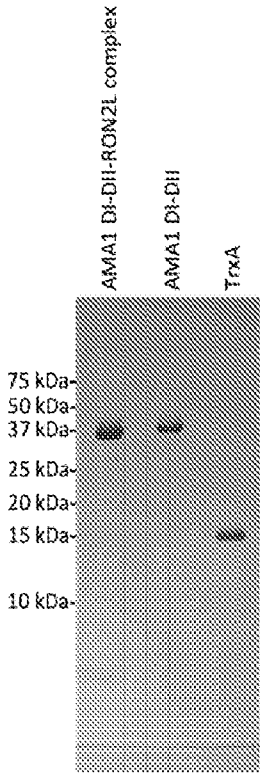
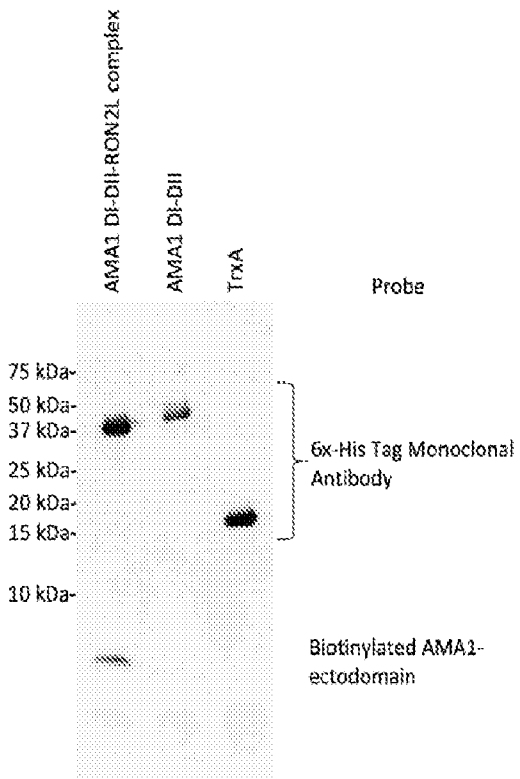


FIG. 14C



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/035244

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 A61P33/02 A61P33/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2015/002959 A1 (US HEALTH [US]) 8 January 2015 (2015-01-08) paragraph [0160] - paragraph [0161]; claim 1; figures 1-6,11; examples 1,3 paragraph [0166]	36,38, 40,43 1-35
X	-& P. SRINIVASAN ET AL: "Immunization with a functional protein complex required for erythrocyte invasion protects against lethal malaria", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 28, 15 July 2014 (2014-07-15), pages 10311-10316, XP055145125, ISSN: 0027-8424, DOI: 10.1073/pnas.1409928111	36,38, 40,43
Y	abstract; figures 1-4	1-35

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 September 2024	04/10/2024

Name and mailing address of the ISA/	Authorized officer
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International application No
PCT/US2024/035244

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