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(71) Applicant: **THE BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA** [US/US]; 660 Parrington Oval, Room 119, Norman, OK 73019 (US).

(72) Inventor: **LI, Jun**; 4405 Pennington Court, Norman, OK 73072 (US).

(74) Agent: **SMITH, Michael, H.**; Hall Estill Hardwick Gable Golden & Nelson, P.C., 100 North Broadway, Suite 2900, Oklahoma City, OK 73102 (US).

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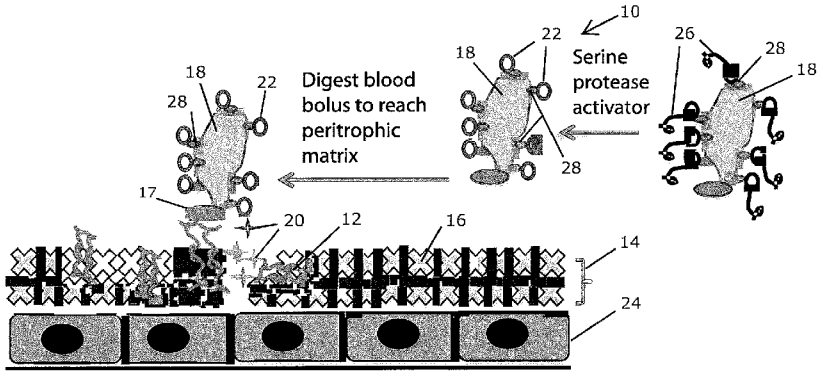

FIG. 9

(57) Abstract: A method and immunogenic composition for blocking transmission of a *Plasmodium* species via an *Anopheles* species. The immunogenic composition, containing a Fibrinogen-Related Protein 1 (FREP1) protein and/or an immunogenic portion thereof, is administered to a mammalian (e.g., human) or avian subject, causing formation of anti-FREPl antibodies in the subject, wherein the anti-FREPl antibodies, when consumed by an *Anophelese* mosquito from blood obtained from the mammalian (e.g., human) or avian subject, prevent or inhibit the infection of the *Anopheles* mosquito by the *Plasmodium* species for stopping malaria transmission.

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IMMUNOGENIC COMPOSITIONS AND METHODS FOR BLOCKING TRANSMISSION OF MALARIA

GOVERNMENT SUPPORT

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BACKGROUND

[0002] Malaria parasites cause more than 225 million clinical cases and over 781,000 deaths per year. *Anopheles gambiae* is one of the most important vectors in Africa transmitting *Plasmodium falciparum*. Parasites have to overcome mosquito physical barriers and other innate immune system to infect mosquitoes before they can infect humans. Identification of naturally-occurring parasite-resistance genes and their functions are of fundamental interest and may be used to develop new malaria control strategies.

[0003] Many mosquito genes related to parasite infection have been reported. Among these are pattern recognition receptors (PRR) including leucine-rich-repeats (LRR) proteins, fibrinogen-related protein family members (FREPs or FBNs), thioester-containing proteins (TEPs) and C-type lectins (CTLs). Some gene products inhibit the development of oocysts, for example, Tep1, APL1C and LRIM1 form a complex that activates the mosquito complement-like system in hemolymph to kill parasites. Other mosquito gene products facilitate parasite development by protecting them from the innate immune attack. The C-type lectins, CTL4 and CTLM2 can protect *Plasmodium* ookinetes from melanization. Moreover, it has been proposed that parasite invasion is mediated by specific proteins, and this hypothesis is supported by experiments that demonstrate the preferential adherence of ookinetes to midguts and the inhibition of parasite invasion by a peptide, SM1.

[0004] The parasite-resistance phenotype is a multigenic trait in natural *An. gambiae* populations and previous genetic mapping studies identified several contributing loci. A major parasite resistance island (PRI) was identified based on segregation in two parental strains with differences in vector competence originating in Eastern and Western Africa. However, identification of the malaria resistance genes within PRI in wild-derived mosquitoes is a significant challenge because the locus is large (>10MB) and it is difficult to acquire sufficient samples for direct-association studies. Synchronizing large numbers of same-age (3-5 days old) wild adult mosquitoes is difficult and low blood-feeding rates (<10%) on membrane feeders make it a logistical challenge to obtain enough samples for

genome-wide direct-association studies. Thus, it is necessary to narrow the size range of the PRI for further analysis.

[0005] As noted above, *Anopheline* mosquitoes transmit malaria pathogens, of which *P. falciparum* is the most dangerous. Female mosquitoes require bloodmeals for egg production. Feeding on *Plasmodium*-infected blood will result in the ingestion of male and female haploid gametocytes that fuse to form diploid ookinetes: a process that initiates *Plasmodium* infection of the mosquito vector. Ookinetes start invading mosquito midgut epithelial cells between 12 to 24 hours after a bloodmeal. A composition and method for inhibiting the infection of mosquitos by *Plasmodium* would be beneficial to block malaria transmission. It is to such compositions and methods that the present disclosure is directed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] Several embodiments of the present disclosure are hereby illustrated in the appended drawings. It is to be noted however, that the appended drawings only illustrate several typical embodiments and are therefore not intended to be considered limiting of the scope of the presently disclosed inventive concepts.

[0007] FIGS. 1A-1D show a direct association analysis of *P. falciparum* infection and genetic variation within candidate genes in wild-derived *An. gambiae*. (A) *AgADA* gene single nucleotide polymorphism (SNP) 427 (2L, 39852810 bp). (B) *FBN30* gene SNP 28 (2L, 39966795 bp). (C) *FREP1* gene SNP 1325 (2L, 41165983 bp). (D) The combined effect of the SNPs in all three genes has higher impact on parasite resistance than individual gene (RRR: all three genes are resistant genotypes in mosquitoes; RRS: two of three genes are resistant genotypes; RSS: one of three genes is resistant genotype; SSS: all three genes are susceptible genotypes). The Y-axis gives the number of oocysts in each mosquito (dots) and the X-axis specifies the genotypes (top) and amino acids (below) for each mosquito. Mosquitoes (n=22) were grouped according to their genotypes and phenotypes, and closely-related phenotypes joined first. *Pi,* mean intensity of infection-associated *p*-value calculated with a t-test. *Pp,* prevalence-associated *p*-value calculated with the Fisher-exact test. Bold letters indicate statistically-significant associations.

[0008] FIGS. 2A-2L show verification of the phenotypes of dsRNA-mediated knock-downs of *FREP1, FBN30,* and *AgADA* mRNA on *Plasmodium* infection. (A, E, I) Quantitative reverse transcription PCR detection of *FREP1, FBN30* and *AgADA* mRNAs in dsRNA-treated experimental and control mosquitoes. Ag S7 mRNA was used as a loading control. (B, F, J) Fluorescent microscopic detection of GFP-*P. berghei* oocysts (green spots)

in mosquitoes treated with the GFP dsRNA. (C, G, K) Fluorescent microscopic detection of GFP-*P. berghei* oocysts in mosquitoes treated with the *FREP1, FBN30* or *AgADA* dsRNA. (D, H, L) Statistical analyses of the number of oocysts in mosquitoes treated with the dsRNA of *FREP1, FBN30* or *AgADA*, respectively, and GFP. The experiments were repeated twice and showed similar results. The black lines in the graphs indicate the means of oocyst number under different treatment groups.

[0009] FIGS. 3A and 3B depict structural characteristics of the FREP1protein. FREP1 is secreted from insect cells and forms tetramers. (A) FREP1 protein has N-terminal signal peptide, three coiled coils, C-terminal FBN domain. (B) Western blot shows that the recombinant FREP1 protein was secreted from High Five cells into culture medium. "S" and "P" represent supernatant and cell pellet respectively. Two replicates have different protein concentrations. (C) Gel filtration chromatography shows that recombinant FREP1 protein forms tetramers. X-axis represents the molecular weight and y-axis represents the absorbance at 405nm of ELISA using anti-FREP1 antibodies.

[0010] FIGS. 4A-4F are micrographs showing the presentation of FREP1 protein in the mosquito midgut peritrophic matrix (PM). (A, C, and E) Negative control of naïve and blood fed *An. gambiae* midgut in which purified pre-immune rabbit Ig G antibody was used to detect FREP1 protein. (B, D, and F) Experimental group of naïve and blood fed *An. gambiae* midgut in which anti-FREP1 rabbit Ig G was used to detect FREP1 protein; E and F are the magnification of areas on C and D (highlighted by rectangles) respectively. Locations of the midgut epithelium, peritrophic matrix (PM), FREP1 protein and blood bolus are annotated on the images.

[0011] FIG. 5 shows micrographs indicating binding between recombinant FREP1 protein (SEQ ID NO:2) and *P. falciparum* by indirect immunofluorescence assays. Images in the first, second and third rows were asexual rings, sexual gametocytes and diploid ookinetes. The first and second column detected FREP1 and nuclei respectively. Merging column one and two generated the third column. Last column are parasites under light microscope. Infected and uninfected cells are annotated on the images directly.

[0012] FIGS. 6A and 6B show binding between FREP1 protein and *P. falciparum* by ELISA. (A) BSA, heat-inactivated insect cell-expressed recombinant FREP1, and insect cell-expressed recombinant FREP1 were used to determine their binding with the same lysate of cultured *P. falciparum*-infected red blood cells (RBC). The OD_{405} values and standard deviations were obtained from 4 repeats. Significant more insect cell-expressed recombinant FREP1 was retained in ELISA plates than the negative control ($p<10^{-5}$). Substituting

recombinant FREP1 with heat-inactivated recombinant FREP1 showed no binding. (B) lysates of normal blood, asexual stage, gametocytes, and ookinetes were used to determine their binding to insect cell-expressed recombinant FREP1 protein. The signals from infected cells were significantly higher than the normal blood ($p<0.02$). Statistical *p* values were calculated using student t-test.

[0013] FIGS. 7A-7D show the impact of dsRNA-mediated knockdown of *FREP1* on *P. falciparum* infection in mosquitoes. (A) Quantification RT-PCR detection of *FREP1* mRNA in dsRNA-treated experimental and control mosquitoes. Lane order (M, 1-4): DNA ladder, *GFP* dsRNA treated mosquitoes amplified with *FREP1* primers, *FREP1* dsRNA treated mosquito with *FREP1* primers, *GFP* dsRNA treated mosquito with *Ags7* primers, *FREP1* dsRNA treated mosquito with *Ags7* primers. All primer sequences are shown in Table 1. (B) *GFP* dsRNA-treated mosquito midgut with oocysts (round red spots) after staining with 0.1% mercury dibromofluresein disodium salt in PBS for 20 minutes. (C) *FREP1* dsRNA-treated mosquito midgut with oocyst. (D) Statistical analyses of the number of oocysts in mosquitoes treated with the dsRNA of *FREP1* and *GFP*, respectively. Y-axis represents the number of oocysts per midgut and x-axis represents two different treatments. The black bars represent the mean oocysts per midgut. The *p*-values between two groups were calculated using student *t*-test. The experiments were repeated twice.

[0014] FIGS. 8A and 8B show results demonstrating that anti-FREP1 antibody blocks *P. falciparum* parasite invasion in mosquitoes. Three-day old female *A. gambiae* were fed with *P. falciparum* infected blood containing 0.2% mature stage V gametocytes that containing 0.5mg/ml purified anti-FREP1 antibodies at one concentration (A) and a series of dilutions of anti-FREP1 antibodies (0.4, 0.2, 0.1, and 0.05mg/ml) (B). The midguts were dissected 7 days post-infection and the number of oocysts in mosquito midguts was counted. The results showed that anti-FREP1 antibody significantly reduced *P. falciparum* infection ($P<0.0001$) (A) and the antibody concentration as low as 0.1mg/ml significantly ($P<0.003$) reduced the malaria infection intensity (B). "N" represents the sample size and the light bars represent the mean oocysts per midgut. The purified pre-immune rabbit antibodies (0.5 and 0.4 mg/ml respectively in A and B) were used as negative controls.

[0015] FIG. 9 is a schematic representation of a hypothetical molecular model for FREP1-mediated *Plasmodium* parasite invasion in *Anopheline* mosquitoes. After a bloodmeal, the FREP1 protein is secreted into mosquito midgut lumen to form PM. Since FREP1 protein is a tetramer, the FBN domains at one side of the tetramer bind chitin in PM. The FBN domains at the other side of the tetramer bind a FREP1-binding partner on parasites. The continuing

secreted chitinase from the anchored ookinete digests chitin in PM and the plasmin digests proteins, which results in disruption of PM for the ookinete to overcome the PM barrier.

[0016] FIGS. 10A and 10B show the relative abundance of *FREP1* mRNA in tissues and post bloodmeal in mosquitoes according to a published microarray data. (A) *FREP1* mRNA in different tissues. (B) The relative mRNA abundance of *FREP1* in whole mosquitoes after a bloodmeal. The time "0" stands for naïve mosquitoes.

DETAILED DESCRIPTION

[0017] Traditionally, insecticides such as DDT, pyrethroids and anti-malaria medicines such as chloroquine are used to control malaria. However, as indicated above, malaria remains a worldwide health crisis because of lack of vaccines against malaria, the fast spread of drug resistant parasites and pesticide-resistant mosquitoes, and environmental concerns. Breaking the *Plasmodium* transmission cycle is considered to be one of the greatest challenges in malarial control. Malaria transmission blocking vaccines (TBV) and other novel approaches to interrupt *Plasmodium* transmission are desperately needed. However, the current list of candidate TBV antigens that could be targeted through vaccination is limited, which is due in part to the poor understanding of the mosquito-*Plasmodium* interactome.

[0018] As explained in further detail below, it has been discovered that a mosquito protein named fibrinogen-related protein 1 (FREP1) plays an important role in *Plasmodium* infection in mosquitoes, and thus is involved in transmission of malaria. Antibodies against FREP1 have been found herein to render a majority of mosquitoes free from malaria infection. Discovery of this protein as a target molecule has enabled development of a novel approach to control malaria by vaccinating *Plasmodium*-infection prone mammals and humans who then develop antibodies against FREP1, whereby the anti-FREP1 antibodies in the blood taken up by the mosquitos during feeding inhibit infection of the mosquito by *Plasmodium*, thereby interrupting the transmission of malaria when the now infection-resistant mosquitos again feed on their next mammalian or human host.

[0019] The present work reveals that the midgut peritrophic matrix (PM) protein FREP1 has a highly conserved C-terminal domain with ~95% identity among the most prevalent human malaria mosquito vectors such as *An. arabiensis, An. funestus, and An. minimus*. Moreover, *in vitro* assays show that recombinant FREP1 binds both *P. berghei*- and *P. falciparum*-infected cells. The data also show that targeting parasite-mosquito midgut FREP1 blocks *Plasmodium* infection in most if not all of relevant vectors. Thus, unlike conventional strategies directed against sexual stage antigens from *P. falciparum* (Pfs25,

Pfs28, Pfs48/45 or Pfs230), or *P. vivax* orthologs (Pvs25, etc.), targeting the mosquito-expressed FREP1 through vaccination can stop transmission of multiple species of *Plasmodium* in multiple species of *Anopheline* mosquitoes.

[0020] Before describing various embodiments of FREP1 immunogenic compositions and methods of their use in inhibiting malarial transmission, in more detail by way of exemplary description, examples, and results, it is to be understood that the presently disclosed inventive concepts are not limited in application to the details of methods and compositions as set forth in the following description. The presently disclosed inventive concepts are capable of other embodiments or of being practiced or carried out in various ways. As such, the language used herein is intended to be given the broadest possible scope and meaning; and the embodiments are meant to be exemplary, not exhaustive. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting unless otherwise indicated as so. Moreover, in the following detailed description, numerous specific details are set forth in order to provide a more thorough understanding of the disclosure. However, it will be apparent to a person having ordinary skill in the art that the presently disclosed inventive concepts may be practiced without these specific details. In other instances, features which are well known to persons of ordinary skill in the art have not been described in detail to avoid unnecessary complication of the description.

[0021] Unless otherwise defined herein, scientific and technical terms used in connection with the presently disclosed inventive concepts shall have the meanings that are commonly understood by those having ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0022] The present application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional application Serial No. 62/074,451, filed November 3, 2014, entitled "VACCINES AND METHODS FOR BLOCKING TRANSMISSION OF MALARIA", which is hereby expressly incorporated herein in its entirety. All patents, published patent applications, and non-patent publications mentioned in the specification are indicative of the level of skill of those skilled in the art to which the presently disclosed inventive concepts pertain. All patents, published patent applications, and non-patent publications referenced in any portion of this application are herein expressly incorporated by reference in their entirety to the same extent as if each individual patent or publication was specifically and individually indicated to be incorporated by reference.

[0023] All of the compositions and methods of production and application thereof disclosed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of the presently disclosed inventive concepts have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit, and scope of the inventive concepts. All such similar substitutes and modifications apparent to those having ordinary skill in the art are deemed to be within the spirit, scope, and concept of the inventive concepts as defined herein.

[0024] As utilized in accordance with the methods and compositions of the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0025] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or when the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." The use of the term "at least one" will be understood to include one as well as any quantity more than one, including but not limited to, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 100, or any integer inclusive therein. The term "at least one" may extend up to 100 or 1000 or more, depending on the term to which it is attached; in addition, the quantities of 100/1000 are not to be considered limiting, as higher limits may also produce satisfactory results. In addition, the use of the term "at least one of X, Y and Z" will be understood to include X alone, Y alone, and Z alone, as well as any combination of X, Y and Z.

[0026] As used in this specification and claims, the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0027] The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB.

Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AAB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0028] Throughout this disclosure, the term "about" is used to indicate that a value includes the inherent variation of error for the composition, the method used to administer the composition, or the variation that exists among the recipients. As used herein the qualifiers "about" or "approximately" are intended to include not only the exact value, amount, degree, or other qualified characteristic or value, but are intended to include some slight variations due to measuring error, manufacturing tolerances, observer error, and combinations thereof, for example. The term "about" or "approximately", where used herein when referring to a measurable value is meant to encompass, for example, variations of ± 20% or ± 10%, or ± 5%, or ± 4%, or ± 3%, or ± 2%, or ± 1%, or ± 0.1% from the specified value, as such variations are appropriate to perform the disclosed methods and as understood by persons having ordinary skill in the art.

[0029] As used herein, the term "substantially" means that the subsequently described event or circumstance completely occurs or that the subsequently described event or circumstance occurs to a great extent or degree. For example, the term "substantially" means that the subsequently described event or circumstance occurs at least 90% of the time, or at least 95% of the time, or at least 98% of the time.

[0030] The term "pharmaceutically acceptable" refers to compounds and compositions which are suitable for administration to humans and/or animals without undue adverse side effects such as toxicity, irritation and/or allergic response commensurate with a reasonable benefit/risk ratio.

[0031] By "biologically active" is meant the ability to modify the physiological system of an organism without reference to how the active agent has its physiological effects.

[0032] As used herein, "pure," or "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other object species in the composition thereof), and particularly a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80% of all macromolecular species present in the composition, more particularly more than about 85%, more than about 90%, more than about 95%, or more than about 99%. The term "pure" or "substantially pure" also refers to preparations where the

object species (e.g., the peptide compound) is at least 60% (w/w) pure, or at least 70% (w/w) pure, or at least 75% (w/w) pure, or at least 80% (w/w) pure, or at least 85% (w/w) pure, or at least 90% (w/w) pure, or at least 92% (w/w) pure, or at least 95% (w/w) pure, or at least 96% (w/w) pure, or at least 97% (w/w) pure, or at least 98% (w/w) pure, or at least 99% (w/w) pure, or 100% (w/w) pure.

[0033] The terms "subject" and "patient" are used interchangeably herein and will be understood to refer to warm blooded animals, for example, mammals and birds, particularly mammals. Non-limiting examples of animals within the scope and meaning of this term include dogs, cats, rats, mice, guinea pigs, chinchillas, horses, goats, cattle, sheep, zoo animals, Old and New World monkeys, non-human primates, and humans, and any other animal susceptible to malaria.

[0034] "Treatment" refers to therapeutic treatments. "Prevention" refers to prophylactic or preventative treatment measures. The term "treating" refers to administering the composition to a subject for therapeutic purposes. The terms "therapeutic composition" and "pharmaceutical composition" refer to an active immunogenic composition that may be administered to a subject by any method known in the art or otherwise contemplated herein, wherein administration of the composition brings about a therapeutic effect as described elsewhere herein. In addition, the compositions of the presently disclosed inventive concept may be designed to provide delayed, controlled, extended, and/or sustained release using formulation techniques which are well known in the art.

[0035] The term "effective amount" refers to an amount of an active agent which is sufficient to exhibit a detectable therapeutic effect without excessive adverse side effects (such as toxicity, irritation and allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of the inventive concepts. The therapeutic effect may include, for example but not by way of limitation, formation of antibodies against the immunogen in a vaccine.

[0036] The term "homologous" or "% identity" as used herein means a nucleic acid (or fragment thereof) or a protein (or a fragment thereof) having a degree of homology to the corresponding natural reference nucleic acid or protein that may be in excess of 70%, or in excess of 80%, or in excess of 85%, or in excess of 90%, or in excess of 91%, or in excess of 92%, or in excess of 93%, or in excess of 94%, or in excess of 95%, or in excess of 96%, or in excess of 97%, or in excess of 98%, or in excess of 99%. For example, in regard to peptides or polypeptides, the percentage of homology or identity as described herein is typically calculated as the percentage of amino acid residues found in the smaller of the two

sequences which align with identical amino acid residues in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to assist in that alignment (as set forth by Dayhoff, in Atlas of Protein Sequence and Structure, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972)). In one embodiment, the percentage homology as described above is calculated as the percentage of the components found in the smaller of the two sequences that may also be found in the larger of the two sequences (with the introduction of gaps), with a component being defined as a sequence of four, contiguous amino acids. Also included as substantially homologous is any protein product which may be isolated by virtue of cross-reactivity with antibodies to the native protein product. Sequence identity or homology can be determined by comparing the sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. In particular, sequence identity may be determined using any of a number of mathematical algorithms. A non-limiting example of a mathematical algorithm used for comparison of two sequences is the algorithm of Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1990, 87, 2264-2268, modified as in Karlin & Altschul, Proc. Natl. Acad. Sci. USA 1993, 90, 5873-5877.

[0037] In one embodiment "% identity" represents the number of amino acids or nucleotides which are identical at corresponding positions in two sequences of a protein having the same activity or encoding similar proteins. For example, two amino acid sequences each having 100 residues will have 95% identity when 95 of the amino acids at corresponding positions are the same.

[0038] Another example of a mathematical algorithm used for comparison of sequences is the algorithm of Myers & Miller, CABIOS 1988, 4, 11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson & Lipman, Proc. Natl. Acad. Sci. USA 1988, 85, 2444-2448.

[0039] Another algorithm is the WU-BLAST (Washington University BLAST) version 2.0 software (WU-BLAST version 2.0 executable programs for several UNIX platforms). This program is based on WU-BLAST version 1.4, which in turn is based on the public domain NCBI-BLAST version 1.4 (Altschul & Gish, 1996, Local alignment statistics, Doolittle ed., Methods in Enzymology 266, 460-480; Altschul et al., Journal of Molecular

Biology 1990, 215, 403-410; Gish & States, Nature Genetics, 1993, 3: 266-272; Karlin & Altschul, 1993, Proc. Natl. Acad. Sci. USA 90, 5873-5877; all of which are incorporated by reference herein).

[0040] In addition to those otherwise mentioned herein, mention is made also of the programs BLAST, gapped BLAST, BLASTN, BLASTP, and PSI-BLAST, provided by the National Center for Biotechnology Information. These programs are widely used in the art for this purpose and can align homologous regions of two amino acid sequences. In all search programs in the suite, the gapped alignment routines are integral to the database search itself. Gapping can be turned off if desired. The default penalty (Q) for a gap of length one is Q=9 for proteins and BLASTP, and Q=10 for BLASTN, but may be changed to any integer. The default per-residue penalty for extending a gap (R) is R=2 for proteins and BLASTP, and R=10 for BLASTN, but may be changed to any integer. Any combination of values for Q and R can be used in order to align sequences so as to maximize overlap and identity while minimizing sequence gaps. The default amino acid comparison matrix is BLOSUM62, but other amino acid comparison matrices such as PAM can be utilized.

[0041] The terms "polynucleotide sequence" or "nucleic acid," as used herein, include any polynucleotide sequence which encodes a FREP1 protein or fragment thereof including polynucleotides in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand. The polynucleotide sequence may be expressed using polynucleotide sequence(s) which differ in codon usage due to the degeneracies of the genetic code or allelic variations.

[0042] Utility

[0043] In at least one embodiment, the presently disclosed inventive concepts are directed to immunogenic compositions, including but not limited to a vaccine formulation, comprising a purified fibrinogen-related protein-1 (FREP1) protein and/or an immunogenic portion thereof in an amount effective to stimulate production of anti-FREP1 antibodies in the subject to which the immunogenic composition is administered. An immunogenic portion of FREP1 protein is any portion or fragment thereof which is effective in generating antibodies against FREP1 protein. The fragments may be at least 10, at least 25, at least 50, at least 75, at least 100, at least 125, at least 150, at least 175, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, at least 650, at least 700, at

least 725, at least 750, or more amino acids in length up to and including a full-length FREP1 protein.

[0044] The purified FREP1 protein or immunogenic portion thereof may be combined with at least one pharmaceutically-acceptable excipient to form the immunogenic (e.g., vaccine) formulation. Pharmaceutically-acceptable excipients include, but are not limited to, carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives, including but are not limited to ethylenediamine tetraacetic acid (EDTA), gelatin, glycerin, glycine, human serum albumen, antibiotics, surfactants, physiological saline solutions, buffered saline solutions at neutral pH such as phosphate buffered saline (PBS), Freund's incomplete adjuvant, Freund's Complete adjuvant, alum, monophosphoryl lipid A, aluminum phosphate or hydroxide, QS-21, salts, i.e., $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)_2$, silica, kaolin, carbon polynucleotides, i.e., poly IC and poly AU. Non-limiting examples of adjuvants include QuilA, Alhydrogel, aluminum hydroxide, aluminum phosphate, muramyl dipeptide, bacterial lipopolysaccharides and derivatives and purified saponins from Quil A, and microparticles such as liposomes or ISCOMs and the like. Optionally, the immunogenic compositions can be administered with, alone or separately, immunomodulators and immunostimulants, such as but not limited to, interleukins, interferons, and the like. Many other vaccine components and formulations are known to those of skill in the art, including for example those described in Remington: The Science and Practice of Pharmacy, 22nd Ed. 2012. When the immunogenic composition is a vaccine, the FREP1 protein or immunogenic portion thereof may comprise a component of a multivalent vaccine that comprises immunogens against other, non-malarial diseases.

[0045] The presently disclosed inventive concepts are further directed to a method of stimulating an immune response against fibrinogen-related protein-1 (FREP1) protein and/or an immunogenic portion thereof. The immunogenic compositions are administered to humans, or to animals which are infected or may become infected by the malarial organisms, including but not limited to primates, rodents (including mice, rats, and guinea pigs), and avian species such as chickens.

[0046] The immunogenic composition (e.g., vaccine) is administered in an amount sufficient to elicit production of antibodies as part of an immunogenic response. Dosage for any given subject depends upon many factors, including the subject's size, general health, sex, body surface area, age, the particular compound to be administered, time and route of administration, and other drugs being administered concurrently. Determination of optimal dosage is well within the abilities of a pharmacologist of ordinary skill. For example, the

amounts of the FREP1 protein or immunogenic portion thereof that can form a protective immune response typically are in, but are not limited to, a unit dosage of about 0.001 μg to 100 mg per kg of body weight, such as but not limited to, .01 μg to 10 mg/kg of body weight, or about 0.1 μg to about 1 mg/kg body weight, given, for example, in a single dose or in multiple doses given, for example at about 1 to 6 week intervals between immunizations. When the pharmaceutical composition is administered parenterally, via the intramuscular or deep subcutaneous route, the protein is preferably admixed or absorbed with an adjuvant to attract or to enhance the immune response. Such adjuvants include but are not restricted to those listed elsewhere herein. The immunogenic compositions may be designed for oral, cutaneous, intramuscular or intranasal administration or any other effective method known in the art.

[0047] As noted, the therapeutically effective and non-toxic dose of the immunogenic composition can be determined by a person of ordinary skill in the art. However the specific dose for any person will depend upon a variety of factors including age, general health, diet of the patient, time and route of administration, synergistic effects with other drugs being administered and whether the immunogenic composition is administered repeatedly. If necessary the composition will be administered repeatedly with one to three month intervals between each dose and with an optional booster dose later in time. Actual methods of preparing the appropriate dosage forms are known, or will be apparent, to those skilled in this art; see, for example, Remington's Pharmaceutical Sciences latest edition.

[0048] The FREP1 protein(s) or immunogenic portion(s) thereof that comprise the immunogenic compositions of the presently disclosed inventive concepts may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the presently disclosed inventive concepts may be glycosylated or may be non-glycosylated. Procedures for the isolation of the individually expressed polypeptides may be isolated by recombinant expression/isolation methods that are well-known in the art. Typical examples for such isolation may utilize an antibody to a conserved area of the protein or to a His tag or cleavable leader or tail that is expressed as part of the protein structure. As used herein, "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. Thus, for example, recombinant

cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention. In other embodiments, the presently disclosed inventive concepts are directed to antibodies formed against FREP1 protein or immunogenic portions thereof.

[0049] In at least one embodiment, the presently disclosed inventive concepts include a method for blocking transmission of a *Plasmodium* species (such as listed elsewhere herein), comprising administering to a mammalian or avian subject an immunogenic composition comprising a FREP1 protein (e.g., having an amino acid sequence as set forth in SEQ ID NO:2) and/or an immunogenic portion thereof, causing formation of anti-FREP1 antibodies in the mammalian or avian subject. In this manner anti-FREP1 antibodies formed in the immunized subject, when consumed by a mosquito from blood obtained from the immunized subject, prevent or reduce the infection of the mosquito by the *Plasmodium* species.

[0050] Examples of embodiments of the presently disclosed inventive concepts are now provided hereinbelow. However, these embodiments and others of the presently disclosed inventive concepts are to be understood as not limited in application to the specific experimentation, results and laboratory procedures described herein. Rather, the Examples are simply provided as various embodiments and are meant to be exemplary, not exhaustive, and it will be appreciated that additional and different embodiments of the teachings of the presently disclosed inventive concepts will doubtless suggest themselves to those of skill in the art; therefore, such other embodiments are considered to have been inferred from the disclosure herein.

[0051] EXAMPLES

[0052] Example 1

[0053] The malaria parasite resistance island (PRI) of the African mosquito vector, *Anopheles gambiae*, was mapped to five genomic regions containing 80 genes using co-expression patterns of genomic blocks. High-throughput sequencing identified 347 non-synonymous single nucleotide polymorphisms (SNPs) within these genes in mosquitoes from malaria endemic areas in Kenya. Direct association studies between non-synonymous SNPs and *Plasmodium falciparum* infection identified three naturally-occurring genetic variations in each of three genes, *An. gambiae* adenosine deaminase gene (*AgADA*), fibrinogen-related protein 30 gene (*FBN30*) and fibrinogen-related protein 1 gene (*FREP1*), that were associated significantly with parasite infection. A role for these genes in the

resistance phenotype was confirmed by RNA interference knockdown assays. Silencing *FBN30* increased parasite infection significantly, while ablation of *FREP1* transcripts resulted in mosquitoes nearly free of parasites.

[0054] MATERIALS AND METHODS

[0055] Mosquito reference genome sequences, oligonucleotide array data, and analytical software. The *An. gambiae* reference genome sequences (version AgamP3.35, PEST strain) and *C. quinquefasciatus* supercontigs (version CpipJ1, Johannesburg JHB strain) were downloaded from Vectorbase (www.vectorbase.org). Affymetrix oligo array (GeneChip *Plasmodium/Anopheles* Genome Array, Affymetrix Inc. Santa Clara, CA) data were obtained from www.angaged.bio.uci.edu (Dissanayake SN, Marinotti O, Ribeiro JM, & James AA (2006) angaGEDUCI: Anopheles gambiae gene expression database with integrated comparative algorithms for identifying conserved DNA motifs in promoter sequences. *BMC Genomics* 7:116). The R (version 2.6.1; www.r-project.org/) and Bioconductor packages (version 2.8, www.bioconductor.org/) were used for statistical analyses. The MUMmer software (version 3.0) was downloaded from sourceforge (mummer.sourceforge.net/). Programs implemented for this project are available for download (http://omics.ou.edu/download/.ExpressionPattern/scripts.zip).

[0056] Detecting synteny between An. gambiae and C. quinquefasciatus.

[0057] Genome sequence comparisons between *An. gambiae* and *C. quinquefasciatus* were used to identify conserved chromosomal domains. The "promer" function (with parameters –mum --coords -c 41 -g 1000 -l 8) in the MUMmer package (Kurtz S, *et al.* (2004) Versatile and open software for comparing large genomes. *Genome Biol* 5(2):R12) was used to detect the conserved genomic regions based on derived protein sequences. Following removal of isolated and small (<150 bp) conserved regions, neighboring conserved regions were joined recursively to form a single, larger region if they were on the same *An. gambiae* chromosome and included in the same *C. quinquefasciatus* contigs. Syntenic blocks were verified manually using the graphic presentation generated by the "mummerplot" in the MUMmer package. Genomic regions between syntenic blocks in *An. gambiae* are not conserved and were included as internal controls in the signature expression profile detection.

[0058] Detecting signature expression profiles of genomic blocks containing immunity-related genes.

[0059] Probes on the Affymetrix array were mapped to the *An. gambiae* reference genome (AgamP3.53) using BLAT (38). Cross-hybridizing probes mapping to multiple genomic regions were removed from further analysis. No infections are required because we used a set

of known genes related to infection as a training set to identify characteristic expression patterns. However, a minimum number of independent genome-wide expression profiles (samples) are needed to obtain high resolution of genomic block expression patterns. Since we identified a total of 2,494 *An. gambiae* genomic blocks and the expression of each block in the samples can be higher, lower or unchanged, more than eight sample conditions are needed for this study (3^8=6561). We used array data from 14 samples of different developmental stages and tissue origins (Dissanayake SN, Marinotti O, Ribeiro JM, & James AA (2006) angaGEDUCI: Anopheles gambiae gene expression database with integrated comparative algorithms for identifying conserved DNA motifs in promoter sequences. *BMC Genomics* 7:116). The probe ID, sequence, and signal data were assembled into one table (http://omics.ou.edu/download/.ExpressionPattern/OligoSignalAndPosition.tab). The array data then were normalized with the quantile normalization procedure (Bolstad BM, Irizarry RA, Astrand M, & Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19(2):185-193) and mapped to the syntenic and non-conserved regions based on their positions in the *An. gambiae* genome. The expression values of probes were condensed to syntenic and non-conserved blocks using the Robust Multichip Average (RMA) algorithm (Irizarry RA, *et al.* (2003) Summaries of affymetrix GeneChip probe level data. *Nucleic Acids Res* 31(4):e15). The hierarchical clustering algorithm (Eisen MB, Spellman PT, Brown PO, & Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *P Natl Acad Sci USA* 95(25):14863-14868) was used to construct an expression tree of genomic blocks.

[0060] Seventy genes known or proposed to have roles in mosquito immune defense (positive or negative) were assessed to detect signature expression profiles in the constructed expression tree. The genes were used to designate genomic blocks as "immunity" blocks. The immunity block enrichment *p*-values based on the binomial distribution for a leaf were calculated at different levels of branches, and the smallest *p*-value was used to represent the "immunity" associated value (*p*) for that leaf. The enrichment scores were generated using the equation: score= -ln(*p*). If a genomic block in a branch contained more than one immune gene, it was counted only once in statistical analysis. The enrichment-score profile was smoothed using the Ramer-Douglas-Peucker line smooth algorithm with a window size of 40 leaves.

[0061] Direct association between genetic variation within candidate genomic blocks and *P. falciparum* infection in wild-derived *An. gambiae*.

[0062] *Anopheles gambiae* larvae were collected from diverse habitats in a malaria-hyperendemic region in highland areas near Kisumu, western Kenya. Fewer than four larvae per habitat were collected and only one female from each habitat was used to avoid the impacts of siblings on the association studies. The distance between any two habitats was ≥10 meters. Morphologically-identified *An. gambiae* larvae were brought to the laboratory at ICIPE and reared to adults. The resulting 3-5 day post-emergence female mosquitoes were challenged with a *P. falciparum*-infected blood drawn from patients with >100 gametocytes per microliter blood (approved IRB protocols NO.163 and 0906M68726). All mosquitoes for the direct-association study were fed simultaneously on the same source of infected human blood. The blood serum was replaced with the same amount of non-immunized human serum (AB-type, Sigma-Aldrich, MO, USA) before mosquito challenges. Fully-engorged mosquitoes were maintained in the laboratory with cotton soaked in an 8% glucose solution, midguts dissected seven days after blood feeding and stained with 0.1% mercurochrome (Sigma-Aldrich, MO, USA), and the number of oocysts counted with light microscopy. Mosquito carcasses were preserved in 75% ethanol for subsequent DNA analysis, including molecular species identification, whole-genome sequencing by an Illumina paired-end approach and DNA amplification followed by Sanger sequencing. The high-throughput sequencing 100 bp reads from individual mosquitoes were mapped to the *An. gambiae* reference genome (AgamP3.35) using the SOAP program (v2.21) (Li R, *et al.* (2009) SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* 25(15):1966-1967), and the "soapsnp" program (v1.02) was used to detect nucleotide variations at each position. The scores for base-calling (phred score) were greater than 30 to avoid SNPs caused by sequencing errors. At least two reads from one mosquito at a SNP site were required. SNPs within the candidate genomic regions were verified visually using GBrowse (v2.26). The genotypes of candidate SNPs were analyzed further by PCR cloning and Sanger sequencing. The highly-conserved regions (no variations in nine wild mosquitoes based on Illumina sequencing) adjacent to candidate SNPs were selected manually to design oligonucleotide primers (Table S6) to clone the genomic regions by DNA amplification. The amplification products were used to genotype the candidate SNPs in individual mosquitoes using Sanger sequencing. Finally, the Fisher-exact test was used to assess the significance of association of SNPs with the prevalence of *P. falciparum* infection. The t- and Wilcox-tests were used to assess the significance of association between SNPs and the *P. falciparum* mean intensities of infection.

[0063] Validation of candidate genes by RNAi

[0064] Challenge experiments with *P. berghei* and RNAi-mediated ablation of candidate gene mRNA accumulation were performed as described in Budd et al. (Michel K, Budd A, Pinto S, Gibson TJ, & Kafatos FC (2005) Anopheles gambiae SRPN2 facilitates midgut invasion by the malaria parasite Plasmodium berghei. *EMBO Rep* 6(9):891-897). Briefly, the *An. gambiae* G3 strain was maintained at 27°C, 80% humidity with a 12 hour alternating day-night cycle and fed on mice for egg production. The coding regions of *AgADA, FBN30* and *FREP1* were amplified from DNA derived from an adult cDNA library using gene-specific primers and a nested iterative strategy, and used as templates for production *in vitro* of double-strand RNA (dsRNA). No off-target sequences for the designed dsRNA were found in the *An. gambiae* genome. dsRNA was injected into the hemocoel of ~100 1-day old adult G3 mosquitoes and specific transcript levels were assayed quantitatively 72 hours later in a sample of five of them. The *An. gambiae* ribosomal S7 gene (AgS7) was used as an internal control for quantitative expression analyses. The remaining mosquitoes were fed the same day with a blood meal containing the GFPcon *P. berghei* strain (MRA-865). Midguts were dissected seven days later, stained with 0.1% mercurochrome and the number of oocysts counted.

[0065] RESULTS

[0066] Syntenic blocks in An. gambiae and C. quinquefasciatus

[0067] *C. quinquefasciatus* supercontigs were compared with the *An. gambiae* reference genome based on translated protein sequences. Neighboring conserved regions were joined to define the boundaries of syntenic blocks. For example, comparison of *C. quinquefasciatus* contig3.1 and the *An. gambiae* chromosome 2L identified nine conserved syntenic blocks. These steps were repeated between 3,171 *C. quinquefasciatus* superc ontigs and the *An. gambiae* chromosomal arms resulting in a total of 1,242 syntenic blocks detected in the latter species. The chromosome arms 2L, 2R, 3L, 3R and X contain 237, 348, 197, 228 and 232 syntenic blocks, respectively, which is similar to a previous report (n=1514) that was based on ortholog analysis. The median size of the syntenic blocks in *An. gambiae* was ~ 37.7 kilo base-pairs (kb) in length with ~12 predicted protein-coding genes. Most individual *C. quinquefasciatus* supercontigs mapped to a single chromosome arm in the *An. gambiae* genome confirming a previous conclusion that few translocation events had occurred between different chromosomes. There were 1,252 non-conserved genomic regions in the *An. gambiae* genome. The major malaria resistance locus, PRI, has 56 conserved syntenic blocks with a median length of ~45kb. The PRI was detected previously in mosquitoes with a chromosomal inversion between 20,528,089 bp and 42,165,532 bp. However, the present map is based on

the PEST strain used as the reference genome (AgamP3.53), which lacks this inversion. This results in the PRI localizing in our map to two non-contiguous domains.

[0068] Expression profiles of syntenic blocks for the innate immunity

[0069] Approximately 186,344 probes on the Affymetrix microarray chip were aligned based on their sequences to the current *An. gambiae* reference genome (AgamP3.53). Among these, 147,549 were mapped uniquely to chromosomes 2, 3 and the X. Detailed information on probe sequence, location and signals can be downloaded in text format from http://omics.ou.edu/download/.ExpressionPattern/OligoSignalAndPosition.tab. A hierarchical expression tree of all genomic blocks constructed based on the probe signal showed that the mRNA abundance resulting from transcription in many genomic regions was lower at 24, 48, and 72 hours post blood feeding when comparing young (three days post emergence) naïve (NBF) and 3 hours post blood feeding mosquitoes (BF3h). These observations are consistent with a physiological switch from host-seeking activity to egg production after a blood meal. In addition, expression in most genomic blocks was different between younger and older (14 days post emergence) animals, and this is consistent with previous observations. Therefore, the genomic block expression tree was consistent with the overall transcript abundance profiles in different conditions.

[0070] QTL discovery is based on the segregation of genetic variations and traits in pedigree analyses. The underlying genetic variations may not alter the expression of that gene and it may not be regulated differentially by variable conditions such as infection. Therefore, known infection-related genes were used as a training set to identify enrichments of characteristic expression patterns. This strategy differs from approaches that determine candidate genes based the differentially-expressed genes under parasite challenge. Seventy known genes were used to label genomic blocks on the expression tree to detect enrichment profiles related to parasite infection. Three branches were enriched significantly with immunity blocks.

[0071] Genetic variation in wild-derived mosquitoes from malaria endemic areas in Kenya.

[0072] More than 10,000 mosquito larvae were collected independently from different habitats in Kenya and reared to adults under laboratory conditions. Approximately 1,000 three- to five-day old morphologically-confirmed *An. gambiae* were fed on *P. falciparum*-infected human blood, with ~10% of them becoming engorged. Whole genome sequencing was used to detect the genetic variation in the wild *An. gambiae* populations. Based on previous reports, the frequency of genetic alleles for PRI is ~0.17, thus >8 mosquitoes are

required to gain the 95% confidence of not missing the candidate genetic alleles. The genomic DNAs of nine confirmed *An. gambiae* (5 un-infected, no oocysts in midguts; 4 infected with 2, 8, 8, 9 oocysts) that fed on the same *P. falciparum* infected human blood were sequenced to detect the genetic variation. The length of each sequence read was 100 bp and the whole-genome coverage for each individual was >36. More than 1.6 X 10^6 SNPs were detected genome-wide in each individual mosquito. This high SNP frequency supports a previous report of ~1 SNP/200 bp. Furthermore, 347 SNPs encoded non-synonymous changes in genes within the candidate genomic regions and were present in at least two mosquitoes. These SNPs were verified manually using GBrowse graphics user interface and the results indicated a low error rate (<1%) caused by sequencing or detection.

[0073] *FREP1, FBN30,* and *AgADA* genes were identified by the association between genetic variation and the *P. falciparum* infection in wild *An. gambiae*

[0074] Eighty candidate genes within PRIs a-e were prioritized by the relationship between the 347 SNPs and the *P. falciparum* infection in the nine mosquitoes. Five SNPs within five genes associated with mean intensity of infection (p-value < 0.05) as well as infection prevalence (p-value <0.12) were chosen for further analysis.

[0075] The five candidate genes from 22 individual wild mosquitoes infected with *P. falciparum*, were cloned and genotyped. The data supported the conclusion that three of five candidate SNPs in three genes (*FREP1, FBN30,* and *AgADA)* are associated significantly with *P. falciparum* infection (FIG. 1). The SNPs, C427T, in *AgADA* (AGAP006906) and C28T in *FBN30* (AGAP006914), are associated significantly with prevalence and mean intensity of infection (FIG. 1A-B). The A1325T SNP in *FREP1* (AGAP007031) is associated significantly with the mean intensity of infection (FIG. 1C). These mutations were designated mosquitoes against *Plasmodium falciparum parasites* (*maplap*) *maplap1*, *maplap2* and *maplap3*, respectively. *maplap1* changes amino acid 143 from arginine to cysteine (R143C), *maplap2* changes amino acid 10 from phenylalanine to leucine (F10L), and *maplap3* changes amino acid 442 from glutamine to leucine (Q442L). As anticipated, the infection intensity in mosquitoes increased when mosquitoes have more susceptible alleles (FIG. 1D).

[0076] Based on the genotypes from 22 mosquitoes, the correlation coefficients between maplap1 and maplap2, maplap1 and maplap3, maplap2 and maplap3 are 0.009, 0.005 and 0.02, respectively, according to Haploview analysis (Barrett JC, Fry B, Maller J, & Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21(2):263-265), supporting the conclusion that the effects of the three genes are independent

of one another and do not result from linkage effects. This result of the small haplotype block size in wild-derived *An. gambiae* populations is consistent with previous reports.

[0077] Impact of FREP1, FBN30, and AgADA dsRNA-mediated knockdown on *Plasmodium* parasite infection

[0078] There is a report of immune-related genes whose products exhibit different effects on *P. falciparum* and *P. berghei* (Mitri C, *et al.* (2009) Fine pathogen discrimination within the APL1 gene family protects Anopheles gambiae against human and rodent malaria species. *PLoS Pathog* 5(9):e1000576). The three genes in this study are associated significantly with *P. falciparum* infection and we used the rodent parasite in RNAi experiments to test whether they function across species. FREP1 mRNA was reduced ~80% in treated mosquitoes following injection of gene-specific dsRNAs (FIG. 2A) and surprisingly, this resulted in a reduction in infection prevalence from 70% and 83% to 30% and 31%, respectively, in two replicates of control and experimental mosquitoes (FIG. 2B-D). In contrast, ablation of FBN30 transcripts to undetectable levels resulted in a two-fold increase in the oocyst mean intensities of infection (FIG. 2E-H) (p-value < 0.004). The prevalence was similar: 82% versus 95% and 95% versus 96%, in two replicates for control and experimental samples, respectively. Knock-down of AgADA transcripts reduced slightly *P. berghei* mean intensity of infection when comparing control and experimental groups (FIG. 2I-L). Prevalence also was lower (82% and 85% to 76% and 70%, respectively) in AgADA dsRNA treated mosquitoes.

[0079] Discussion

[0080] Several quantitative trait loci (QTL) for *Plasmodium* parasite infection of *An. gambiae* have been identified. However, the high genetic variation and small available sample size make the identification of natural resistance genes a major challenge. Microarray-based differential expression often is used to detect candidate genes. However, the genes underlying QTLs may not be regulated by a parasite infection. Multiple independent expression profiles were integrated to find immune signature patterns using a set of training genes and thereby unlinked the basal parasite-resistance phenotype from that elicited in response to infection. Microarray data at the gene level also may be imprecise due to the co-existence of genuine biological variation. Combining related genes as modules was used previously to find robust signatures. Here syntenic chromosomal domains were used as higher-level modules to detect the immunity-signature expression patterns against malaria parasites in *An. gambiae*, and narrowed the candidate genomic regions.

[0081] Three SNPs, (maplap1, maplap2, maplap3) were found in *AgADA, FBN30* and *FREP1*, respectively, related to parasite infection in *An. gambiae*. maplap1 in *AgADA* is associated significantly with *P. falciparum* mean intensity of infection and prevalence in wild-derived mosquitoes from Kenya. It is interesting to note that mutations in adenosine deaminase also cause severe immunodeficiency in humans, supporting the conclusion that this gene is conserved in metazoan immunity. However, knock-down of *AgADA* did not alter greatly mosquito susceptibility to *P. berghei* as anticipated. This may result from differences between the local *P. falciparum* isolates and rodent malaria *P. berghei* or physiological differences between the naturally-occurring mutation and experimental transient down-regulation of *AgADA* expression.

[0082] The fibrinogen-related gene family has been found to defend against pathogens in invertebrates as pattern recognition proteins, and there are 59 putative fibrinogen-related genes (FREPs or FBNs) in *An. gambiae*. Fibrinogen-related genes were screened previously by ablating several genes simultaneously in the immunity against parasites and it was proposed that FBN9 was the most-potent anti-*Plasmodium* FREP protein. The present one-by-one analysis of the candidate genes supports the conclusion that FBN30 and the FREP1 also play important roles during parasite infection.

[0083] Although FBN30 and FREP1 proteins are members of FBNs, and both have signal peptides and fibrinogen-like domains at their N- and C-termini, respectively, they differ in length and expression profiles, and have contrasting effects against parasites. FREP1 and FBN30 have 738 and 280 amino acids, respectively. FBN30 is expressed highly in mosquito fat body tissues and inhibits parasite development, supporting the hypothesis that FBN30 proteins may be involved in humoral innate immunity. In contrast, the FREP1 protein is expressed highly in midguts and it facilitates parasite infection, supporting the conclusion that FREP1 acts as a receptor of *Plasmodium* parasite to facilitate ookinete invasion. The detailed molecular mechanisms of these proteins against *Plasmodium* require further elucidation. In conclusion, PRI in *An. gambiae* populations from malaria endemic areas were analyzed, and identified and confirmed that the *AgADA, FBN30,* and *FREP1* genes play roles in the *Plasmodium* parasite infections in the *An. gambiae. FREP1* was determined to be essential for the *Plasmodium* invasion of *An. gambiae.*

[0084] Example 2

[0085] As shown above, a mosquito gene, *FREP1*, has been identified that is implicated in *Plasmodium* infection in mosquitoes. Genetic polymorphisms in *FREP1* are significantly associated with *P. falciparum* infection intensity levels in wild *An. gambiae* populations from

Kenya. FREP1 protein is a member of the fibrinogen-related protein family (FREPs or FBNs) that contains a highly conserved C-terminal interacting fibrinogen-like (FBN) domain among mosquito species. In invertebrates, FREP/FBNs are common pattern recognition receptors (PRR). Previous work examining the role and function of FREP/FBN family members in *Anopheles* mosquitoes has shown that two family members, FBN9 and FBN30, appear to restrict *Plasmodium* infection of midgut epithelial cells. Indeed, silencing the expression of either FBN9 or FBN30 in mosquitoes increased *Plasmodium* infection. Here, the role and function of a third FREP/FBN family member, FREP1, during *P. falciparum* infection of Anopheles mosquitoes is demonstrated. Genetic and biochemical assays reveal that FREP1 functions as a critical molecular anchor in the PM that is required for *Plasmodium* ookinetes to invade and infect of mosquito midguts. In contrast to FBN9 and FBN30 that inhibit *Plasmodium* infection, these results show that FREP1 is an essential host factor that mediates the major human pathogen, *P. falciparum* to infect mosquito midguts. Antibodies against FREP1 protein inhibit the infection of *P. falciparum* in mosquitoes. Collectively, these data reveal new insight into *Plasmodium-Anopheles* interactions, and demonstrate that FREP1 as an efficient antigen to elite antibodies and inhibit malaria transmission.

[0086] MATERIALS AND METHODS

[0087] Rearing *An. gambiae* mosquitoes

[0088] *An. gambiae* G3 strain was maintained at 27°C, 80% humidity with a 12-hour day-night cycle. Larvae were reared on ground KOI fish food supplements (0.1 mg per larvae per day). Adult mosquitoes were maintained with 8% sucrose and fed with mouse blood (mice were anesthetized with ketamine/xylazine) for egg production.

[0089] Generating anti-FREP1 polyclonal antibody

[0090] *FREP1* was cloned using PCR with primers shown in Table 1 from *An. gambiae* mosquito cDNA library that was generating by reversely transcribing from total RNA. The PCR product and pQE30 plasmid were digested with restriction enzymes XmaI and Hind III. After ligation and transformed into E. coli JM109, the positive plasmid verified by PCR was transformed into E. coli M15 strain. One μM IPTG was used to induce gene expression in E. coli M15 strain. The expressed cells were lysed in buffer B (8 M Urea, 100mM NaH_2PO_4, 10mM Tris·Cl, pH 8.0). Since there was a 6xHis tag at the N-terminal of expressed protein, the recombinant FREP1 protein was purified by Ni-NTA column using a standard protocol (QIAGEN (2003) The QIAexpressionist: A handbood for high-level expression and purification of 6XHis-tagged proteins. QIAGEN: QIAGEN). The purity of recombinant

FREP1 protein was confirmed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. The purified recombinant FREP1 protein was then used as an antigen to generate customized polyclonal antibody against FREP1 in rabbits (Thermo Fisher Scientific, Rockford, IL, USA). Rabbits were boosted three times in 2-week intervals, after which anti-FREP1 antibody in anti-serum was purified with affinity chromatograph protein A-agarose and suspended in PBS.

[0091] Expressing recombinant FREP1 protein in insect cells

[0092] A *FREP1* complete coding sequence (SEQ ID NO:1) was obtained by PCR with primers shown in Table 1 from adult *An. gambiae* cDNA library, and was cloned into plasmid pIB/V5-His (Life Tech, Grand Island, NY) to generate plasmid pIB-FREP1. After amplified in E. coli DH5, the plasmid was purified with endotoxin-free plasmid preparation kits (Sigma-Aldrich, St. Louis, MO). The cabbage looper ovarian cell-derived High Five cells (Wickham TJ, Davis T, Granados RR, Shuler ML, Wood HA (1992) Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol Prog 8: 391-396) was used to express recombinant FREP1 protein (SEQ ID NO:2). In brief, endotoxin-free recombinant pIB-FREP1 plasmids were mixed with Cellfectin® Reagent (1μl Cellfectin/μg plasmids, Invitrogen) in 5-6ml Express Five® SFM medium (Invitrogen, Grand Island, NY). The cells were cultured in 25cm^2 CELLSTAR® cell culture flask (Greiner Bio-One, Monroe, NC) for 48 hours at 27°C. Medium and cells were separated by centrifugation at 300 g for 5 minutes. The proteins in medium were concentrated using Amicon® ULTRA-4 Centrifugal Filter Devices (Milipore, Billerica, MA) by centrifugation at 5,000 g for 10 minutes.

TABLE 1: PCR Primers

Purpose	Primer name	Primer sequence (SEQ ID NO:)
In vitro synthesis of *FREP1* dsRNA	Forward	5'-TAATACGACTCACTATAGGAGCTCGAGGTGAAGCAGAG-3' (3)
	Reverse	5'-TAATACGACTCACTATAGGTTCTCCAGCCGGTTGTGT-3' (4)
Verify *FREP1 mRNA* expression	Forward	5'-ACAGGGCAAGTTCGAGAAGA-3'(5)
	Reverse	5'-AAGTCAACCGTACCGTCCTG-3' (6)
AgS7 gene	Forward	5'-GGCGATCATCATCTACGTGC-3' (7)
	Reverse	5'- GTAGCTGCTGCAAACTTCGG-3' (8)
In vitro synthesis of *GFP* dsRNA	Forward	5'-TAATACGACTCACTATAGGCAAGTTTGAAGGTGATACCC-3'(9)
	Reverse	5'-TAATACGACTCACTATAGGCTTTTCGTTGGGATCTTTCG-3'(10)
Clone *FREP1* gene into pQE30	Forward	5'-A*CCCGGGCAC*TGCCCTGAACGGTGCAG-3' (11)
	Reverse	5'-GGC*AAGCTT*CGCGAACGTCGGCACAGTC-3' (12)
Clone *FREP1* gene into pIB/V5-His	Forward	5'-TCA*AAGCTT***CACC**ATGGTGAATTCATTCGTGTCG-3' (13)
	Reverse	5'-AC*TCTAGA*TTACGCGAACGTCGGCACAGTCGTG-3' (14)

The italic and underlined sequences denote restriction recognition sites. The underlined sequences are T7 promoter. The bold sequence is Kozak consensus sequences. The primers were synthesized through Integrated DNA Technologies Inc. (USA).

[0093] Gel Filtration Chromatography to determine the FREP1 protein size

[0094] Similar to the previous description (Duong-Ly KC, Gabelli SB (2014) Gel filtration chromatography (size exclusion chromatography) of proteins. Methods Enzymol 541: 105-114), about 0.1mg recombinant FREP1 protein in 0.5ml culture medium was applied onto fast protein liquid chromatography gel filtration (Bio-Rad, Hercules, CA) with sephadex G-200 column (60 cm in length, 0.5 cm in diameter) with flow rate controlled to 0.2 ml/min. Sephadex G-200 can separate proteins ranging from 5KD to 600KD. Fractions of approximately 0.1 ml were collected. ELISA and western blot were used to detect recombinant FREP1 protein in the fractions. Standard curve of molecule weight for the gel filtration column at each fraction was obtained using a set of proteins with known molecular weights.

[0095] Immunohistochemistry assay to determine protein distribution in mosquito tissues

[0096] Midguts from 3-5 day-old naïve and blood-fed female mosquito were dissected in PBS. Tissues were embedded completely in optimal cutting temperature (OCT) compound and immediately frozen in liquid nitrogen. Frozen midguts were sectioned (8-10 μm) with a cryostat. The sections were mounted on super frost plus slides (positively charged), air dried for 30 minutes at room temperature and fixed in 4% paraformaldehyde-PBS for 20 minutes and stored at -20°C until use. Prior to staining, sections were re-hydrated in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6) with 0.05% tween-20 (TBST) for 10 minutes. Sections were blocked with 1-3 drops of blocking solution (5% dry milk in TBST) for 30 minutes, and then next incubated with 1:2,000 diluted rabbit anti-FREP1 antibodies in blocking solution for two hours. Control sections were incubated with blocking solution containing pre-immune rabbit antibodies. Samples were washed 3 times for 5 minutes with TBST. Next 100 μl of 1:20,000 diluted goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) in blocking solution was incubated with each sample for 30 minutes at room temperature. Slides were then washed 3 times in TBST. Finally, the sections were developed with 100 μl of BCIP/NBT Chromogen Solution (Sigma-Aldrich; St. Louis, MO) for 10-20 minutes, rinsed with water, and examined under a microscope at 64 and 640x magnification.

[0097] Preparation of *P. falciparum* gametocytes and ookinetes

[0098] *P. falciparum* parasites (NF54 strain from MR4) were added into O^+ fresh human blood (4% RBC, 0.5-1.0% parasitemia). Cultures were maintained in 6-well plates (Corning Incorporated Costar) with 5.0 ml complete RPMI-1640 medium supplemented with 10% heat-inactivated human AB-type serum (Interstate blood bank, Memphis) and 12.5 μg/ml hypoxanthine. The plates were maintained under 37 °C in a candle jar, and medium was replaced daily until day 15-17. The parasitemia or gametocytemia was checked every other day by Giemsa staining of thin blood smears. To prepare ookinetes, the cultured *P. falciparum* cultures harboring stage V gametocytes were diluted 10-fold in complete RPMI-1640 (no sodium bicarbonate). The cultures were incubated at room temperature (RT) for 24 hours to simulate the formation of zygotes and ookinetes. The culturing and infection experiments of *P. falciparum* were conducted in the biosafety level 2 lab at the University of Oklahoma.

[0099] Indirect immunofluorescence assays to examine the binding between parasites and insect cell-expressed recombinant FREP1 protein

[0100] *P. falciparum* cultures were deposited on premium cover slip glass slides (Fisher Scientific) to make blood smear. Dry smears were fixed in 4% paraformaldehyde in PBS at RT for 30 mins, and then sequentially incubated in PBS containing 10 mM glycine for 20 mins, dry ice cold methanol for 5 mins, 0.2% BSA in PBS for 30 mins, anti-FREP1 antibody (1:1000 dilution in PBS containing 0.2% BSA) for two hours, enhancer (Alexa Fluor ® 594 Goat Anti-Mouse SFX kit, Invitrogen) for 30 minutes, and secondary antibody (Alexa Fluor ® 594 Goat Anti-rabbit Antibody, 1:1000 dilution in 0.2% BSA, Life Technologies Inc.). Between each incubation, the smears were washed 3 times for 3 minutes in PBS containing 0.2% BSA. Cover slips were rinsed in distilled water for 20 seconds, and 50 μl vectashield mounting media (Vector Laboratories, Burlingame, CA) was added onto the cover slip and the cover slip was covered onto slides. After incubating for at least 2 hours in dark, the cells were examined using Nikon Eclipse Ti-S fluorescence microscope.

[0101] Binding assay between FREP1 and *Plasmodium* parasites by ELISA

[0102] Cultured *P. falciparum* infected RBC and healthy human RBC were collected and washed three times with incomplete RPMI-1640, and then re-suspended (1:3 in volume) in 1XPBS containing 0.2% Tween-20 (PBST). The cells were homogenized by ultrasonication (10 seconds sonication followed by 50 seconds cooling down on ice for 6 times). The insoluble materials or intact cells were removed by centrifugation at 8,000 g for 2 minutes. The protein concentration in supernatant was measured using Bradford protein assay kit

(Thermo Scientific, Rockford, IL). About 100µl lysate containing 2mg/ml protein concentration was added per well into immunoGrade microplate (Brand, Wertheim, Germany), and the plates were incubated over night at 4 ^{0}C. Microplates were blocked with 100 µl 0.2mg/ml BSA in PBS per well for 1.5 hours, followed sequential incubation of 100 µl insect cell expressed recombinant FREP1 protein (7.5 µg/ml) for 1 hour, 100 µl anti-FREP1 antibody (diluted 5000-fold with PBS-0.2% BSA) for 1 hour, 100 µl alkaline phosphatase-conjugated anti-rabbit Ig G (1:20000 dilution with PBS-0.2% BSA) for 1 hour. The wells were washed three times with PBST between incubations. The wells were developed with 100 µl of pNPP solution (Sigma-Aldrich, St. Luis, MO). When colors in wells were visible, the optical density absorbance (405 nm) was measured.

[0103] Ablating FREP1 expression by dsRNA to verify its function on susceptibility to *P. falciparum* infection in *An. gambiae* mosquitoes

[0104] *FREP1* was cloned from *An. gambiae* mosquito cDNA library as described above. Briefly, nested-PCR using the primers listed in Table 1 was used to generate a DNA template for in vitro synthesis of dsRNA. The non-mosquito sequence, amplified from Aequoria green fluorescent protein (GFP) gene using primers listed in Table 1, was used as a negative control. Double-stranded RNA (dsRNA) was synthesized from these gene fragments using the in vitro transcription system T7 Megascript (Amibon, TX). The dsRNA was purified with Qiagen RNA purification kit. About 207 ng dsRNA in a 69 nl solution were injected into the hemocoel of each cold-anesthetized 1-day old *An. gambiae* G3 female mosquito. About 100 mosquitoes per treatment were used for RNAi knockdown experiments. Thirty-six hours after dsRNA injection, the treated mosquitoes were fed with *P. falciparum* infected blood that contained 0.2% gametocytes through membrane feeding following an established protocol. Seven days post infection and feeding, treated mosquitoes were dissected in phosphate buffer saline (PBS, pH7.2) and oocysts numbers were counted under light microscope after staining with 0.1% mercury dibromofluresein disodium salt in PBS. In the negative control group, ~100 mosquitoes injected with GFP dsRNA were also exposed to the same cultured parasites, and all procedures were the same as the experimental group. The transcript knockdown efficiency was confirmed by quantitative RT-PCR in five treated mosquitoes that were taken randomly 12 hours after infection.

[0105] Antibody blocking assays of *P. falciparum* infection in *A. gambiae* mosquitoes.

[0106] *P. falciparum* infected blood cultures containing mature stage V gametocytes were diluted with fresh O+ type human blood to get the 0.2% final concentration of stage V gametocytes. An equal volume of heat-inactivated (65 °C for 15 minutes) AB-type human

serum was added. Serial dilutions of rabbit polyclonal anti-FREP1 antibody (5mg/ml, 4mg/ml, 2mg/ml, 1mg/ml, 0.5mg/ml) in PBS were added to the gametocyte cultures (1/10 volume of blood). Artificial membrane feeding was conducted using 3-day old female naive mosquitoes. After feeding for 15 minutes, the engorged mosquitoes were maintained with 8% sugar in a BSL-2 insectary (28 °C, 12-hour light/dark cycle, 80% humidity). Seven days after infection, mosquitoes were dissected and the midguts were stained with 0.1% mercurochrome and examined using light microscopy to count the number of oocysts. The purified pre-immune rabbit antibody (Thermo Scientific, IL, USA) was used as controls.

[0107] Results

[0108] Recombinant FREP1 protein is secreted from insect cells into culture medium, and form tetramers

[0109] To understand the basic biochemical characteristics of FREP1 protein, its functional domains were examined. According to our previous genome annotation (Li J, Ribeiro JM, Yan G (2010) Allelic gene structure variations in *Anopheles gambiae* mosquitoes. PLoS One 5: e10699), the full length of FREP1 protein has 738 amino acids, including a 22-amino acid signal peptide at the N-terminal, three coiled-coil regions, and a conserved ~200-amino acid FBN domain at the C-terminal (FIG. 3A). All six cysteine amino acid residuals are within FBN domain.

[0110] Cellular *FREP1* expression patterns in vitro were examined next. We cloned and expressed the full-length FREP1 protein in High Five insect cells under the OplE2 promoter by the plasmid pIB/V5-His and then used a reducing SDS-PAGE and western blotting analysis to detect FREP1 in secreted or cellular fractions. The results show that FREP1 protein was exclusively detected in the cell culture supernatant, and no FREP1 protein was detected in the cell pellet (FIG. 3B). Therefore, FREP1 is a secreted protein.

[0111] To determine whether FREP1 assumes distinct quaternary structures, non-reducing SDS-PAGE and gel filtration chromatography was used. The result of non-reducing SDS-PAGE and western blotting was the same as FIG. 3B, indicating that insect cell-expressed recombinant FREP1 protein exists as either monomers or multimers that associate via non-covalent interaction. Gel filtration chromatography was utilized to separate recombinant FREP1 protein and protein complexes, and ELISA was subsequently used to quantify the recombinant FREP1 protein in each fraction. Based on the gel-filtration standard curve, the size of recombinant FREP1 protein appeared to be between 293KD and 353KD (FIG. 3C). Because the unit molecular weight of FREP1 protein is about 80KD, data support that FREP1

can exist as a tetramer (~320KD). Together, these data show that FREP1 is a secreted protein that can form tetramers through hydrophobic intermolecular interactions.

[0112] FREP1 protein localizes to the peritrophic matrix in mosquito midguts after blood feeding

[0113] Next, the localization of FREP1 protein in mosquito midguts was determined. Consistent with previously reported microarray-based transcriptome data, FREP1 mRNA abundance in midguts is about double of other tissues (FIG. 10A). Because the microarray data support that FREP1 is highly expressed in mosquito midguts, FREP1 protein in mosquito midguts were further analyzed by immunohistochemistry (IHC) assays using polyclonal anti-FREP1 rabbit antibodies (FIG. 4). Comparisons between the negative control sections (FIG. 4A, pre-immune antibody) and the experimentally stained sections (FIG. 4B, anti-FREP1 antibody) of naïve mosquito midguts only showed a weak FREP1 signal (purple color on IHC sections). However, 12 hours post bloodmeal, the FREP1 signal (purple color) in the experimental sections (FIG. 4D) is significantly more intense than that in the control sections (FIG. 4C), indicating that the FREP1 protein becomes detectable in mosquito midguts after bloodmeal. This result is consistent with microarray-based expression data showing up-regulation of FREP1 mRNA expression in mosquitoes following bloodmeal feeding (FIG. 10B). Furthermore, two portions of stained midgut sections (rectangles in FIGS. 4C and 4D) were magnified 640X to show a more detailed structure (FIGS. 4E and 4F, respectively). Strikingly, the majority of FREP1 protein was found to be localized in the mosquito PM that resides within the midgut lumen. Consistent staining patterns and FREP1 localization were observed in more than 3 independent experiments. Together, the data from the microarray analyses and our new IHC studies consistently show that *FREP1* gene is up-regulated after blood feeding and that FREP1 protein is secreted into mosquito midgut lumen and associated with the PM.

[0114] FREP1 protein binds *P. falciparum*

[0115] Next it was analyzed whether FREP1 protein could interact with *P. falciparum* parasites or parasite-infected red blood cells (RBC). *P. falciparum* infected RBC at various developmental stages was fixed on a cover glass, and then probed the cells with insect cell-expressed recombinant FREP1 protein. Anti-FREP1 antibody and fluorescence conjugated secondary antibodies were used sequentially to determine whether FREP1 protein bound to cells. In fluorescence assays, the bound FREP1 protein appears red and cell nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) appear purple. The results (FIG. 5) showed that the recombinant FREP1 protein bound to asexual stage (ring stage trophozoite)

P. falciparum-infected cells (FIG. 5, first row), gametocytes (FIG. 5, middle row), and ookinetes (FIG. 5, third row). Of note, non-infected RBC do not have nuclei and cannot be stained by DAPI. Among more than 50 independent fields examined by microscopy, approximately 80% of DAPI-positive (parasite-infected) cells showed FREP1 binding intensities that were at least double that of uninfected cells. Notably, it was also found that as the parasites matured and developed from rings to gametocytes to ookinetes, the FREP1 staining patterns became more distinctly localized to specific regions on the parasite surface (FIG. 5, first to third row). Indeed, diffuse FREP1 staining was observed on rings, whereas gametocytes and ookinetes exhibited discreet, intense regions of FREP1 binding. Collectively, these data support that FREP1 associated with RBC infected with either asexual or sexual stage *P. falciparum* parasites. Moreover, the FREP1 binding patterns transition from spatially diffuse to more distinct as the parasites mature from asexual stages into sexual stage parasites or diploid ookinetes.

[0116] As a complementary approach, an ELISA assay was used to confirm the interaction between insect cell-expressed recombinant FREP1 and parasites. The lysates of cultured *P. falciparum* parasites (~10% parasitemia) were used to coat ELISA plates, and plates were then incubated with insect cell-expressed FREP1 protein. Bovine serum albumin (BSA) or heat-inactivated recombinant FREP1 protein were used to substitute FREP1 as negative controls. The results (FIG. 6A) indicate that significantly more ($p<10^{-5}$) recombinant FREP1 protein was retained in wells than the negative control in three replicates. Importantly, the ELISA signal disappeared if the insect cell-expressed FREP1 protein was denatured at 65 °C for 15 minutes before use (FIG. 6B). Because the polyclonal anti-FREP1 antibody can recognize denatured FREP1, the diminished ELISA signal of heat-inactivated recombinant FREP1 protein confirmed a conformation-dependent interaction between insect cell-expressed recombinant FREP1 protein and parasites.

[0117] Finally, to confirm that FREP1 protein binds parasites and not uninfected RBC, ELISA plates were coated with lysates from parasite-infected RBC that were enriched with parasites representing each developmental stage. For asexual stage *P. falciparum*, we used 3-day old cultured *P. falciparum* infected cells with ~90% uninfected RBC, ~10% trophozoites and schizonts, and no detectable gametocytes. For sexual stage *P. falciparum*, 14-day old cultured *P. falciparum* infected cells with 79%±5% uninfected cells, 15%±3% schizonts, and 6%±3% gametocytes were used. For ookinetes, 14-day old *P. falciparum* infected cells were cultured at room temperature for two days, resulting in cells containing ~0.5% ookinetes. The lysate of naive RBC was used as a negative control. The total protein concentrations in these

lysates were normalized prior to coating the ELISA plates. As shown in FIG. 6B, the *P. falciparum* infected cells from asexual stage parasites, gametocytes, and ookinetes display significantly higher binding signals than the negative control ($p<0.02$), confirming that FREP1 protein can bind parasites of all stages. The ELISA signals also increased from ring stages to ookinetes, indicating the concentrations of FREP1-binding partners increased with the increase of the number of parasites in cultures. These results from IHC and ELISA collectively demonstrate that FREP1 protein can specifically bind to constitutively parasite-expressed molecule(s) at multiple developmental stages.

[0118] Ablating FREP1 expression reduced *P. falciparum* infection

[0119] To determine whether FREP1 directly regulates *P. falciparum* infection of Anopheles mosquito vectors, a standard dsRNA-mediated gene-silencing assay was used to knockdown *FREP1*, and analyzed the impact of this silencing on *P. falciparum* infection of mosquito midguts. One-day old *An. gambiae* G3 adult female mosquitoes were injected with FREP1 dsRNA, and were subsequently fed on cultured *P. falciparum* gametocytes 36 hours post- dsRNA injection. Because blood up-regulates *FREP1* expression, the FREP1 mRNA abundance 12 hours post bloodmeal infection was analyzed. The quantitative RT-PCR results show that FREP1 mRNA abundance 12 hours post bloodmeal was barely detectable, e.g. 20-fold lower, in FREP1 dsRNA treated mosquitoes (FIG. 7A, lane 2) than that in the control mosquitoes that were treated with GFP dsRNA (FIG. 7A, lane 1). The AgS7 gene, which is constitutively expressed in mosquitoes, was used as a loading control in the quantitative RT-PCR assays (FIG. 7A, lane 3 and 4). Seven days post infection, the number of oocysts between FREP1 dsRNA treated and control GFP dsRNA treated mosquitoes was compared. The results show that significantly ($p < 0.005$) fewer oocysts developed in FREP1 depleted *An. gambiae* midguts (FIG. 7B), compared to the GFP dsRNA treated mosquitoes (FIG. 7C), indicating that FREP1 facilitates *P. falciparum* infection of the *An. gambiae* midgut. Consistent results for two independent dsRNA-mediated gene expression-silencing experiments (FIG. 7D) were obtained. Collectively, these results show that FREP1 associated with *P. falciparum* as a parasite agonist in mosquitoes.

[0120] Anti-FREP1 antibody blocks *P. falciparum* parasite infection in mosquitoes

[0121] If FREP1 exerts its function through direct interaction with *Plasmodium* parasites in the PM, interfering with this interaction would be expected to inhibit parasite infection of the mosquito midgut. To test this hypothesis, rabbit anti-FREP1 polyclonal antibody was mixed with cultured *P. falciparum* gametocytes (0.2% gametocytes, 0.5 mg/ml antibody) prior to use in membrane feeding-based infection assays. A purified pre-immune rabbit

antibody was used as a negative control. Strikingly, co-ingestion of gametocytes with the anti-FREP1 antibody significantly ($p<0.0001$) reduced *P. falciparum* infection as indicated by a reduction of average 10 and 12 oocysts per midgut to 0.4 and 0.2 oocysts per midgut of the pre-immune rabbit antibody control treatments respectively in two replicate experiments. The infection prevalence also decreased from 85% and 82% to 35% and 16% between the experimental and control mosquito cohorts respectively in two replicates (FIG. 8A). To examine the effect of anti-FREP1 antibody concentration on blocking *P. falciparum* infection, a dilution series (0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml) of antibodies was tested. The results (FIG. 8B) show that as the concentration of anti-FREP1 antibody decreased, the number of oocysts in mosquito midguts increased. Notably, even the antibody concentrations as low as 0.1 mg/ml, which is 5-times lower than the concentration of antibody in the original undiluted rabbit serum, still mediated significant ($p<0.003$) reductions in *Plasmodium* parasite infection of mosquitoes.

[0122] Discussion

[0123] As demonstrated in Example 1, polymorphisms within the *FREP1* gene are associated with reduced *P. falciparum* infection intensity. It was desired herein to investigate the molecular mechanisms and functions of FREP1 protein during infection of mosquitoes by the human malaria pathogen *P. falciparum*. Firstly, it was demonstrated that FREP1 protein is expressed in the mosquito midgut PM and exerts its effects on *Plasmodium* infection in the PM. It is well known that PM formation occurs ubiquitously in the midguts of hematophagous insects and serves as an important physical barrier to resist or prevent invasion by pathogens in blood. Previous studies showed that the *FREP1* gene was up-regulated by bloodmeal. Here it was shown that FREP1 protein is secreted into the mosquito midgut lumen after a bloodmeal and is associated with the PM. Another important component in PM is chitin, a polymer of N-acetylglucosamine (NAG). FBN domains in FREPs can recognize carbohydrates and their derivatives, and FBN domains can also bind acetyl groups within molecular complexes such as NAG [23].

[0124] Secondly, it was demonstrated that the quaternary structure of FREP1 is a tetramer. Invertebrate FREP/FBN family members tend to multimerize in order to exert their physiological functions. For example, the *An. gambiae* mosquito FBN9 protein forms dimers that interact with Gram-positive and Gram-negative bacteria. Moreover, the functional forms of horseshoe crab TL-5A and TL-5B proteins form propeller-like structures with each blade corresponding to a disulfide-linked dimer. Similarly, the present results show that FREP1 forms tetramers through hydrophobic forces, instead of disulfide-bonds, because non-

reducing SDS-PAGE showed that insect cell-expressed FREP1 is a monomer and gel-filtration showed that FREP1 forms a tetramer. Consistent with this, coiled-coil motifs in proteins have been reported to mediate protein homodimer complexes and tetramer complex formation. Therefore, the interaction of coiled-coil domains in FREP1 may also keep FREP1 monomers together as tetramers.

[0125] Thirdly, both indirect immunofluorescence assays and ELISA assays showed that the FREP1 protein interacts with parasites at different stages, ranging from asexual ring stage trophozoites to sexual stage gametocytes and diploid ookinetes. Interestingly, indirect immunofluorescence assays also showed that the putative parasite-expressed FREP1-binding partners tend to become localized to specific regions as the parasites develop from asexual stages to sexual stages to ookinetes. The redistribution of FREP1 protein binding partner(s) expressed on *Plasmodium* parasites might increase the affinity of interaction between FREP1 proteins and the ookinete. The redistribution of FREP1 protein binding partner on the parasite surface may also facilitate the ability of ookinetes to orient themselves towards midgut epithelial cells for efficient invasion. Since FREPs were proposed to act as pattern recognition molecules, the C-terminal FBN domain within FREP1 is likely responsible for mediating interactions between FREP1 proteins and *Plasmodium* parasites.

[0126] Finally, the in vivo function of FREP1 during *P. falciparum* infection of mosquitoes was examined. As indicated in Example 2, ablation of FREP1 expression in *An. gambiae* mosquitoes resulted in significant reduction of the number of rodent malaria pathogen *P. berghei* oocysts. These data were extended to investigate the influence of FREP1 on *P. falciparum* infection of mosquitoes using RNA interference. Importantly, the data show that FREP1 is also critical for *P. falciparum* infection. Knockdown of *FREP1* expression significantly reduced *P. falciparum* infection intensity and prevalence in *An. gambiae*. Collectively, FREP1 regulates infection by both *P. berghei* and *P. falciparum*, supporting the conclusion that FREP1 functions as a conserved host factor of *Plasmodium* invasion of the mosquito midgut. Thus, targeting *FREP1* or FREP1 can be used to block or limit the transmission and infectivity of multiple *Plasmodium* species.

[0127] Based on these experimental data, and without wishing to be bound by theory, a molecular model 10 for FREP1 activity during *Plasmodium* invasion is proposed (FIG. 9). *FREP1* 12 is up-regulated and expressed in midguts after bloodmeal and ultimately secreted into the mosquito midgut lumen. In the lumen, the FREP1 protein forms tetramers through interaction of coiled-coil regions of FREP1 monomers, and tetramers are integrated into the mosquito PM 14 by binding NAG unit in chitin 16. FREP1 12 is thus likely to be a structural

component of the PM 14. The tetramerization of FREP1 12 increases the binding affinity between FREP1 12, chitin 16 in PM 14, and parasites. A FREP1 binding partner 17 binds the FREP1 12 and ookinetes 18. The interaction between ookinetes 18 and FREP1 12 in PM 14 localizes and positions ookinetes on PM, and the enzymatic activities of the chintinase 20 secreted by ookinetes 18 and the active plasmin 22 on ookinete 18 surface may result in a disruption of the PM 14 structure for parasite invasion. After ookinetes crossing PM 14 and midgut epithelium cells 24, other proteins in mosquito hemolymph will interact with parasites and impact infection intensity.

[0128] According to this model, a treatment method in which *FREP1* gene or FREP1 protein is blocked would significantly reduce the capacity for mosquitoes to transmit malaria, which is consistent with experimental data reported herein. For example, if the concentration of gametocytes in a bloodmeal is below a certain threshold, e.g. less than 100 oocysts per midgut, blocking FREP1 by RNAi or antibodies could render a majority of mosquitoes resistant to *P. falciparum* (FIG. 8A) and *P. berghei*. When the density of gametocytes in blood is above a threshold, the infection intensity is still significantly lower in mosquitoes treated with dsRNA (FIG. 7D) or anti-FREP1 antibodies than that in the control groups (FIG. 8B). Importantly, because the number oocysts in wild *An. gambiae* when infected with clinical *P. falciparum* isolates in malaria endemic areas is usually very low, e.g. less than 10 oocysts per midgut, FREP1 remains a critical component of pathways of mosquito invasion at physiological parasite densities.

[0129] Ookinetes 18 have to overcome and exit the blood bolus barrier before reaching PM 14. Previous reports showed that mammalian plasminogen 26 can be captured by enolase 28 on the surface of ookinetes 18 (FIG. 9). Plasminogen is an essential serine protease precursor in vertebrate blood. After activation, plasminogen becomes plasmin that can cleave fibrin or fibronectin to free ookinetes in the blood bolus thereby facilitating their migration to the midgut PM. After traversing the PM, many ookinetes will manage to invade and cross the mosquito midgut epithelia through multiple pathways. Parasite penetration-induced apoptosis of midgut epithelial cells will further determine survival and/or successful infection by ookinetes. Therefore, interactions between PM and *Plasmodium* parasites (i.e., the 'interactome') mediated through FREP1 is important for parasite invasion, and targeting this interaction may prove effective for limiting malaria transmission. It is worth emphasizing that proteins in the PM are readily accessible by antibodies in blood. Thus, FREP1 can serve as an excellent antigenic target for inclusion in malaria transmission

blocking vaccines. Consistent with this notion, and as demonstrated h erein, anti-FREP1 antibodies significantly reduced the *Plasmodium* loads in mosquitos.

[0130] In at least one embodiment, the presently disclosed inventive concepts are directed to a method for blocking transmission of a *Plasmodium* species by administering to a mammalian or avian subject an immunogenic composition comprising a Fibrinogen-Related Protein 1 (FREP1) protein and/or an immunogenic portion thereof, causing formation of anti-FREP1 antibodies in the mammalian or avian subject, wherein the anti-FREP1 antibodies, when consumed by a mosquito from blood obtained from the mammalian or avian subject, prevent or inhibit the infection of the mosquito by the *Plasmodium* species. The FREP1 protein of the immunogenic composition may comprise the amino acid sequence set forth in SEQ ID. NO:2. The FREP1 protein of the immunogenic composition may have at least 70% identity with the amino acid sequence as set forth in SEQ ID NO:2. The immunogenic composition may comprise an excipient. The excipient may be at least one of a group consisting of carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives. The mosquito in an *Anopheles* species.

[0131] In at least one embodiment, the presently disclosed inventive concepts are directed to an immunogenic composition comprising an amount of a Fibrinogen-Related Protein 1 (FREP1) and/or an immunogenic portion thereof, which is immunogenically-effective in causing formation of anti-FREP1 antibodies in a mammalian or avian subject; and an excipient. The FREP1 protein may comprise the amino acid sequence set forth in SEQ ID. NO:2. The FREP1 protein may have at least 70% identity with the amino acid sequence as set forth in SEQ ID NO:2. The excipient may be at least one of a group consisting of carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives.

[0132] In at least one embodiment, the presently disclosed inventive concepts are directed to a polyclonal antibody composition, comprising polyclonal anti-FREP1 antibodies formed by the method of immunizing a mammalian or avian subject with an immunogenic composition comprising a FREP1 protein and/or an immunogenic portion thereof, wherein the immunogenic composition further comprises an excipient. The FREP1 protein of the immunogenic composition used to form the polyclonal antibodies may comprise the amino acid sequence set forth in SEQ ID. NO:2. The FREP1 protein of the immunogenic composition used to form the polyclonal antibodies may have at least 70% identity with the amino acid sequence as set forth in SEQ ID NO:2. The immunogenic composition used to form the polyclonal antibodies may comprise an excipient. The excipient may be at least one

of a group consisting of carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives.

[0133] While the presently disclosed inventive concepts have been described herein in connection with certain embodiments so that aspects thereof may be more fully understood and appreciated, it is not intended that the presently disclosed inventive concepts be limited to these particular embodiments. On the contrary, it is intended that all alternatives, modifications and equivalents are included within the scope of the presently disclosed inventive concepts as defined herein. Thus the examples described above, which include particular embodiments, will serve to illustrate the practice of the presently disclosed inventive concepts, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of particular embodiments of the presently disclosed inventive concepts only and are presented in the cause of providing what is believed to be the most useful and readily understood description of procedures as well as of the principles and conceptual aspects of the inventive concepts. Changes may be made in the formulation of the various compositions described herein, the methods described herein or in the steps or the sequence of steps of the methods described herein without departing from the spirit and scope of the presently disclosed inventive concepts.

What is claimed is:

1. A method for blocking transmission of a *Plasmodium* species, comprising: administering to a mammalian or avian subject an immunogenic composition comprising a Fibrinogen-Related Protein 1 (FREP1) protein and/or an immunogenic portion thereof, causing formation of anti-FREP1 antibodies in the mammalian or avian subject, wherein the anti-FREP1 antibodies, when consumed by a mosquito from blood obtained from the mammalian or avian subject, prevent the infection of the mosquito by the *Plasmodium* species.

2. The method of claim 1, wherein the FREP1 protein of the immunogenic composition comprises the amino acid sequence set forth in SEQ ID. NO:2.

3. The method of claim 1, wherein the FREP1 protein of the immunogenic composition has at least 70% identity with the amino acid sequence as set forth in SEQ ID NO:2.

4. The method of claim 1, wherein the immunogenic composition further comprises an excipient.

5. The method of claim 4, wherein the excipient is at least one of a group consisting of carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives.

6. The method of claim 1, wherein the mosquito in an *Anopheles* species.

7. An immunogenic composition comprising an amount of a Fibrinogen-Related Protein 1 (FREP1) and/or an immunogenic portion thereof, which is immunogenically-effective in causing formation of anti-FREP1 antibodies in a mammalian or avian subject; and an excipient.

8. The immunogenic composition of claim 7, wherein the FREP1 protein comprises the amino acid sequence set forth in SEQ ID. NO:2.

9. The immunogenic composition of claim 7, wherein the FREP1 protein has at last 70% identity with the amino acid sequence as set forth in SEQ ID NO:2.

10. The immunogenic composition of claim 7, wherein the excipient is at least one of a group consisting of carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives.

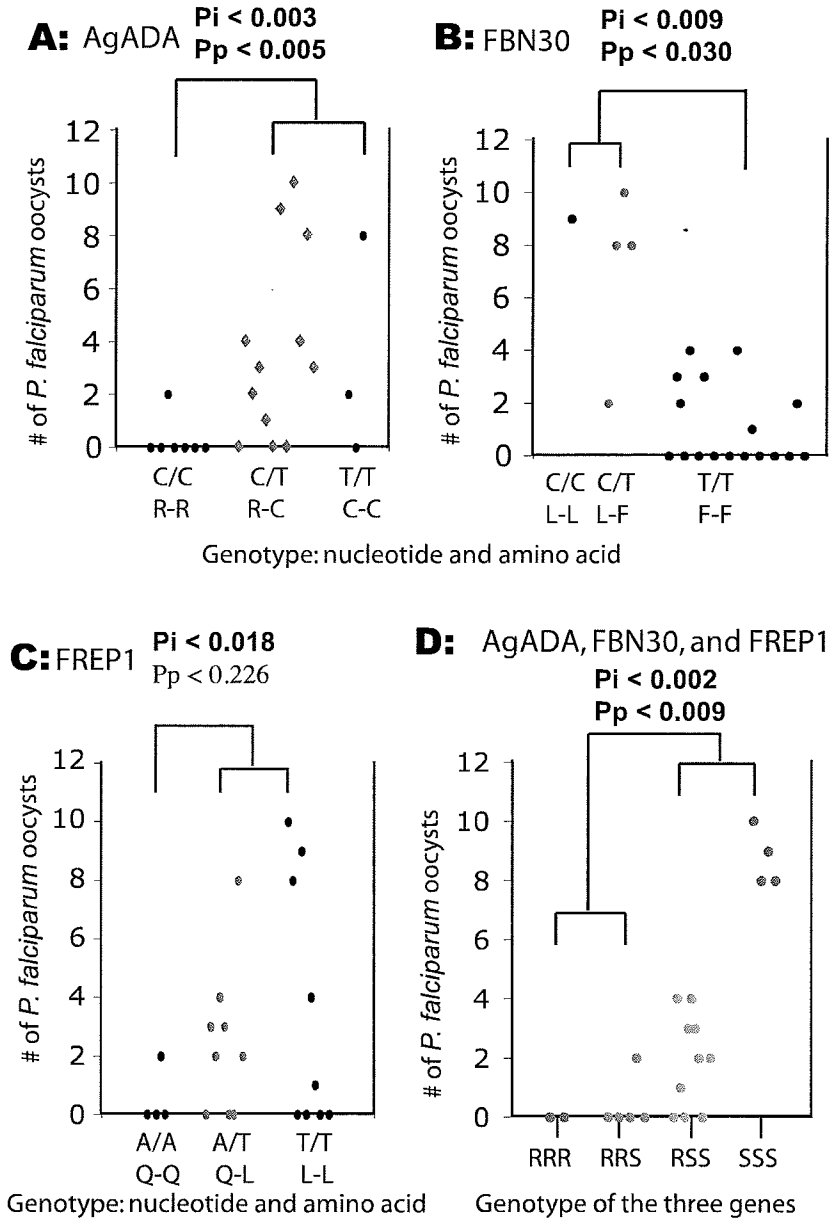
11. A polyclonal antibody composition, comprising polyclonal anti-FREP1 antibodies formed by the method of immunizing a mammalian or avian subject with an immunogenic composition comprising a FREP1 protein and/or an immunogenic portion thereof, wherein the immunogenic composition further comprises an excipient.

12. The polyclonal antibody composition of claim 11, wherein the FREP1 protein of the immunogenic composition comprises the amino acid sequence set forth in SEQ ID. NO:2.

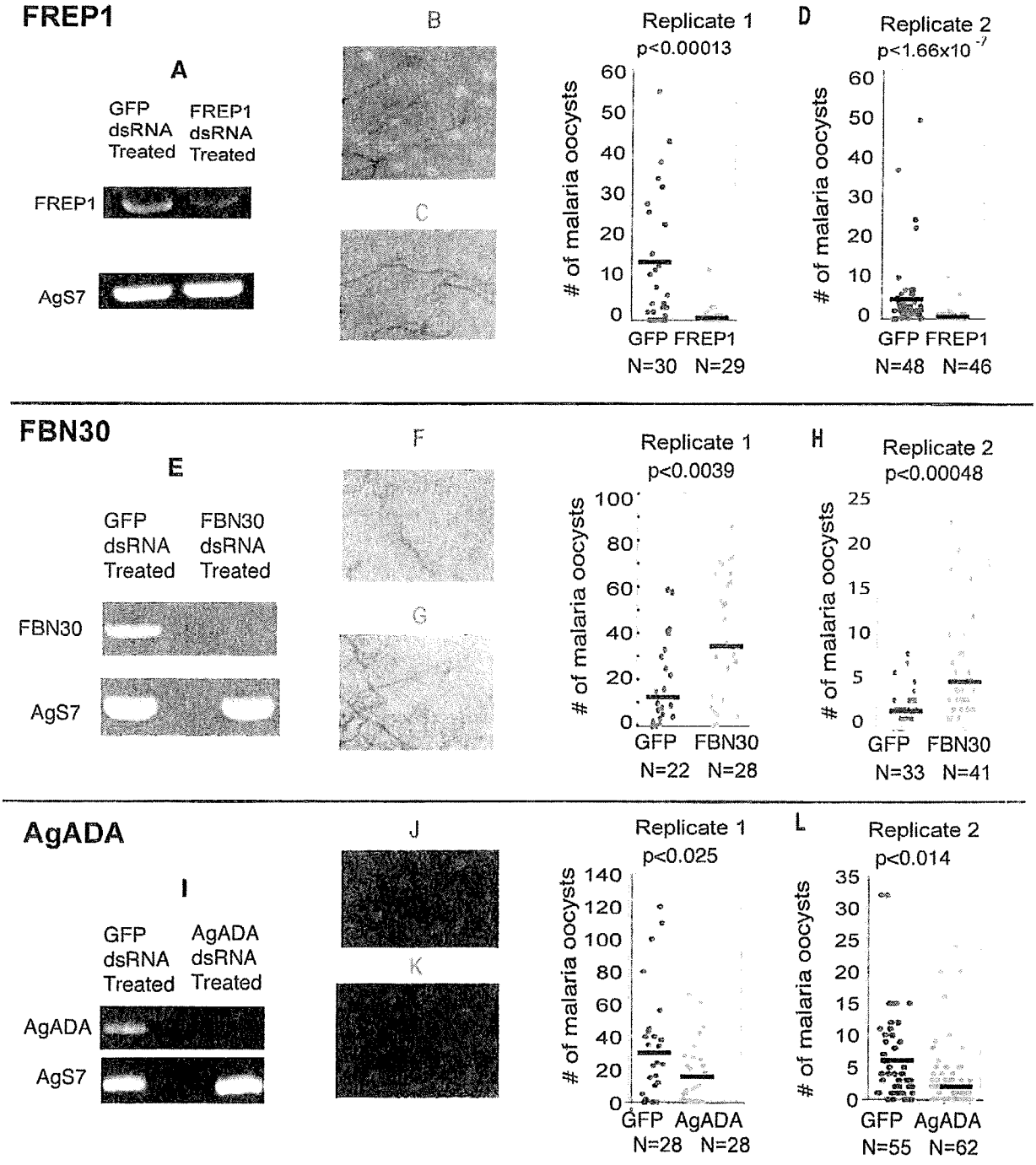
13. The polyclonal antibody composition of claim 11, wherein the FREP1 protein of the immunogenic composition has at least 70% identity with the amino acid sequence as set forth in SEQ ID NO:2.

14. The polyclonal antibody composition of claim 11, wherein the immunogenic composition further comprises an excipient.

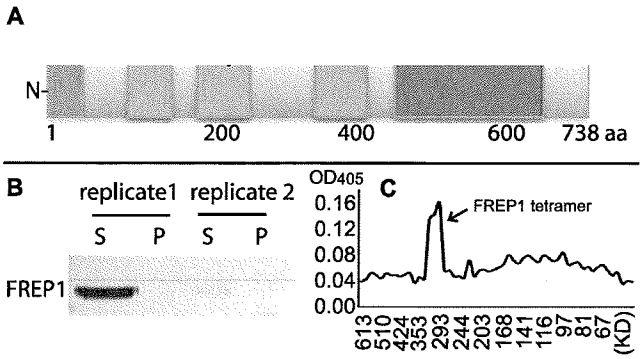
15. The immunogenic composition of claim 14, wherein the excipient is at last one of a group consisting of carriers, delivery vehicles, adjuvants, solvents, stabilizers, and preservatives.


A: AgADA
Pi < 0.003
Pp < 0.005
B: FBN30
Pi < 0.009
Pp < 0.030
# of P. falciparum oocysts
0
2
4
6
8
10
12
C/C R-R
C/T R-C
T/T C-C
C/C L-L
C/T L-F
T/T F-F
Genotype: nucleotide and amino acid
C: FREP1
Pi < 0.018
Pp < 0.226
A/A Q-Q
A/T Q-L
T/T L-L
Genotype: nucleotide and amino acid
D: AgADA, FBN30, and FREP1
Pi < 0.002
Pp < 0.009
RRR
RRS
RSS
SSS
Genotype of the three genes

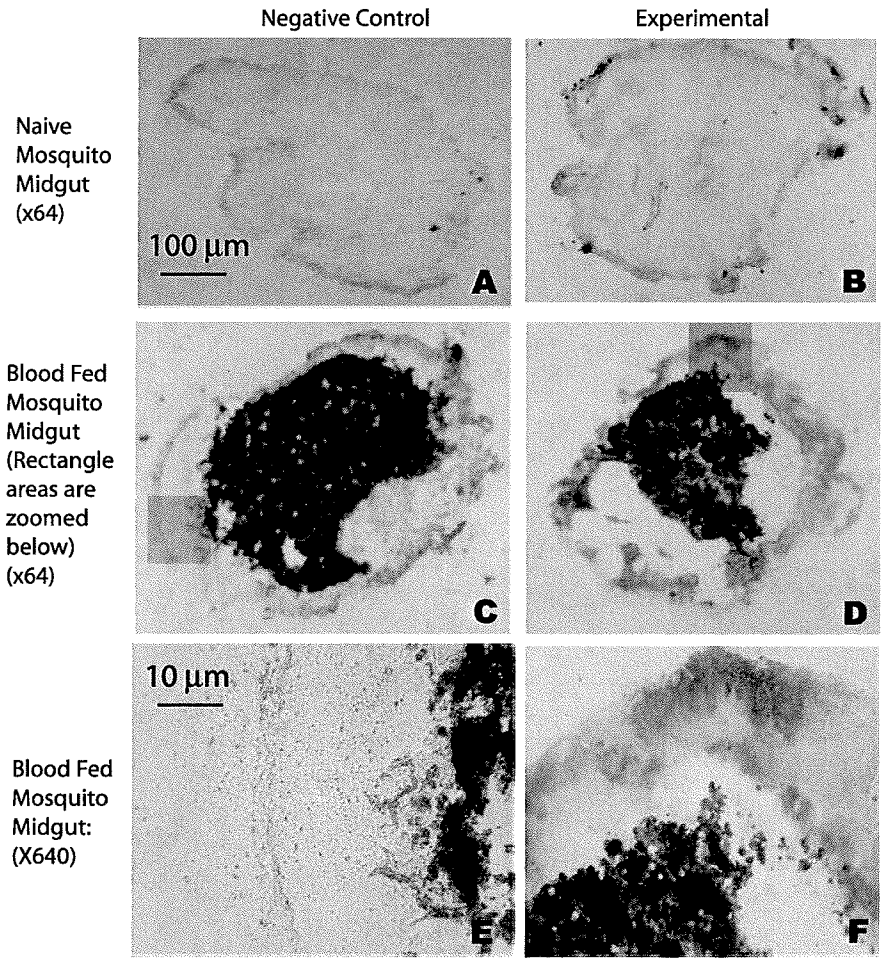
FIGS. 1A-1D


FREP1
A
GFP dsRNA Treated
FREP1 dsRNA Treated
FREP1
AgS7
B
C
Replicate 1
p<0.00013
# of malaria oocysts
GFP FREP1
N=30 N=29
D
Replicate 2
p<1.66x10 -7
# of malaria oocysts
GFP FREP1
N=48 N=46
FBN30
E
GFP dsRNA Treated
FBN30 dsRNA Treated
FBN30
AgS7
F
G
Replicate 1
p<0.0039
# of malaria oocysts
GFP FBN30
N=22 N=28
H
Replicate 2
p<0.00048
# of malaria oocysts
GFP FBN30
N=33 N=41
AgADA
I
GFP dsRNA Treated
AgADA dsRNA Treated
AgADA
AgS7
J
K
Replicate 1
p<0.025
# of malaria oocysts
GFP AgADA
N=28 N=28
L
Replicate 2
p<0.014
# of malaria oocysts
GFP AgADA
N=55 N=62

FIGS. 2A-2L



FIGS. 3A and 3B



FIGS. 4A-4F

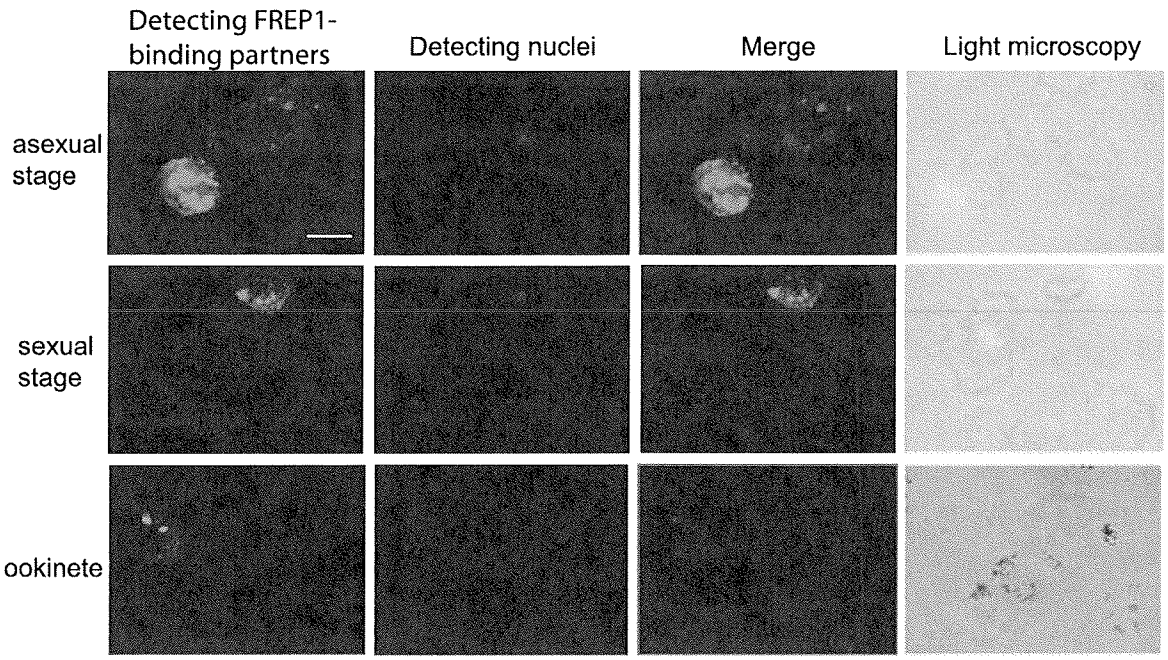
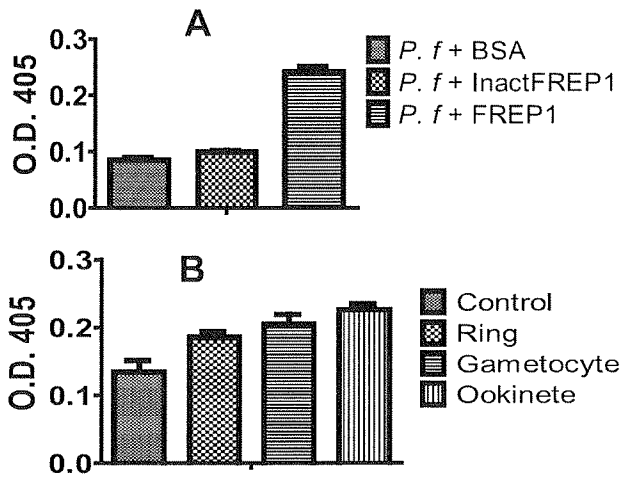
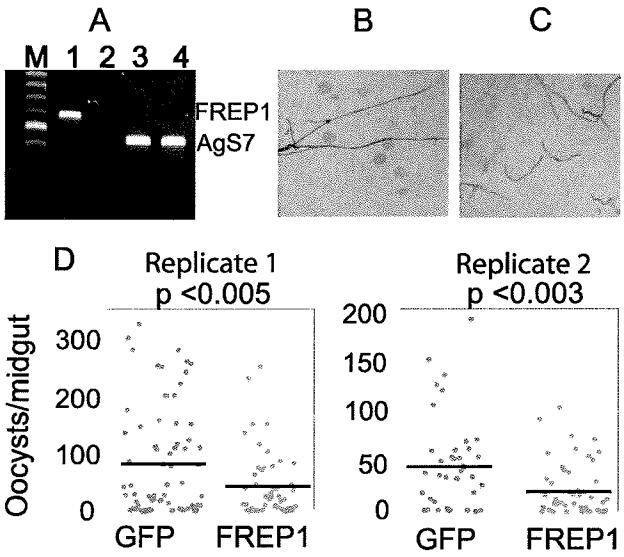


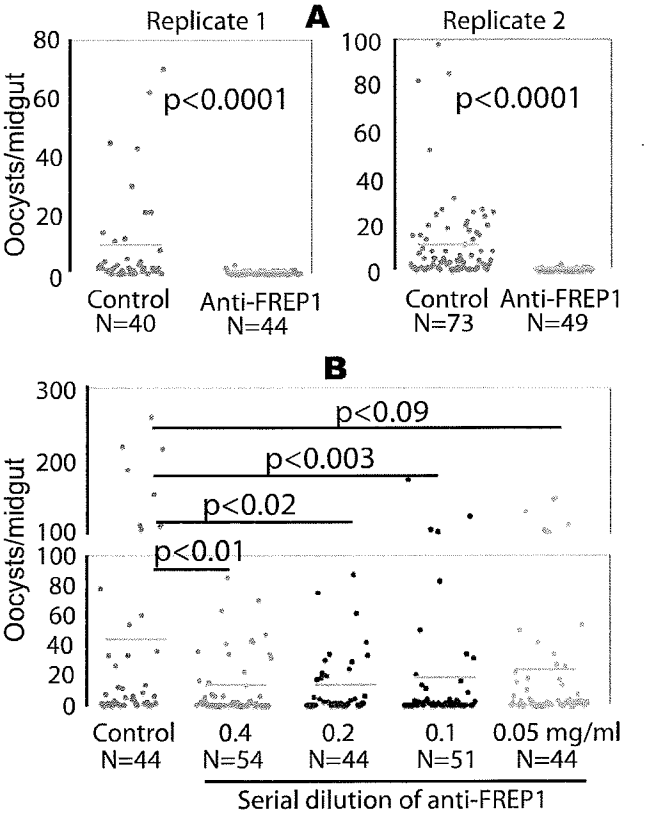
FIG. 5



FIGS. 6A and 6B



FIGS. 7A-7D



FIGS. 8A and 8B

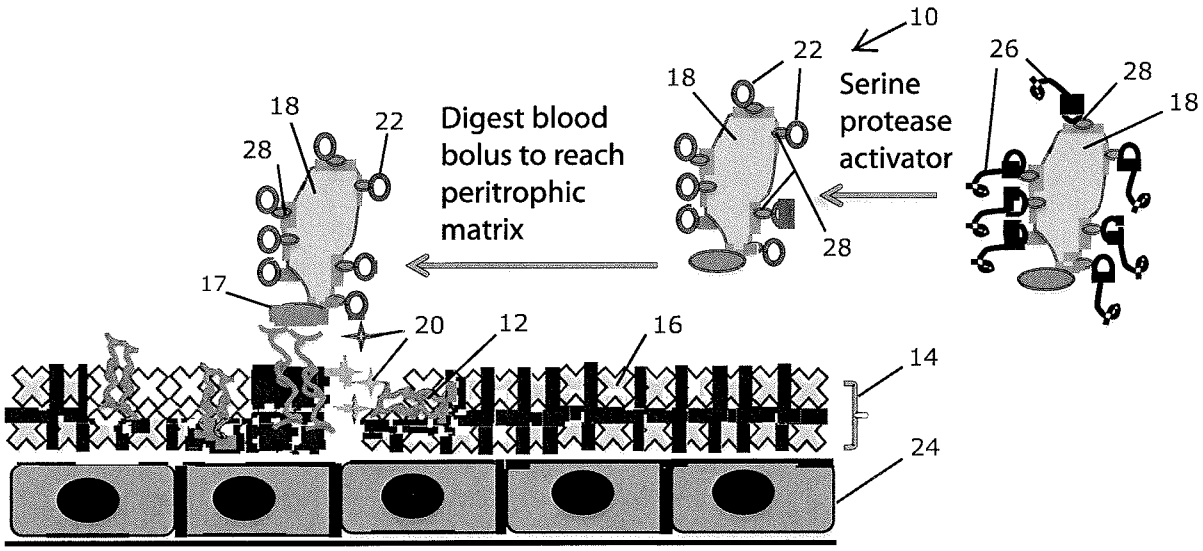
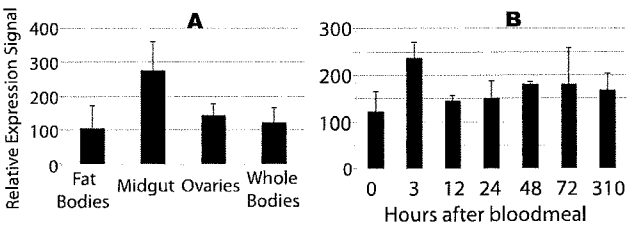


FIG. 9



FIGS. 10A and 10B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/58637

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, C12N 1/10, A61K 39/00 (2016.01)
CPC - C12Q 1/689, C12R 1/90
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)
IPC (8): C12Q 1/68, C12N 1/10, A61K 39/00 (2016.01)
CPC: C12Q 1/689, C12R 1/90

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/6.15, 435/258.2, 424/185.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Google patents, Google scholar, Google web, PatBase, Proquest Dialog
vaccine; block/inhibit/reduce; transmission; Plasmodium/malaria; Anopheles/mosquito; immunogenic/antigenic; Fibrinogen-Related Protein 1/FREP1; antibodies

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2002/011757 A2 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 14 February 2002 (14.02.2002) Abstract; claim 6; page 4, ln 22-32; page 13, ln 6-11; page 15, ln 8-15; page 21, ln 32-41	1-15
Y	Ll et. al. Genome-block expression-assisted association studies discover malaria resistance genes in Anopheles gambiae. PNAS. 17 December, 2013. Vol. 110. No. 51. pp 20675-20680. especially, Abstract; page 20678, Col. 2, para 2; page 20679, Col. 1, para 1	1-15
Y	GenBank Accession No. XP-565171.3 [Anopheles gambiae str. PEST] 28 September 2011 [online] [Retrieved on 21 February 2016]. Retrieved from website<URL: http://www.ncbi.nlm.nih.gov/protein/158286408#feature_158286408> Entire document	2-3, 8-9, 12-13

☐ Further documents are listed in the continuation of Box C. ☐

* 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
21 January 2016 (21.01.2016)	05 FEB 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/58637

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: