TREATMENT OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA, HEMOLYTIC ANEMIAS AND DISEASE STATES INVOLVING INTRAVASCULAR AND EXTRAVASCULAR HEMOLYSIS

RELATED APPLICATION

[0001] This application claims priority benefit of U.S. Provisional Patent Application Serial No. 61/280,567, filed on November 5, 2009, the content of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to methods and materials for treatment of subjects suffering disorders that involve abnormal lysis of red blood cells, including intravascular and extravascular hemolysis, utilizing inhibitors of the complement alternative pathway and complement component C3 fragment activity. More particularly, the present invention relates to methods and materials for treatment of subjects with paroxysmal nocturnal hemoglobinuria, aHUS, hemolytic anemias and other disorders that involve complement-mediated hemolysis, and which may involve an extravascular component which is not adequately treated by terminal inhibitors of complement, such as inhibitors of C5 activity.

BACKGROUND OF THE INVENTION

[0003] Complement-mediated hemolytic anemia is a significant health problem, and contributes to numerous disorders of the red blood cells, such as paroxysmal nocturnal hemoglobinuria (PNH). PNH is a hematological disorder characterized by the clonal expansion of one or a few hematopoietic stem cells which are incapable of glycosylphosphatidylinositol (GPI)-anchor biosynthesis, due to an acquired somatic mutation in the phosphatidylinositol glycan class A (*PIG-A*) gene. Affected progeny cells are deficient in all GPI-anchored surface proteins, including complement regulators CD55 and CD59. Thus, PNH red blood cells (RBCs) are vulnerable to activated complement, and particularly to the membrane attack complex (MAC), resulting in chronic intravascular hemolysis with recurrent exacerbations. Other complement-mediated disorders that affect red blood cells include atypical hemolytic uremic syndrome (aHUS); chronic hemolytic anemia; antibody-mediated autoimmune hemolytic anemia; anemia caused by hemoglobinopathies such as

sickle cell disease; anemia caused by infection such as malaria; anemia due to transfusion reaction; and cold agglutinin disease (CAD).

[0004] Treatment with eculizumab, a monoclonal antibody (Mab) directed against complement component 5 (C5), has been partially effective in PNH and other hematological disorders. However, a significant subpopulation of patients with PNH exhibit suboptimal hematological response to treatment with anti-C5 Mab. In this subpopulation, little improvement of anemia is observed, and some still require blood transfusion, with continuing signs of persistent hemolysis (reticulocytosis, elevated unconjugated bilirubin). Risitano and Rotoli, Biologics, 2:205-222 (2008). The recurrence may be characterized as "breakthroughs" where hemolytic activity may persist despite treatment with terminal complement inhibitors. Hill et al., Blood, 106:2559-65 (2005). For these subjects, a need remains for additional methods and materials for the effective treatment of PNH.

[0005] Complement inhibitors are known in the art, and a new class of targeted complement inhibitors has been developed, which allows treatment in a fashion which results in high localized concentrations of inhibitor at the tissue sites where complement is activated, while minimizing potentially adverse systemic effects. This class of inhibitors includes, for example, TT30 (SEQ ID NO:3), TT31 and TT32. TT30 is an immunomodulatory compound which inhibits the complement alternative pathway. TT30 comprises a complement alternative pathway inhibitory portion of Factor H protein, which is targeted to sites of complement activation and inflammation through fusion with a portion of the complement receptor 2 protein (CR2 or CD21) which is known to bind to tissue/cell-fixed fragments of the complement component 3 (C3). TT31 is similar to TT30, but contains an additional copy of the complement alternative pathway inhibitory portion of Factor H protein. TT32 comprises a complement inhibitory portion of complement receptor 1 (CR1), targeted through fusion with the same portion of the CR2 protein. CR1 is known to be a broader inhibitor of complement than is Factor H. TT32 will therefore inhibit not only the complement alternative pathway, but will locally inhibit both the classic and lectin pathways of complement as well. Suitable targeted inhibitors are described in Gilkeson et al., US Patent Publication 2008/0221011, the disclosure of which is hereby specifically incorporated herein by reference.

SUMMARY OF THE INVENTION

[0006] Modulation of the complement system represents a therapeutic modality for numerous pathologic conditions associated with complement activation. As summarized above, however, a significant subpopulation of subjects with PNH and other forms of anemia do not optimally respond to treatment with treatment with terminal complement inhibitors, such as the anti-C5 Mab, eculizumab, due in whole or in part to extravascular hemolysis.

[0007] The present inventors have found that subjects suffering from complement-mediated disorders that affect red blood cells, such as PNH, that are not effectively or optimally treated with a terminal complement inhibitor surprisingly may be effectively treated with a composition that inhibits the complement alternative pathway, and through this activity block the the formation and activity of amplifying C3 convertase on the surface of PNH red blood cells. In certain embodiments, the compositions of the present invention which inhibit the activation of complement component C3 may thereby inhibit not only the amplification loop of the complement alternative pathway, but may also partially inhibit alternative pathway activation via spontaneous C3 'tickover.' In certain, embodiments, in addition to inhibition of the complement alternative pathway, the compositions of the present invention may further exhibit inhibitory effects on other complement pathways, such as the classical and lectin activation pathways.

[0008] The present inventors have discovered that subjects with suboptimal hematological response to terminal complement inhibitors, such as eculizumab, may exhibit extravascular hemolysis mediated by complement effector mechanisms other than MAC. Based on flow cytometric analysis of complement fraction 3 (C3) on RBCs, we provide evidence of selective C3 opsonization of GPI-negative red cells. The extent of this phenomenon tends to correlate with the clinical response to eculizumab, and may be the manifestation of a novel phenomenon in the pathophysiology of PNH.

[0009] While not being bound by any one theory, targeting the terminal complement at the level of C5 may not protect red blood cells from damage through the early complement components (i.e., C3), which may lead to extravascular hemolysis. It is also believed that treatment with terminal complement inhibitors, such as eculizumab, may allow low-level intravascular hemolysis to continue, sufficient to keep LDH in the high normal range and HgB low normal, possibly by a mechanism that involves accumulation of C5 convertases that

eventually can out-compete the terminal complement inhibitor for C5 during normal on-off cycling of the monoclonal antibody. This is supported by the occurrence of pharmacokinetic 'breakthroughs' of hemolysis, which have been reported to occur in patients whose blood level of eculizumab dips below 'trough' concentrations of 35 ug/mL. The inventors believe that breakthroughs may also be caused by other crisis circumstances, such as viral infection or other causes of increased complement activation, which may lead to a disturbance of the balance between eculizumab, C5 and C5 convertase. The inventors believed that the accumulation of C3 fragments on PNH red blood cells contributes to viral-associated breakthrough through activation of cells in the reticuloendothelial system. In addition, though, by binding to C5 at the cell surface interface, treatment with terminal complement inhibitors of hemolytic anemias such as PNH may contribute to the accumulation of C3, C3 convertases, C3 fragments and C5 convertases, which can contribute to the inability of subjects to achieve and stably maintain fully normal serum LDH and HgB levels. Accordingly, the present invention provides methods for the treatment of subjects with complement-mediated hemolysis such as those suffering from PNH. Such methods target early complement activation, and are able to control intravascular hemolysis, as well as reduce or avoid possible extravascular hemolysis resulting from uncontrolled C3 activation and opsonization. The methods and compositions of the present invention may therefore be more effective in treating patients suffering from PNH or other hemolytic anemias, can effectively achieve and maintain normal serum LDH and HgB levels and reduce or eliminate the occurrence of 'breakthrough' hemolytic crises that have been observed in patients treated with terminal complement inhibitors. Thus, the methods and compositions of the present invention may also be more effective for treatment of patients suffering from PNH, while reducing or avoiding the occurrence of such 'breakthrough' hemolytic crises.

[0010] In certain embodiments, the present invention comprises a method of treating a subject having paroxysmal nocturnal hemoglobinuria (PNH) or other complement-mediated hemolytic disorder affecting red blood cells, the method comprising administering an effective amount of a composition that inhibits (such as selectively inhibits) the activity of the complement alternative pathway, wherein the composition inhibits local activation of complement component C3 (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase and opsonization of red blood cells by fragments of C3.

[0011] In other embodiments, the invention comprises a method of treating a subject exhibiting extravascular hemolysis, which may be due to a complement-mediated hemolytic disorder affecting red blood cells, such as PNH, the method comprising administering an effective amount of a composition that inhibits (such as selectively inhibits) the activity of the complement alternative pathway, wherein the composition inhibits local activation of complement component (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase and opsonization of red blood cells by fragments of C3.

[0012] The terminal complement inhibitor selectively inhibits cleavage of complement protein C5, and may be for example, a humanized anti-C5 antibody or antigen-binding fragment thereof, such as eculizumab or pexelizumab. Thus, in certain embodiments, the present invention comprises treatment of a subject having paroxysmal nocturnal hemoglobinuria, wherein the subject has previously been treated with an anti-C5 antibody, such as eculizumab or pexelizumab, but whose PNH disease state and/or symptoms persist.

[0013] In certain embodiments, the methods of the present invention comprise treating a subject having a complement-mediated hemolytic disorder affecting red blood cells, such as paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS); chronic hemolytic anemia; and cold agglutinin disease (CAD), wherein the subject exhibits at least one of the following characteristics, which characteristics may be symptoms of residual anemia and/or complement-mediated extravascular hemolysis and/or incomplete control of intravascular hemolysis:

a. the subject exhibits signs or symptoms continued loss of red blood cells by ongoing or intermittent intravascular hemolysis and/or extravascular hemolysis;
b. the subject has red blood cells opsonized by fragments of C3;
c. the subject requires periodic blood transfusions;
d. the subject has low normal or below normal levels of hemoglobin;
e. the subject has low normal or below normal levels of platelets;
f. the subject has high normal or above normal reticulocytes;
g. the subject has high normal or above normal bilirubin; or
h. the subject has iron overload or is at risk of iron overload.

[0014] The above characteristics can also be used to monitor the subject's progress in response to treatment with complement alternative pathway inhibitors in accordance with the present invention, and to modify the dosage regime if deemed clinically appropriate. In certain embodiments, the subject having a complement-mediated hemolytic disorder affecting red blood cells, such as PNH, has previously been treated with a terminal complement inhibitor, but persists in exhibiting at least one of the above characteristics. In such cases, the present invention provides methods and compositions for avoiding or reducing the occurrence and/or severity of at least one of the above characteristics.

[0015] In another aspect, the present invention comprises methods of rendering transfusion independent a subject having a complement-mediated hemolytic disorder affecting red blood cells, such as PNH, where that subject exhibits a suboptimal response to treatment with a terminal complement inhibitor. Such suboptimal response may include the subject exhibiting at least one of the above characteristics of residual anemia and/or complement-mediated extravascular hemolysis. The method comprises administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, wherein the composition inhibits activation of complement component C3 (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase and opsonization of red blood cells by fragments of C3.

[0016] In another aspect, the present invention comprises methods of reducing hemolysis in a subject having PNH, the method comprising administering an effective amount of a composition that inhibits activation of complement component C3 (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase, and opsonization of PNH red blood cells by fragments of C3.

[0017] In yet another aspect, the present invention comprises methods of reducing residual anemia in a subject having PNH, the method comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, wherein the composition inhibits activation of complement component C3 (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase, and opsonization of red blood cells by fragments of C3.

[0018] In yet another aspect, the present invention provides methods of increasing the proportion of PNH red blood cells which are able to survive complement attack, the method comprising administering an effective amount of a composition that inhibits activation of complement component C3 (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase, and opsonization of PNH red blood cells by fragments of C3.

[0019] In certain aspects of the present invention, in addition to inhibiting the complement alternative pathway, the composition may also inhibit one or more of the classical and lectin complement pathways. For example, complement receptor 1 (CR1), is expected to have inhibitory effects on all three of the complement pathways. While not directly an inhibitor of the complement alternative pathway, antibodies to MASP-1 may also be useful in the present invention. This is because removing MASP-1 from the system can reduce the amount of C3b that is produced via the classical and lectin pathways. With less C3b available, the amplification loop of the complement alternative pathway may be at least partially inhibited. Accordingly, for purposes of the present invention MAP1 and antibodies to MASP-1 are considered to be complement alternative pathway inhibitors that may be useful in certain embodiments of the invention. See, Skjodt et al., Molecular Immunology, 47:2229-30 (2010); and Petersen et al., Molecular Immunology 38:133-149 (2001).

[0020] In yet another aspect, the present invention provides methods of reducing or avoiding the occurrence of 'breakthrough' hemolytic crises in subjects who are treated for hemolysis using terminal complement inhibitors.

[0021] As preferred embodiments useful to accomplish the above methods, the present invention provides agents and compositions that inhibit the activity of the complement alternative pathway. Such agents and compositions may comprise fusion proteins comprising a complement receptor 2 (CR2) protein or biologically active fragment thereof; and modulator of complement activity which acts by modulating the presence of complement component C3 or its cleavage fragments, and/or C3 convertases, for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase, halting further progression of the complement cascade to form the membrane attack complex, and preventing or reducing the binding of such C3 molecules or fragments thereof to red blood cells, and in particular, preventing or reducing the opsonization of PNH red blood cells by fragments of C3.

[0022] In preferred embodiments, the inhibitor of the complement alternative pathway may comprise a fusion of the CR2 protein, or a fragment comprising at least the first two amino terminal short consensus repeat (SCR) domains of CR2, fused to factor H (FH) protein, or a biologically active fragment thereof. The biologically active fragment of FH protein may comprise at least the first four amino terminal SCR domains of FH. One such complement inhibitor is TT30 (SEQ ID NO:3), a fusion protein that comprises the first four amino-terminal short consensus repeat (SCR) domains of CR2 fused to the first five amino-terminal SCR domains of FH. Another such complement inhibitor comprises the first two amino terminal SCR domains of CR2 fused to the first four amino-terminal SCR domains of FH. The linkage can be at either end of the domains, such that the fusion protein may be described, proceeding from amino to carboxy termini, as either CR2-FH or FH-CR2.

[0023] In other preferred embodiments, the inhibitor of the complement alternative pathway may comprise a fusion of the CR2 protein, or a targeting fragment thereof comprising at least the first two amino terminal short consensus repeat (SCR) domains of CR2, fused to a complement inhibitor selected from the group consisting of Crry, DAF, MCP, complement factor I, compstatin or CR1, or biologically active fragments thereof. In other preferred embodiments, the inhibitory portion of the fusion protein may comprise an antibody to a factor selected from the group consisting of Factor B, Factor D or properdin; or an antigen-binding fragment thereof. As mentioned above, although not direct inhibitors of the complement alternative pathway, inhibitors of MASP-1 may effectively reduce the amount of C3b present, thereby at least partially inhibiting the amplification loop of the complement alternative pathway. Accordingly, inhibitos of MASP1 protein, such as antibodies to MASP1 and the endogenous MASP1 inhibitor MAP1 may be useful in certain embodiments of the present invention.

[0024] In a preferred embodiment, the inhibitor of the complement alternative pathway comprises a fusion of the CR2 protein, or a targeting fragment thereof comprising at least the first two amino terminal short consensus repeat (SCR) domains of CR2, fused to an antibody to Factor B within the third SCR domain and prevents formation of the C3bBb complex, or an antigen-binding fragment thereof. Suitable antibodies are described, for example, in Holers et al., US Patent Publication 2005/0260198 and in Emblen et al., US Patent Publication 2008/0299114. The disclosure of these documents is hereby specifically incorporated herein by reference. The linkage can be at either end of the domains, such that

the fusion protein may be described, proceeding from amino to carboxy termini, as either CR2-complement inhibitor or complement inhibitor-CR2.

[0025] In additional embodiments, the inhibitor of the complement alternative pathway may comprise a fusion of a monoclonal antibody portion fused to a complement inhibitory portion. The monoclonal antibody portion comprises a monoclonal antibody, or a binding fragment thereof, which is directed toward complement component 3 (C3) or which will bind to the C3 fragment comprising one or more binding domains selected from the group consisting of C3b, iC3b, C3dg and C3d. The complement inhibitory portion comprises a complement inhibitor, or a biologically active fragment thereof, selected from the group consisting of Factor H protein, the group consisting of Crry, DAF, MCP, complement factor I, compstatin or CR1, or biologically active fragments thereof. In other preferred embodiments, the inhibitory portion of the fusion protein may comprise an antibody to a factor selected from the group consisting of Factor B, Factor D, MASP1, or the endogenous MASP1 inhibitor MAP1; or an antigen-binding fragment thereof. The linkage can be at either end of the domains, such that the fusion protein may be described, proceeding from amino to carboxy termini, as either antiC3-complement inhibitor or complement inhibitor-antiC3.

[0026] In further embodiments, the present invention comprises methods and materials for the treatment of subjects with a complement-mediated hemolytic disorder that affects red blood cells, such as PNH, wherein the method comprises administering to a subject afflicted with such disorder both a terminal complement inhibitor and an inhibitor of the complement alternative pathway. In this method, the complement inhibitors may be administered simultaneously or sequentially in either order. The terminal complement inhibitor may comprise an anti-C5 monoclonal antibody, such as eculizumab or pexelizumab, or another terminal complement inhibitor which inhibits C5 or other components of the Membrane Attack Complex (MAC), which comprises C6 through C9. For example, CD59, or TT33, which is a targeted CD59 fusion protein is useful as a terminal complement inhibitor. In certain embodiments, the inhibitor of the complement alternative pathway may inhibit the activity of the complement alternative pathway, wherein the composition inhibits activation of complement component C3 (C3), C3 fragments and/or C3 convertases, for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase, and opsonization of PNH red blood cells by fragments of C3. In other embodiments, the inhibitor of the complement alternative

pathway may additionally inhibit other complement pathways, such as the classical and lectin mediated pathways.

[0027] In another aspect, the present invention provides compositions for the treatment of PNH, or of other diseases which involve a complement-mediated extravascular hemolysis component, which compositions comprise a combination of:

a) a terminal complement inhibitor; and
b) an inhibitor of the complement alternative pathway.

[0028] The terminal complement inhibitor may preferably comprise an anti-C5 antibody, such as eculizumab or pexelizumab. The inhibitor of the complement alternative pathway may preferably comprise fusion proteins comprising a complement receptor 2 (CR2) protein or biologically active fragment thereof; and modulator of complement activity which acts by modulating the presence of complement component C3, its cleavage fragments and/or C3 convertases and preventing or reducing the binding of such C3 molecules or fragments thereof to red blood cells, and in particular, preventing or reducing the opsonization of red blood cells by fragments of C3. In particularly preferred embodiments, the inhibitor of the complement alternative pathway may comprise a fusion of the CR2 protein, or a fragment comprising at least the first two amino terminal SCR domains of CR2, fused to factor H protein, or a biologically active fragment of FH. One such complement alternative inhibitor comprises a CR2 targeting domain portion fused to a complement inhibitory portion of factor H comprising the first four N-terminal SCR domains of human Factor H. In a particular preferred embodiment, the inhibitor of the complement alternative pathway is TT30 (SEQ ID NO:3), which comprises the first four N-terminal SCR domains of CR2 fused to the first five N-terminal SCR domains of FH. In another preferred embodiment, the inhibitor of the complement alternative pathway is TT31, which comprises the first four N-terminal SCR domains of CR2 fused to two copies of the first five N-terminal SCR domains of FH.

[0029] In other preferred embodiments, the inhibitor of the complement alternative pathway may comprise the CR2 protein, or a fragment comprising at least the first two amino terminal short consensus repeat (SCR) domains of CR2, fused to a complement inhibitory portion selected from the group consisting of Anti-Factor B antibody, Anti-Properdin antibody, Anti-Factor D antibody, Factor I protein, compstatin, Crry, DAF, MCP or CR1, or biologically active fragments thereof. One such preferred embodiment is TT32, which

comprises the first four SCR domains of CR2 fused to the first ten SCR domains of CR1. Because of CR1's ability to inhibit not only the complement alternative pathway, but also the classical and lectin mediated complement pathways, inhibitors such as TT32 may find more wide-ranging use in other disease states which involve both an intravascular and extravascular component of hemolysis, as well as in autoimmune diseases and related conditions.

[0030] In other preferred embodiments, the inhibitor of the complement alternative pathway may comprise a fusion of a monoclonal antibody portion fused to a complement inhibitory portion. The monoclonal antibody portion comprises a monoclonal antibody, or a binding fragment thereof, which is directed toward complement component 3 (C3) or which will bind to the C3 fragment comprising one or more binding domains selected from the group consisting of C3b, iC3b, C3dg and C3d. The complement inhibitory portion comprises a complement inhibitor, or a biologically active fragment thereof, selected from the group consisting of Factor H protein, Anti-Factor B antibody, Anti-Properdin antibody, Anti-Factor D antibody, Factor I protein, compstatin, Anti-MASP1 antibody, anti-MAP1 antibody, Crry, DAF, MCP or CR1, or biologically active fragments thereof. In preferred embodiments, the complement inhibitory portion comprises (a) the first four SCR domains of human Factor H protein; (b) the first five N-terminal SCR domains of Crry; or (c) the first ten SCR domains of CR1; or; (d) MCP, comprising the first three N-terminal SCR domains; soluble DAF, comprising SCR domains 2-4, with or without the serine-threonine rich region, but without the glycophosphatidyl anchor.

[0031] The present invention provides in one aspect a method of treating a subject having a complement-mediated hemolytic disorder affecting blood cells, the method comprising administering an effective amount of a composition that inhibits activation of the complement alternative pathway, wherein the composition inhibits activation of complement component C3 (C3) and opsonization of red blood cells by fragments of C3.

[0032] In another aspect provided herein is a method of treating complement-mediated hemolysis in a subject, the method comprising administering an effective amount of a composition that inhibits activation of the complement alternative pathway, wherein the composition maintains normal serum levels of lactate dehydrogenase and hemoglobin.

[0033] In certain embodiments in any of the methods described herein, the composition selectively inhibits the complement alternative pathway. In certain embodiments in any of the methods described herein, the composition that inhibits the activity of the complement alternative pathway comprises a fusion protein comprising a complement receptor 2 (CR2) protein or biologically active fragment thereof; and a factor H (fH) protein or biologically active fragment thereof. In certain embodiments in any of the methods described herein, the fusion protein comprises the first four amino-terminal short consensus repeat (SCR) domains of CR2 fused to the first five amino-terminal SCR domains of fH.

[0034] In certain embodiments in any of the methods described herein, the method further comprises administering a terminal complement inhibitor to the subject. In certain embodiments in any of the methods described herein, the terminal complement inhibitor inhibits cleavage of complement protein C5 (C5). In certain embodiments in any of the methods described herein, the terminal complement inhibitor is a humanized anti-C5 antibody or antigen-binding fragment thereof. In certain embodiments in any of the methods described herein, the terminal complement inhibitor is eculizumab.

[0035] In certain embodiments in any of the methods described herein, the subject has paroxysmal nocturnal hemoglobinuria (PNH) and the subject's red blood cells are opsonized by fragments of C3 in the absence of the composition. In certain embodiments in any of the methods described herein, the subject has previously been treated with a terminal complement inhibitor. In certain embodiments in any of the methods described herein, the subject was not responsive, partially responsive, or has progressed on the treatment of the terminal complement inhibitor.

[0036] In certain embodiments of any of the methods described herein, the subject has one or more of the following characteristics:

a. the subject exhibits signs or symptoms continued loss of red blood cells by ongoing or intermittent intravascular hemolysis and/or extravascular hemolysis;
b. the subject has red blood cells opsonized by fragments of C3;
c. the subject requires periodic blood transfusions;
d. the subject has low normal or below normal levels of hemoglobin;
e. the subject has low normal or below normal levels of platelets;
f. the subject has high normal or above normal reticulocytes;

g. the subject has high normal or above normal bilirubin; or

h. the subject has iron overload or is at risk of iron overload,

wherein the method comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway.

[0037] In certain embodiments of any of the methods described herein, the subject requires periodic blood transfusions. In certain embodiments of any of the methods described herein, the subject is thereby rendered transfusion independent. In certain embodiments of any of the methods described herein, the subject has below normal levels of hemoglobin.

[0038] In certain embodiments of any of the methods described herein, the composition increases the survival of red blood cells in the subject. In certain embodiments of any of the methods described herein, the complement-mediated hemolytic disorder is sickle cell anemia.

[0039] It is understood that aspect and embodiments of the invention described herein include "consisting" and/or "consisting essentially of" aspects and embodiments.

[0040] Reference to "about" a value or parameter herein includes (and describes) variations that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X".

[0041] As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise.

[0042] The term "subject" refers to a mammal, including humans. A subject includes, but is not limited to, human, bovine, horse, feline, canine, rodent, or primate.

BRIEF DESCRIPTION OF THE FIGURES

[0043] **FIGURE 1** illustrates the results of single color flow cytometry in measuring C3 binding on red blood cells in PNH subjects. Along the Y axis are plotted the red blood cell counts; along the X axis are plotted Anti-C3 fluorescent isothiocyanate (FITC) counts. **Figure 1a** illustrates the result for normal control subjects; Figure **1b** illustrates the result for subjects with cold agglutinin disease (CAD); **Figure 1c** illustrates the results for PNH subjects who have not been treated with a terminal complement inhibitor; **Figure 1d**

illustrates the results for two PNH subjects who have been treated with the terminal complement inhibitor eculizumab, an antibody to C5.

[0044] **FIGURE 2** illustrates the results of double color flow cytometry measuring C3 binding on RBCs in PNH subjects who have previously been treated with eculizumab. Along the Y axis are plotted the number of red blood cells which are CD59+ [normal]; or CD59- [PNH]. Along the X axis are plotted the number of cells which are C3+ [coated with C3]; or C3- [uncoated]. The upper left quadrant represents CD59+/C3- cells, i.e., normal red blood cells. The upper right quadrant represents CD59+/C3+ cells, i.e., normal red blood cells coated by C3. The lower left quadrant represents CD59-/C3- cells, i.e., PNH red blood cells, uncoated by C3. The lower right quadrant represents CD59-/C3+ cells; i.e., PNH red blood cells coated by C3. **Figure 2a** illustrates the result for normal control subjects; **Figure 2b** illustrates the result for subjects with cold agglutinin disease (CAD); **Figure 2c** illustrates the results for PNH subjects who have not been treated with a terminal complement inhibitor; **Figure 2d** illustrates the results for four PNH subjects who have previously been treated with the terminal complement inhibitor eculizumab (Ecu), an antibody to C5.

[0045] **FIGURE 3** illustrates the correlation of C3 binding on red blood cells in PNH subjects (Y axis), and the percent of total RBCs which are PNH (CD59-)(X axis). It can be noted that the percent of C3-coated RBCs correlates with the PNH RBC population size.

[0046] **FIGURE 4** illustrates the percentage of C3+ RBCs within the PNH population in patients who either have not been treated, or have been previously treated with Ecu. It can be noted that C3 is bound on PNH RBCs only in those subjects receiving the terminal complement inhibitor eculizumab, an antibody to C5, with broad heterogeneity among such subjects.

[0047] **FIGURE 5** illustrates the kinetics of C3 binding on PNH RBCs. **Figure 5a** illustrates the appearance of C3 binding after treatment with eculizumab. It can be noted that C3 binding appears a few weeks after starting anti-C5 antibody treatment. **Figure 5b** illustrates the binding of C3 over long term (two years). It can be noted that C3 binding remains very stable over this time period.

[0048] **FIGURE 6** illustrates the correlation of C3 binding on PNH RBCs with the hematological response of the PNH subjects who have previously been treated with the

terminal complement inhibitor eculizumab, an antibody to C5. It can be noted that subjects who achieved the best hematological response to eculizumab have less C3-bound RBCs.

[0049] **FIGURE 7** illustrates the correlation of C3 binding on PNH RBCs (Y axis) with absolute reticulocyte count (ARC)(X axis). It can be noted that C3 binding correlates with measures of persisting hemolysis, such as ARC, but not with LDH. Accordingly, it is theorized that the persisting hemolysis is in significant part due to complement-mediated extravascular hemolysis, and therefore is largely unaffected by the terminal complement inhibitor.

[0050] **FIGURE 8** illustrates the *in vivo* survival of 51Cr labeled red blood cells and their uptake in spleen (solid line) and liver (dotted line) from two subjects. In the first subject (**Figure 8a**), measurements were taken for seven days. In the second subject, measurements were taken for twenty days (Figure 8b). Excess counts refers to excess over control. It should also be noted that increased entrapment of RBCs in spleen and liver was detected in all subjects studied.

[0051] **FIGURE 9** illustrates the complement-mediated hemolysis of PNH RBCs from a previously untreated subject, and in particular the survival of PNH RBCs treated with various concentrations of TT30, a potent inhibitor of C3 convertase. Data represent the percent of RBCs surviving at various time points versus the concentration of TT30 administered. It can be noted that there is greater survival of PNH RBCs with higher doses of TT30.

[0052] **FIGURE 10** illustrates the survival of PNH RBCs from a previously untreated subject, with various treatments over the course of five days. Data represents the percent of PNH RBCs surviving in cells treated with acidified serum + magnesium (AcS+Mg) (a) with no inhibitors; (b) with TT30 (TT) at 3000 nM; and (c) with TT30 (TT) at 4500 nM.

[0053] **FIGURE 11** illustrates the survival of PNH RBCs from a previously untreated subject, for treatment with various concentrations of TT30 over 5 days. Data represents the percent of surviving PNH RBCs with various concentrations of TT30 vs time of treatment. It can be noted that the percent of surviving PNH RBCs increases with higher doses of TT30.

[0054] **FIGURE 12** illustrates the complement-mediated hemolysis of PNH RBCs from a previously untreated subject, and in particular the survival of PNH RBCs treated with TT30, a potent inhibitor of C3 convertase. Data represents the percent of RBCs lysed at various

time points versus the concentration of TT30 administered compared with the RBCs lysed by acidified serum. It can be noted that the percent of PNH RBCs that are lysed decreases with higher doses of TT30.

[0055] **FIGURE 13** illustrates the survival of PNH RBCs from a subject who had been previously treated with eculizumab, with various treatments over the course of five days. Data represents the percent of PNH RBCs surviving in cells treated with acidified serum + magnesium (AcS+Mg) (a) with no inhibitors; (b) with TT30 (TT) at 3000 nM; and (c) with TT30 (TT) at 4500 nM

[0056] **FIGURE 14** illustrates the survival of PNH RBCs from a subject who had been previously treated with eculizumab, for treatment with various concentrations of TT30 over 5 days. Data represents the percent of surviving PNH RBCs with various concentrations of TT30 vs time of treatment. It can be noted that the percent of surviving PNH RBCs increases with higher doses of TT30.

[0057] **FIGURE 15** illustrates the complement-mediated hemolysis of PNH RBCs from a subject who had been previously treated with eculizumab, and in particular the survival of PNH RBCs treated with TT30, a potent inhibitor of C3 convertase. Data represents the percent of RBC lysis at various time points versus the concentration of TT30 administered compared with RBC lysis by acidified serum. It can be noted that the percent of PNH RBCs that are lysed decreases with higher doses of TT30.

[0058] **FIGURE 16** illustrates the percent inhibition of hemolysis of PNH RBCs at 24 hours after treatment with varying concentrations of TT30. The results are pooled from seven independent experiments from a total of four subjects.

[0059] **FIGURE 17** illustrates the fate of PNH RBCs from a previously untreated subject after various timepoints. Measurements were taken over a 24 hour period. The treatments were (**Figure 17a):** and acidified serum (AcS); (**Figure 17b):** AcS + 3000 nM TT30. Cells were characterized for lysis (red) and survival; surviving cells were further characterized for C3-coating: C3-positive (yellow) and C3-negative (green).

[0060] **FIGURE 18** illustrates the fate of PNH RBCs from a second previously untreated subject after various timepoints. Measurements were taken over a 24 hour period. The treatments were (**Figure 18a):** acidified serum (AcS); and (**Figure 18b):** AcS + 3000 nM

TT30. Cells were characterized for lysis (red) and survival; surviving cells were further characterized for C3-coating: C3-positive (yellow) and C3-negative (green).

[0061] **FIGURE 19** illustrates the fate of PNH RBCs from a subject who has previously untreated been treated with eculizumab, after various timepoints. Measurements were taken over a 24 hour period. The treatments were (Figure **19a):** Acidified serum (AcS); and (**Figure 19b):** AcS + 3000 nM TT30. Cells were characterized for lysis (red) and survival; surviving cells were further characterized for C3-coating: C3-positive (yellow) and C3-negative (green).

[0062] **FIGURE 20** illustrates the sequence of complement alternative pathway activation on a red blood cell in the absence of protection.

[0063] **FIGURE 21** illustrates the sequence of normal protection of a red blood cell from complement alternative pathway activation.

[0064] **FIGURE 22** illustrates the sequence of protection of a red blood cell with an anti-C5 monoclonal antibody.

[0065] **FIGURE 23** illustrates the sequence of inhibition of complement alternative pathway activation on a red blood cell as achieved by the targeted CAP inhibitors of the present invention.

[0066] **FIGURE 24** illustrates the binding of TT30 and anti-C3b monoclonal antibody to asthmatic human and cynomolgus monkey.

[0067] **FIGURE 25** illustrates the concentration-dependent inhibition of RBC lysis by TT30 in a rabbit red blood cell hemolysis assay.

[0068] **FIGURE 26** illustrates that human serum effectively lyses rabbit RBCs in the absence of EDTA. Cell counts indicate that approximately 95% of detected cells stain positively for the presence of C3d fragments in rabbit RBCs in the absence of EDTA.

[0069] **FIGURE 27** illustrates that, with addition of increasing concentrations of TT30, at a TT30 concentration of 0.46 uM, TT30 is bound to approximately 70% of the detected cells (upper right quadrant).

[0070] **FIGURES 28 through 30** illustrate that the TT30 is present on the surface of 70% of detected RBCs at T=0, and that significant numbers of RBCs continue to stain positively for TT30 after 24 hours.

[0071] **FIGURE 31** shows the comparison of C3 fragment accumulation and survival of CD59- PNH RBCs incubated with eculizumab or TTT30 in an in vitro hemolysis assay. The percentage of CD59- PNH RBCs surviving and becoming coated with C3 fragments (C3frag+) when incubated in serum from a patient treated with eculizumab (left; estimated concentration ~200 μg/mL) or serum spiked with TT30 (right; 195 μg/mL) are depicted.

[0072] **FIGURE 32** shows the effect of TT30 on CD59- PNH RBC survival or hemolysis in an in vitro hemolysis assay. The percentage of CD59- PNH RBCs surviving (left) or undergoing hemolysis (right) after incubation for various times at TT30 concentrations ranging from 0.195 to 195 μg/mL are depicted. Data were from a single individual but are representative of results for multiple individuals.

[0073] **FIGURE 33** shows the predicted amino acid sequence of TT30 (SEQ ID NO:3). Each SCR is in a separate line. SCRs from CR2 and Factor H are defined and connecting sequences between SCRs are underlined. Consensus glycosylation sites are Asn101, Asn107, and Asn454.

DETAILED DESCRIPTION OF THE INVENTION

[0074] The present application relates to treatment of complement-mediated hemolytic disorders such as paroxysmal nocturnal hemoglobinuria (PNH). The application is based on the finding that modulation of the complement alternative pathway (CAP), specifically, with a targeted construct comprising CR2 and factor H (TT30; SEQ ID NO:3), is more effective than inhibition with a terminal complement inhibitor, namely, an anti-C5 antibody (eculizumab), at preventing hemolysis of PNH red blood cells (RBCs). Without being bound by a theory, it is hypothesized that the in vitro hemolysis of RBCs from PNH patients on anti-C5 antibody despite the presence of protective concentrations of the antibody may be related to increased susceptibility to lysis of C3 fragment coated RBCs.

[0075] Accordingly, the application in one aspect provides a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, particularly in a subject who has hemolytic anemia, or

who exhibits one or more of the following: symptoms of residual anemia and/or complement-mediated extravascular hemolysis and/or incomplete control of intravascular hemolysis. These features are referred to herein collectively as "hemolytic markers." In another aspect, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, wherein the subject has previously been treated with a terminal complement inhibitor (such as an anti-C5 antibody). The methods are carried out by administering an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway. Particularly suitable complement activation pathway inhibitors are targeted constructs (such as targeted constructs described herein) that comprise a targeting moiety which directs the construct to a site of complement activation and an active moiety which has complement inhibitory activity. In another aspect, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject: a) an effective amount of a terminal complement inhibitor (such as an anti-C5 antibody) and b) an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway. "Terminal complement inhibitor" refers to an inhibitor of one or more complement pathways that inhibits the activity of a component that is downstream of the C3 convertase. These include, for example, inhibitor of the C3 convertase, blocking of C5 (for example an anti-C5 antibody), or an inhibitor that blocks the MAC (membrane attack complex) formation.

[0076] Accordingly, in some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has hemolytic anemia or exhibits one or more of the following: symptoms of residual anemia and/or complement-mediated extravascular hemolysis and/or incomplete control of intravascular hemolysis. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject exhibits signs or symptoms of continued loss of red blood cells by ongoing or

intermittent intravascular hemolysis and/or extravascular hemolysis. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has red blood cells opsonized by fragments of C3. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject requires periodic blood transfusions. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has low normal or below normal levels of hemoglobin. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has low normal or below normal levels of platelets. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has high normal or above normal reticulocytes. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has high normal or above normal bilirubin. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorders (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has iron overload or is

at risk of iron overload. In some embodiments, the composition inhibits activation of complement component C3 (C3) and opsonization of red blood cells by fragments of C3. In some embodiments, the composition maintains normal serum levels of lactate dehydrogenase and hemoglobin. In some embodiments, the complement activation pathway inhibitors is a targeted construct (such as targeted constructs described herein) that comprise a targeting moiety which directs the construct to a site of complement activation and an active moiety which has complement inhibitory activity. In some embodiments, the targeted construct comprises a CR2 or a fragment thereof and a factor H or a fragment thereof. In some embodiments, the targeted construct comprises the first four SCR domains of CR2 and the first five SCR domains of factor H (such as TT30). In some embodiments, the targeted construct is selected from TT30, TT31, and TT32.

[0077] In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has previously been treated with a terminal complement inhibitor (such as an anti-C5 antibody). In some embodiments, the subject is non-responsive to the treatment of a terminal complement inhibitor (such as an anti-C5 antibody). In some embodiments, the subject is partially responsive to the treatment of a terminal complement inhibitor (such as an anti-C5 antibody). In some embodiments, the subject is initially responsive to the terminal complement inhibitor (such as an anti-C5 antibody) but becomes non-responsive after a certain period (such as a month, two months, three months, four months, six months) of treatment with the anti-C5 antibody. In some embodiments, the individual exhibits one or more of the hemolytic markers discussed above upon treatment with the terminal complement inhibitor (such as anti-C5 antibody). In some embodiments, the composition inhibits activation of complement component C3 (C3) and opsonization of red blood cells by fragments of C3. In some embodiments, the composition maintains normal serum levels of lactate dehydrogenase and hemoglobin. In some embodiments, the complement activation pathway inhibitors is a targeted construct (such as targeted constructs described herein) that comprise a targeting moiety which directs the construct to a site of complement activation and an active moiety which has complement inhibitory activity. In some embodiments, the targeted construct comprises a CR2 or a fragment thereof and a factor H or a fragment thereof. In some embodiments, the targeted

construct comprises the first four SCR domains of CR2 and the first five SCR domains of factor H (such as TT30). In some embodiments, the targeted construct is selected from TT30, TT31, and TT32. In some embodiments, the subject was terminated with the treatment of the terminal complement inhibitor for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks prior to the treatment with the inhibitor of the alternative complement pathway.

[0078] In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject: a) an effective amount of a terminal complement inhibitor (such as an anti-C5 antibody) and b) an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject: a) an effective amount of an anti-C5 antibody (such as eculizumab) and b) an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject: a) an effective amount of an inhibitor that prevents the formation of the MAC (such as CD59) and b) an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway. In some embodiments, the composition inhibits activation of complement component C3 (C3) and opsonization of red blood cells by fragments of C3. In some embodiments, the composition maintains normal serum levels of lactate dehydrogenase and hemoglobin. In some embodiments, the complement activation pathway inhibitors is a targeted construct (such as targeted constructs described herein) that comprise a targeting moiety which directs the construct to a site of complement activation and an active moiety which has complement inhibitory activity. In some embodiments, the targeted construct comprises a CR2 or a fragment thereof and a factor H or a fragment thereof. In some embodiments, the targeted construct comprises the first four SCR domains of CR2 and the first five SCR domains of factor H (such as TT30). In some embodiments, the targeted construct is selected from TT30, TT31, and TT32. TT30 comprises a complement alternative pathway inhibitory portion of Factor H protein, which is targeted to sites of complement activation and inflammation through fusion with a portion of the complement receptor 2

protein (CR2 or CD21) which is known to bind to tissue/cell-fixed fragments of the complement component 3 (C3). TT31 is similar to TT30, but contains an additional copy of the complement alternative pathway inhibitory portion of Factor H protein. TT32 comprises a complement inhibitory portion of complement receptor 1 (CR1), targeted through fusion with the same portion of the CR2 protein. CR1 is known to be a broader inhibitor of complement than is Factor H. TT32 will therefore inhibit not only the complement alternative pathway, but will locally inhibit both the classic and lectin pathways of complement as well. Other suitable targeted inhibitors are described in Gilkeson et al., US Patent Publication 2008/0221011, the disclosure of which is hereby specifically incorporated herein by reference.

[0079] The present invention provides methods and compositions for the treatment of subjects having any of a number of complement-mediated disease states which affect red blood cells. Among these are subjects having paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS); chronic hemolytic anemia; antibody-mediated autoimmune hemolytic anemia; anemia caused by hemoglobinopathies such as sickle cell disease; anemia caused by infection such as malaria; anemia due to transfusion reaction; and cold agglutinin disease (CAD). In particular embodiments, the present invention provides methods and compositions for the treatment of subjects with hemolytic anemia, which may be caused by the above disease states, or who exhibit at least one characteristic, which characteristics may be symptoms of residual anemia and/or complement-mediated extravascular hemolysis and/or incomplete control of intravascular hemolysis:

a. the subject exhibits signs or symptoms continued loss of red blood cells by ongoing or intermittent intravascular hemolysis and/or extravascular hemolysis;
b. the subject has red blood cells opsonized by fragments of C3;
c. the subject requires periodic blood transfusions;
d. the subject has low normal or below normal levels of hemoglobin;
e. the subject has low normal or below normal levels of platelets;
f. the subject has high normal or above normal reticulocytes;
g. the subject has high normal or above normal bilirubin; or
h. the subject has iron overload or is at risk of iron overload.

[0080] The indications discussed above (also referred to as "hemolytic markers") can also be used to assess responsiveness to treatment, predict responsiveness to treatment, monitoring progress of treatment, determining suitability of the subject for treatment, determining non-suitability of the subject for treatment, selecting subject for treatment, and/or selecting subject for continued treatment. Thus, for example, in some embodiments, there is provided a method for assessing responsiveness, identifying subjects, and/or selecting subjects having paroxysmal nocturnal hemoglobinuria (PNH) or other complement-mediated hemolytic disorder affecting red blood cells for treatment comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway.

[0081] In some embodiments, there is provided a method of assessing whether a subject having paroxysmal nocturnal hemoglobinuria (PNH) or other complement-mediated hemolytic disorder affecting red blood cells will likely respond to treatment comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, the method comprising assessing at least one hemolytic markers described herein, wherein presence of one or more of these characteristics indicate that the subject will likely be responsive to the treatment. In some embodiments, the method further comprises administering the effective amount of a composition that inhibits the activity of the complement alternative pathway to the subject who is likely to respond to the treatment.

[0082] In some embodiments, there is provided a method of identifying a subject suitable for treatment comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, wherein the subject has paroxysmal nocturnal hemoglobinuria (PNH) or other complement-mediated hemolytic disorder affecting red blood cells, the method comprising assessing at least one hemolytic markers described herein, wherein the presence of one or more of these characteristics identify the subject for being suitable for treatment. In some embodiments, the method further comprises administering the effective amount of a composition that inhibits the activity of the complement alternative pathway to the subject who may be suitable for treatment.

[0083] A subject who "may be suitable", which includes a subject who "is suitable" for treatment(s) described herein, is a subject who is more likely than not to benefit from administration of said treatments. Conversely, a subject who "may not be suitable" or "may be unsuitable", which includes a subject who is "unsuitable" for treatment(s) described

herein, is a subject who is more likely than not to fail to benefit from administration of said treatments.

[0084] In addition, methods are provided herein of selecting or not selecting a subject with paroxysmal nocturnal hemoglobinuria (PNH) or other complement-mediated hemolytic disorder affecting red blood cells more likely suitable or less likely suitable for treatment comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, the method comprising (A) assessing at least one hemolytic markers described herein,; and (B) selecting the subject having one or more of these characteristics. In some embodiments, the method further comprises administering the effective amount of a composition that inhibits the activity of the complement alternative pathway to the subject who may be suitable for treatment.

[0085] Provided herein are also methods for marketing a therapy described herein comprising informing a target audience about the use of the compositions described herein for such uses.

[0086] The present invention also provides methods of monitoring responsiveness of a subject with paroxysmal nocturnal hemoglobinuria (PNH) or other complement-mediated hemolytic disorder affecting red blood cells to treatment comprising administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, the method comprising assessing at least one hemolytic marker described herein.

[0087] In certain embodiments, the subject has paroxysmal nocturnal hemoglobinuria (PNH). PNH is a consequence of clonal expansion of one or more hematopoietic stem cells with mutant *PIG-A*. The extent to which the *PIG-A* mutant clone expands varies widely among patients. In some embodiments, more than 90% of peripheral blood cells in the subject are GPI-AP deficient. In certain embodiments, more than any one of 80%, 70%, 60%, 50%, 40%, 30%, 20% or 10% of peripheral blood cells in the subject are GPI-AP deficient. In certain embodiments, less than 10% of peripheral blood cells in the subject are GPI-AP deficient. Another feature of PNH is its phenotypic mosaicism based on the *PIG-A* genotype that determines the degree of GPI-AP deficiency. For example, PNH III cells are completely deficient in GPI-APs, PNH II cells are partially (~90%) deficient, and PNH I cells, which are progeny of residual normal stem cells, express GPI-AP at normal density. In certain embodiments, the subject has only type I and type III cells. In certain embodiments, the

subject has type I, type II, and type III cells. In certain embodiments, the subject has type I and type II cells.

[0088] In certain embodiments, the subject has classic PNH. Classic PNH is characterized by a large population of GPI-AP deficient PMNs, cellular marrow with erythorid hyperplasia and normal or near-normal morphology and frequent or persistent florid macroscopic hemoglobinuria. In certain embodiments, the subject has PNH in the setting of another bone marrow failure syndrome. PNH in the setting of another bone marrow failure is characterized by a relatively small percentage (< 30%) of GPI-AP deficient PMNs, evidence of a concomitant bone marrow failure syndrome and intermittent or absent mild to moderate macroscopic hemoglobinuria. In certain embodiments, the subject has subclinical PNH. Subclinical or latent PNH is characterized by a small (< 1%) population of GPI-AP deficient PMNs, evidence of a concomitant bone marrow failure syndrome and no clinical or biochemical evidence of intravascular hemolysis.

[0089] In certain embodiments, the subject has atypical hemolytic uremic syndrome (aHUS). In certain embodiments, the subject has chronic hemolytic anemia. In some embodiments, the subject has cold agglutinin disease (CAD). In certain embodiments, the subject exhibits signs or symptoms continued loss of red blood cells by ongoing or intermittent intravascular hemolysis and/or extravascular hemolysis. In certain embodiments, the subject has PNH red blood cells opsonized by fragments of C3. In certain embodiments, subject requires periodic blood transfusions. In some embodiments, the subject has any one of the following: paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS); chronic hemolytic anemia; antibody-mediated autoimmune hemolytic anemia; anemia caused by hemoglobinopathies such as sickle cell disease; anemia caused by infection such as malaria; anemia due to transfusion reaction; and cold agglutinin disease (CAD).

[0090] The methods described herein are also useful for treating complement-mediated hemolysis, reducing intravascular hemolysis, reducing extravascular hemolysis, and increasing survival of red blood cells in a subject.

[0091] In a particular embodiment of the present invention, the subject exhibits iron overload. Transfusions and other treatment of anemia may contribute to or exacerbate iron overload. In addition, treatment of PNH with a terminal complement inhibitor such as

eculizumab or pexelizumab or another terminal complement inhibitor which inhibits C5 or other components of the Membrane Attack Complex (MAC), which comprises C6 through C9, leads to the accumulation of C3 fragments on the surface of PNH RBC, as well as subsequent EVH and iron accumulation by the liver. Accordingly, a subject having PNH or another complement-mediated red blood cell disorder who exhibits symptoms of iron overload or who is approaching iron overload may not be optimally treated by further transfusions. In such cases, treatment in accordance with the methods and compositions of the present invention may be especially beneficial in reducing or controlling anemia without contributing to or exacerbating iron overload. Alternatively, treatment in accordance with the methods and compositions of the present invention may be especially beneficial in treating subjects having complement-mediated hemolytic disorders, such as PNH, while preventing the iron overload that can be observed with treatment with a terminal complement inhibitor, or preventing iron overload and thus allowing for continued administration of the terminal complement inhibitor.

[0092] For purposes of the present invention, a subject is considered to be suffering from iron overload if their serum iron levels are in excess of about 350 ug/dL (mild iron toxicity); preferably in excess of about 500 ug/dL (serious iron toxicity). A subject is considered to be at risk of iron overload if their serum iron levels are high normal or above normal ranges. Normal iron range is considered to be from about 40 to about 220 ug/dL; and more preferably approximately from 50 to about 160 ug/dL for adult males. Normal iron ranges for adult females are approximately 5 to 10 percent lower than that for adult males. 'High normal' iron concentration is considered to be in the upper quarter (25%) of the normal range; preferably in the upper tenth (10%) of the normal range. See, Jacobs & DeMott, Laboratory Test Handbook, 5th ed., (LexiComp Inc, Hudson, OH)(2001) at p. 203-205). As is known to one skilled in the art, 'normal ranges' of iron and iron binding capacity can vary depending upon the specific laboratory and test.

[0093] In some embodiments, the serum iron level of an adult male subject is higher than about 220 ug/dL. In some embodiments, the serum iron level of an adult male subject is higher than about 160 ug/dL. In some embodiments, the serum iron level of an adult male subject is between about 175 ug/dL and about 220 ug/dL. In some embodiments, the serum iron level of an adult male subject is between about 130ug/dL and about 160 ug/dL. In some embodiments, the serum iron level of an adult male subject is between about 200 ug/dL and

about 220 ug/dL. In some embodiments, the serum iron level of an adult male subject is between about 150ug/dL and about 160 ug/dL. In some embodiments, the serum iron level of an adult female subject is higher than about 200 ug/dL. In some embodiments, the serum iron level of an adult female subject is higher than about 145 ug/dL. In some embodiments, the serum iron level of an adult female subject is between about 160 ug/dL and about 200 ug/dL. In some embodiments, the serum iron level of an adult female subject is between about 120 ug/dL and about 145 ug/dL. In some embodiments, the serum iron level of an adult female subject is between about 185 ug/dL and about 200 ug/dL. In some embodiments, the serum iron level of an adult female subject is between about 135 ug/dL and about 145 ug/dL.

[0094] In addition, the methods and compositions of the present invention may be useful for the treatment of other disorders involving complement-mediated extravascular hemolysis, and complement-related disorders which involve one or more of the above characteristics. These disorders may be characterized by high serum lactate dehydrogenase (LDH) levels and/or low serum hemoglobin (HgB) levels. Such disorders may include, for example, atypical hemolytic uremic syndrome (aHUS); chronic hemolytic anemia; and cold agglutinin disease (CAD). Jacobson et al., American J. Medicine, 54:514-21 (1973).

[0095] Because terminal complement inhibitors such as eculizumab, which is a monoclonal antibody that binds C5, must competitively bind to C5 and prevent the enzymatic cleavage of C5 by C5 convertase, it may be difficult, if not impossible, for terminal complement inhibitors to treat a subject experiencing red blood cell lysis to effectively achieve and stably maintain normal ranges of such markers of hemolytic lysis, such as LDH and HgB, with reduced risk of occurrence of breakthrough hemolytic lysis. The effect of such inhibitors depends upon the ability of the antibody to C5 to completely block the cleavage of C5 by C5 convertase. However, because antibodies typically achieve an equilibrium level of binding with their antigen, in this case C5, there will periodically or intermittently be some level unbound C5 present in the blood and at the red blood cell surface. Unbound C5 may be irreversibly cleaved by C5 convertase present on the red blood cells. The amount of unbound C5 present will increase if either (a) serum inhibitor concentration is reduced; or (b) serum C5 concentration in the blood is increased. Further, because the terminal complement inhibitor competes with C5 convertase, a natural ligand for C5, which will irreversibly cleave C5, the effectiveness of the terminal complement inhibitor in maintaining control of hemolysis and preventing breakthrough is affected by increases in serum concentrations of

C5 convertase. As noted previously, the accumulation of C3 fragments and C3 convertase in the blood serum of subjects who are receiving terminal complement inhibitors will naturally tend to increase the serum levels of C5 convertase, limiting the efficacy of the inhibitor.

[0096] LDH and HgB: With the targeted inhibitors of the present invention, the above situations leading to 'breakthrough' lysis may be reduced or avoided because the targeted inhibitors are tethered to C3d fragments, on the red blood cell surface, while the complement inhibitor end of the fusion protein is able to act locally at the red blood cell surface to inhibit activation of the complement alternative pathway. Accordingly, a more stable inhibition of complement may be achieved. Thus, in certain embodiments of the present invention, it is anticipated that a subject suffering from PNH, aHUS, CAD or other hemolytic anemia may be effectively treated such that serum concentration levels of lactate dehydrogenase (LDH) and hemoglobin (HgB) may be maintained within normal concentration ranges. In general, LDH in the serum is an indication of red blood cell lysis, and high levels are an indicator for hemolysis. See, Kato et al., Blood, 107:2279-85 (2006). HgB is a measurement of hemoglobin in the serum, and low levels are an indicator for anemia. See Crosby and Ackroyd, Am. J. Medicine, 13:273-83 (1952); Dameshek, Am. J. Medicine, 18:315-25 (1955).

[0097] The presence of hemolytic anemias may be indicated by high serum levels of LDH concomitant with low serum levels of HgB. For purposes of the present invention, the 'normal range' of LDH serum concentration is considered to be up to approximately 350 IU/l; preferably from about 105 to 333 IU/l; preferably from approximately 140 to 280 IU/L; and in other embodiments, up to about 190 U/L. 'High normal' LDH concentrations would be the upper half (50%) of the normal range, preferably the upper quarter (25%) of the normal range, and most preferably, the upper tenth (10%) of the normal range. For purposes of the present invention, the 'normal range' of serum HgB concentration is considered to be within the range of approximately 13.5 to 18.0 gm/dL for adult males; preferably from about 13.8 to 18.0; and more preferably from about 14.0 to about 17.0 for males; and approximately 11.0 to 16.2 gm/dL for females; more preferably about 12.0 to 16.0 gm/dL for adult females. 'Low normal' HgB concentrations would be the lower half (50%) of the normal range, preferably the lower quarter (25%) of the normal range, and most preferably, the lower tenth (10%) of the normal range. As is known to one skilled in the art, 'normal ranges' of LDH and HgB can vary depending upon the specific laboratory and test. [See, WorldWideWeb at

nlm.nih.gov; Jacobs & DeMott, Laboratory Test Handbook, 5th ed., (LexiComp Inc, Hudson, OH)(2001) at p. 206-208; 319-422].

[0098] In certain embodiments, the subject has low normal or below normal levels of hemoglobin. In some embodiments, the hemoglobin levels are below about 13.5 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are below about 13.8 gm/dL in an adult male subject. n some embodiments, the hemoglobin levels are below about 14 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 13.5 gm/dL and about 15.75 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 13.5 gm/dL and about 14.6 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 13.5 gm/dL and about 13.9 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 13.8 gm/dL and about 15.9 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 13.8 gm/dL and about 14.9 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 13.8 gm/dL and about 14.2 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 14 gm/dL and about 15.5 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 14 gm/dL and about 14.75 gm/dL in an adult male subject. In some embodiments, the hemoglobin levels are between about 14 gm/dL and about 14.3 gm/dL in an adult male subject.

[0099] In some embodiments, the hemoglobin levels are below about 11.0 gm/dL in an adult female subject. In some embodiments, the hemoglobin levels are below about 12.0 gm/dL in an adult female subject. In some embodiments, the hemoglobin levels are between about 11.0 gm/dL and about 13.6 gm/dL in an adult female subject. In some embodiments, the hemoglobin levels are between about 11.0 gm/dL and about 12.3 gm/dL in an adult female subject. In some embodiments, the hemoglobin levels are between about 11.0 gm/dL and about 11.5 gm/dL in an adult female. In some embodiments, the hemoglobin levels are between about 12.0 gm/dL and about 14.0 gm/dL in an adult female subject. In some embodiments, the hemoglobin levels are between about 12.0 gm/dL and about 13.0 gm/dL in an adult female subject. In some embodiments, the hemoglobin levels are between about 12.0 gm/dL and about 12.2 gm/dL in an adult female subject.

[0100] In some embodiments, the subject further has LDH levels higher than about 350 IU/l. In some embodiments, the subject further has LDH levels higher than about 280 IU/l. In

some embodiments, the subject further has LDH levels between about 219 IU/l and 333 IU/l. In some embodiments, the subject further has LDH levels between about 276 IU/l and 333 IU/l. In some embodiments, the subject further has LDH levels between about 310 IU/l and 333 IU/l. In some embodiments, the subject further has LDH levels between about 210 IU/l and 280 IU/l. In some embodiments, the subject further has LDH levels between about 245 IU/l and 280 IU/l. In some embodiments, the subject further has LDH levels between about 266 IU/l and 280 IU/l.

[0101] Platelets: Another characteristic that may be indicative of hemolytic anemia is a serum platelet level below normal range. For purposes of the present invention, the 'normal range' of serum platelet concentration is considered to be within the range of approximately 130 to about 410 (x 10^9/L); preferably within the range of approximately 150 to about 400 (x 10^9/L); and more preferably from about 210 to about 330 (x 10^9/L) for adult males. For adult females, the 'normal range' of serum platelet concentration may be considered to be about 5 to 10% higher than for adult males. 'Low normal' platelet concentrations would be the lower half (50%) of the normal range, preferably the lower quarter (25%) of the normal range, and most preferably, the lower tenth (10%) of the normal range. [See, WorldWideWeb at nlm.nih.gov and at questdiagnostics.com; Jacobs & DeMott, Laboratory Test Handbook, 5th ed., (LexiComp Inc, Hudson, OH)(2001) at p. 471-472]. As is known to one skilled in the art, 'normal ranges' of platelets can vary depending upon the specific laboratory and test.

[0102] In certain embodiments, the subject has low normal or below normal levels of platelets. In some embodiments, the serum platelet level is below about 130 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is below about 150 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is below about 210 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 130 (x 10^9/L) and about 270 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 130 (x 10^9/L) and about 200 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 130 (x 10^9/L) and about 158 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 150 (x 10^9/L) and about 275 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 150 (x 10^9/L) and about 112.5 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 150 (x 10^9/L) and about 175 (x 10^9/L) in an adult male subject. In some

embodiments, the serum platelet level is between about 210 (x 10^9/L) and about 260 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 210 (x 10^9/L) and about 240 (x 10^9/L) in an adult male subject. In some embodiments, the serum platelet level is between about 210 (x 10^9/L) and about 222 (x 10^9/L) in an adult male subject.

[0103] In some embodiments, the serum platelet level is below about 140 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is below about 165 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is below about 230 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 140 (x 10^9/L) and about 295 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 140 (x 10^9/L) and about 220 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 140 (x 10^9/L) and about 170 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 165 (x 10^9/L) and about 300 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 165 (x 10^9/L) and about 235 (x 10^9/L) in an adult fmale subject. In some embodiments, the serum platelet level is between about 165 (x 10^9/L) and about 195 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 230 (x 10^9/L) and about 295 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 230 (x 10^9/L) and about 265 (x 10^9/L) in an adult female subject. In some embodiments, the serum platelet level is between about 230 (x 10^9/L) and about 245 (x 10^9/L) in an adult female subject.

[0104] **Reticulocytes:** Another characteristic that may be indicative of hemolytic anemia is a serum reticulocyte level above normal range. Reticulocytes are young red blood cells from which the nucleus has been extruded, but which retain some remnants of ribosomal RNA. Reticulocyte count rises when there is significant blood loss or red blood cells are destroyed prematurely, for instance, through lysis. For purposes of the present invention, the 'normal range' of serum reticulocyte concentration is considered to be within the range of about 0.5 to about 2.0 percent of total red blood cell count; preferably from about 0.5 to about 1.5%; most preferably from about 1.0% to about 1.5%. Reticulocyte counts as a percentage of red blood cells may be higher when hemoglobin levels are low. 'High normal' HgB concentrations would be the upper half (50%) of the normal range, preferably the upper quarter (25%) of the normal range, and most preferably, the upper tenth (10%) of the normal

range. [See, WorldWideWeb at nlm.nih.gov and at questdiagnostics.com; Jacobs & DeMott, Laboratory Test Handbook, 5th ed., (LexiComp Inc, Hudson, OH)(2001) at p. 481-482]. As is known to one skilled in the art, 'normal ranges' of reticulocytes can vary depending upon the specific laboratory and test.

[0105] In some embodiments, the subject has increased reticulocytes. In some embodiments, the subject has a serum reticulocyte concentration of higher than about 1.5% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration of higher than about 2.00 % of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.0% to 1.5% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.25% to 1.5% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.375% to 1.5% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.4% to 1.5% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.45% to 1.5% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.25% to 2.0% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.625% to 2.0% of total red blood cell count. In some embodiments, the subject has a serum reticulocyte concentration between about 1.85% to 2.0% of total red blood cell count.

[0106] Bilirubin: Another characteristic that may be indicative of hemolytic anemia is a serum bilirubin level above normal range. For purposes of the present invention, the 'normal range' of serum bilirubin concentration is considered to be within the range of approximately 0.3 to 1.9 mg/dL; preferably within the range of approximately 0.3 to 1.0 mg/dL. 'High normal' bilirubin concentrations would be the upper half (50%) of the normal range, preferably the upper quarter (25%) of the normal range, and most preferably, the upper tenth (10%) of the normal range. [See, WorldWideWeb at nlm.nih.gov and at questdiagnostics.com; Jacobs & DeMott, Laboratory Test Handbook, 5th ed., (LexiComp Inc, Hudson, OH)(2001) at p. 471-472]. As is known to one skilled in the art, 'normal ranges' of bilirubin can vary depending upon the specific laboratory and test.

[0107] In certain embodiments, the subject has increased bilirubin. In some embodiments, the subject has a serum bilirubin level of higher than about 1.9 mg/dL. In some embodiments, the

subject has a serum bilirubin level of higher than about 1.0 mg/dL. In some embodiments, the subject has a serum bilirubin level between about 1.1 mg/dL and 1.9 mg.dL. In some embodiments, the subject has a serum bilirubin level between about 1.5 mg/dL and 1.9 mg.dL. In some embodiments, the subject has a serum bilirubin level between about 1.75 mg/dL and 1.9 mg.dL. In some embodiments, the subject has a serum bilirubin level between about 0.65 mg/dL and 1.0 mg.dL. In some embodiments, the subject has a serum bilirubin level between about 0.825 mg/dL and 1.0 mg.dL. In some embodiments, the subject has a serum bilirubin level between about 0.93 mg/dL and 1.0 mg.dL.

[0108] In certain embodiments, the subject having a complement-mediated hemolytic disorder affecting red blood cells, such as PNH, has previously been treated with a terminal complement inhibitor, but persists in exhibiting at least one of the above characteristics of residual anemia and/or complement-mediated extravascular hemolysis. In such cases, the present invention provides methods and compositions for avoiding or reducing the occurrence and/or severity of at least one of the above characteristics.

[0109] In certain embodiments, the subject having a complement-mediated hemolytic disorder affecting red blood cells, such as PNH exhibits a suboptimal response to treatment with a terminal complement inhibitor. Such suboptimal response may include the subject exhibiting at least one of the above characteristics of residual anemia and/or complement-mediated extravascular hemolysis. The method comprises administering an effective amount of a composition that inhibits the activity of the complement alternative pathway, wherein the composition inhibits activation of complement component C3 (C3), for example by inhibiting alternative pathway activation by initiation C3 convertase and/or by inhibiting formation and/or activity of amplifying C3 convertase and opsonization of red blood cells by fragments of C3.

[0110] In some embodiments, the subject having a complement-mediated hemolytic disorder affecting red blood cells, such as PNH has previously been treated with a terminal complement inhibitor, being initially responsive to such treatment, and experiencing recurrence. In some embodiments, there is provided a method of treating a complement-mediated hemolytic disorder (such as hemolytic disorder affecting red blood cells for example PNH) in a subject, comprising administering to the subject an effective amount of a composition that inhibits (such as selectively inhibits) activation of the complement alternative pathway, wherein the subject has previously been treated with a terminal

complement inhibitor (such as an anti-C5 antibody). In some embodiments, the subject is non-responsive to the treatment of a terminal complement inhibitor (such as an anti-C5 antibody). In some embodiments, the subject is partially responsive to the treatment of a terminal complement inhibitor (such as an anti-C5 antibody). In some embodiments, the subject is initially responsive to the terminal complement inhibitor (such as an anti-C5 antibody) but becomes non-responsive after a certain period (such as a month, two months, three months, four months, six months) of treatment with the anti-C5 antibody. In some embodiments, the individual exhibits one or more of the hemolytic markers discussed above upon treatment with the terminal complement inhibitor (such as anti-C5 antibody). In some embodiments, the complement activation pathway inhibitors is a targeted construct (such as targeted constructs described herein) that comprise a targeting moiety which directs the construct to a site of complement activation and an active moiety which has complement inhibitory activity. In some embodiments, the targeted construct comprises a CR2 or a fragment thereof and a factor H or a fragment thereof. In some embodiments, the targeted construct comprises the first four SCR domains of CR2 and the first five SCR domains of factor H (such as TT30). In some embodiments, the subject was terminated with the treatment of the terminal complement inhibitor for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks prior to the treatment with the inhibitor of the alternative complement pathway. In some embodiments, the subject has progressed on a prior therapy (for example progressed after any of about 3, 6, 9, or 12 months upon initiation of the prior therapy).

[0111] In another aspect, the invention provides for methods of treating a subject having paroxysmal nocturnal hemoglobinuria (PNH) or another disease which involves a complement-mediated extravascular hemolysis component, the method comprising administering a composition comprising a combination comprising (i) an effective amount of an inhibitor of the complement alternative pathway and (ii) an effective amount of a terminal complement inhibitor. Additional complement inhibitors (such as inhibitors of the complement alternative pathway can also be administered.

[0112] The composition comprising an inhibitor of the complement alternative pathway and a terminal complement inhibitor can be administered simultaneously. The term "simultaneous administration," as used herein, means that the composition comprising an inhibitor of the complement alternative pathway and terminal complement inhibitor in a combination therapy are administered with a time separation of no more than about 15 minutes, such as no more

than about any of 10, 5, or 1 minutes. When the drugs are administered simultaneously, the composition comprising an inhibitor of the complement alternative pathway and the terminal complement inhibitor may be contained in the same composition (e.g., a composition comprising both the inhibitor of the complement alternative pathway and the terminal complement inhibitor) or in separate compositions (e.g., the composition comprising an inhibitor of the complement alternative pathway is contained in one composition and the terminal complement inhibitor is contained in another composition).

[0113] Alternatively, the inhibitor of the complement alternative pathway and the terminal complement inhibitor are administered sequentially, i.e., the composition comprising the inhibitor of the complement alternative pathway is administered either prior to or after the administration of the terminal complement inhibitor. As used herein, the term "sequential administration" means that the composition comprising an inhibitor of the complement alternative pathway and the terminal complement inhibitor are administered with a time separation of more than about 15 minutes, such as more than about any of 20, 30, 40, 50, 60, or more minutes. Either the composition comprising an inhibitor of the complement alternative pathway or the terminal complement inhibitor may be administered first. The composition comprising an inhibitor of the complement alternative pathway and the terminal complement inhibitor are contained in separate compositions, which may be contained in the same or different packages or kits. In some embodiments, the composition comprising the inhibitor of the complement alternative pathway is administered prior to the administration of the terminal complement inhibitor. In some embodiments, the composition comprising the inhibitor of the complement alternative pathway is administered after the administration of the terminal complement inhibitor.

[0114] In some embodiments, the administration of the composition comprising the inhibitor of the complement alternative pathway and the complement inhibitor are concurrent, i.e., the administration period of the composition comprising the inhibitor of the complement alternative pathway and that of the terminal complement inhibitor overlap with each other. In some embodiments, the composition comprising the inhibitor of the complement alternative pathway is administered for at least one cycle (for example, at least any of 2, 3, or 4 cycles) prior to the administration of the terminal complement inhibitor. In some embodiments, the terminal complement inhibitor is administered for at least any of one, two, three, or four weeks.

[0115] In some embodiments, the administrations of the composition comprising the inhibitor of the complement alternative pathway and the terminal complement inhibitor are non-concurrent. For example, in some embodiments, the administration of the composition comprising the inhibitor of the complement alternative pathway is terminated before the terminal complement inhibitor is administered. In some embodiments, the administration of the terminal complement inhibitor is terminated before the composition comprising the inhibitor of the complement alternative pathway is administered. In some embodiments, the time period between these two non-concurrent administrations can range from about one day to about eight weeks. In some embodiments, the time period between these two non-concurrent administrations is about one day. In some embodiments, the time period between these two non-concurrent administrations is more than about one day, such as about two, three, four, five, six days. In some embodiments, the time period between these two non-concurrent administrations is at least about one week. In some embodiments, the time period between these two non-concurrent administrations is at least about two weeks. In some embodiments, the time period between these two non-concurrent administrations is at least about four weeks. In some embodiments, the time period between these two non-concurrent administrations is at least about eight weeks.

[0116] The dosing frequency of the composition comprising the inhibitor of the complement alternative pathway and the terminal complement inhibitor may be adjusted over the course of the treatment, based on the judgment of the administering physician. When administered separately, the composition comprising the inhibitor of the complement alternative pathway and the terminal complement inhibitor can be administered at different dosing frequency or intervals. For example, the composition comprising the inhibitor of the complement alternative pathway can be administered weekly, while a terminal complement inhibitor can be administered more or less frequently. In some embodiments, sustained continuous release formulation of the composition comprising the inhibitor of the complement alternative pathway and/or terminal complement inhibitor may be used. Various formulations and devices for achieving sustained release are known in the art.

[0117] The composition comprising the inhibitor of the complement alternative pathway and the terminal complement inhibitor can be administered using the same route of administration or different routes of administration. In some embodiments (for both simultaneous and sequential administrations), the composition comprising the inhibitor of the complement

alternative pathway and the terminal complement inhibitor are administered at a predetermined ratio.

TARGETING TO COMPLEMENT ACTIVATED TISSUE

[0118] The compositions described herein preferably are targeted for increased association to tissue which has been injured, damaged or has become inflamed by physical, chemical or other insult or injury. Targeting can be accomplished by tethering, fusing or otherwise associating an active agent to a targeting moiety. In some embodiments, the targeting moiety binds to a binding partner associated with complement activation. "A binding partner associated with complement association" refers to a molecule or epitope that is present at the site of complement activation. In some embodiments, the molecule or epitope is only present when a complement pathway is activated. In some embodiments, the molecule or epitope is available for binding to the targeting moiety only when a complement pathway is activated. In some embodiments, the targeting moiety binds to tissue-associated complement component 3 (C3) or one or more fragments of C3, including, but not limited to: C3b, iC3b, C3d and C3dg. In preferred embodiments, the targeting moiety will bind to complement component 3 (C3) or one or more fragments of C3, including, but not limited to: C3b, iC3b, C3d and C3dg. Preferred targeting moieties include, for example, complement receptor 2 (CR2) or fragments thereof that retain the ability to bind to one or more fragments of C3; monoclonal antibodies to C3, C3b, iC3b, C3d, C3dg, or other fragments of C3. It is also possible to use some of the non-regulatory fragments of Factor H that retain the ability to bind to one or more fragments of C3, including C3b, iC3b and C3d or other fragments of C3. These fragments potentially include fragments comprising SCR domains 5-8 and SCR domains 19-20. It should be noted that the term "CR2-targeted" may be used in the present invention to mean that a molecule will specifically bind to one or more fragments of C3, such as C3, C3b, iC3b, C3d and C3dg, in a manner analogous to the natural binding of CR2. Thus, for example, both a fusion protein that comprises either (a) a targeting moiety derived from CR2 or (b) an antibody to C3d; fused to complement Factor H may be referred to as a "CR2-targeted Factor H."

[0119] In some embodiments, the construct is a fusion protein comprising the targeting moiety and the active moiety. In some embodiments, the targeting moiety and the active moiety are fused through a peptide linker. In some embodiments, the targeting moiety and the active moiety are joined by a non-peptide linker. In some embodiments, the targeting moiety

is fused to the N-terminus of the active moiety. In some embodiments, the targeting moiety is fused to the C-terminus of the active moiety. In some embodiments the targeting moiety is inserted in the middle of the active moiety.

[0120] Exemplary fusion proteins include, but are not limited to, polypeptides in which the C-terminal portion of a targeting moiety is fused to the N-terminal portion of the active moiety, the N-terminal portion of a targeting moiety is fused to the C-terminal portion of the active moiety, two or more targeting moieties fused to both the N- terminal and the C-terminal portions of the active moiety, the targeting moiety inserted in the middle of the active moiety, and the like.

[0121] In some embodiments, the molecule comprises two or more (such as any of two, three, four, five, or more) CR2 portions (or any other target moiety portion, or combinations thereof). These CR2 portions (or other target moiety portions) may be the same or different, for example in terms of amino acid sequences, structures, and/or functions.

[0122] In some embodiments, the molecule comprises two or more (such as any of two, three, four, five, or more) active moiety portions. These active moiety portions may be the same or different, for example in terms of amino acid sequences, structures, and/or functions.

[0123] In some embodiments, the molecule (such as a fusion protein) comprises: 1) two or more CR2 portions comprising a CR2 or a fragment thereof, and 2) two or more active moieties

[0124] In some embodiments, the targeting moiety exhibits high avidity for its binding partner. In some embodiments, the targeting moiety exhibits high avidity but low affinity to its binding partner. Binding avidity is a measure of the strength of the initial process by which a targeting moiety, such as an antibody or ligand, will seek out, locate and bind with its binding partner, and is the initial process involved in binding affinity. Binding affinity, on the other hand, is a broader measure which also takes into account not only avidity, but other characteristics of binding such as strength of interaction and coefficients of dissociation.

[0125] Methods have been developed for the measurement and modification of binding attributes, such as avidity and affinity. For example, see Lee et al., Molecular Immunology, 47:816-24 (2010); Kaymakcalan et al., Clinical Immunology, 131:308-16 (2009); Konstandin et al., J Immunol. Methods, 310:67-77 (2006); and Oda et al., Molecular Immunology,

37:1111-22 (2000). Accordingly, the present invention may include methods for assessing the binding avidity and affinity of a targeting moiety; mutating or modifying the targeting moiety and assessing the effects of such mutation or modification, in order to obtain targeting moieties with improved targeting characteristics, e.g., binding avidity.

[0126] Although certain sections herein discuss CR2-targeted constructs, it is understood that the same applies to other targeting moieties described herein. The different targeting moieties are described herein in more detail.

CR2 and CR2 Fragments.

[0127] The use of complement receptor 2 (CR2), or functional fragments thereof, to target complement modulators to tissue which exhibit or express C3, or fragments of C3 to which the CR2 is able to bind, including C3b, iC3b, C3d and C3dg, is described in US 2008/0267980, Tomlinson and Holers, the disclosure of which is hereby incorporated herein by reference. Such CR2 molecules, and functional fragements thereof, can be used in the present invention as the targeting moiety. In particularly preferred embodiments, the first two N-terminal short concensus repeat domains (SCRs), comprising an active binding site for C3dg, can be used in the present invention as the targeting moiety. The present inventors have found that, while red blood cells are normally privileged from complement attack, the complement-mediated blood disorders treatable by the present invention may lead to abnormal coating of the RBC surface with C3 and/or C3 fragments, rendering the affected RBCs vulnerable to complement attack and opsonization.

[0128] Human complement receptor 2, also referred to as CD21 (CR2/CD21)(SEQ ID NO:1 and SEQ ID NO:2), is a ~145 kD transmembrane protein of the C3 binding protein family comprising 15 or 16 short consensus repeat (SCR) domains, structural units characteristic of such proteins. CR2 is expressed on mature B cells and follicular dendritic cells, and plays an important role in humoral immunity. J. Hannan et al., Biochem. Soc. Trans. (2002) 30:983-989; K.A. Young et al., J. Biol. Chem. (2007) 282(50):36614-36625. CR2 protein does not bind intact C3 protein, but binds its breakdown products, including the C3b, iC3b, and C3d cleavage fragments, via a binding site located within the first two amino-terminal short consensus repeats ("SCRs 1-2") of the CR2 protein. Consequently, the SCR1-2 domain of CR2 discriminates between cleaved (i.e., activated) forms of C3 and intact circulating C3. As a targeting group, SCRs 1-2 of CR2 are therefore able to discriminate between circulating C3

and the C3 fragments generated during complement activation. While the affinity of CR2 for C3d is only 620-658 nM (J. Hannan et al., Biochem. Soc. Trans. (2002) 30:983-989; J.M. Guthridge et al., Biochem. (2001) 40:5931-5941), the avidity of CR2 for clustered C3d makes it an effective method of targeting molecules to sites of complement activation.

[0129] CR2 contains an extracellular portion having 15 or 16 repeating units known as short consensus repeats (SCR domains). The SCR domains typically have a framework of highly conserved residues including four cysteines, two prolines, one tryptophan and several other partially conserved glycines and hydrophobic residues. SEQ ID NO:1 represents the full-length human CR2 protein sequence having 15 SCR domains. Amino acids 1-20 of SEQ ID NO:1 comprise the leader peptide, amino acids 23-82 of SEQ ID NO:1 comprise SCR1, amino acids 91-146 of SEQ ID NO:1 comprise SCR2, amino acids 154-210 of SEQ ID NO:1 comprise SCR3, amino acids 215-271 of SEQ ID NO:1 comprise SCR4. The active site (C3d binding site) is located in SCR1-2 (the first two N-terminal SCR domains)(SEQ ID NO:2). These SCR domains are separated by short sequences of variable length that serve as spacers. It is understood that species and strain variations exist for the disclosed peptides, polypeptides, and proteins, and that the CR2 or a fragment thereof described herein encompasses all species and strain variations.

[0130] In certain embodiments, the CR2 portion comprises a polypeptide that contains some or all of the ligand binding sites of the CR2 protein, and includes, but is not limited to, full-length CR2 proteins (such as human CR2 as shown in SEQ ID NO:1), soluble CR2 proteins (such as a CR2 fragment comprising the extracellular domain of CR2), other biologically active fragments of CR2, a CR2 fragment comprising SCR1-2 (SEQ ID NO:2), or any homolog of a naturally occurring CR2 or fragment thereof, as described in detail below. In some embodiments, the CR2 portion has at least one of the following properties or CR2: (1) the ability to bind to C3d, (2) the ability to bind to iC3b, (3) the ability to bind to C3dg, (4) the ability to bind to C3d, and (5) the ability to bind to one or more cell-bound fragments of C3b that bind to the two N-terminal SCR domains of CR2.

[0131] In certain embodiments, the CR2 portion comprises the first two N-terminal SCR domains of CR2 (SEQ ID NO:2). In certain embodiments, the CR2 portion comprises the first three N-terminal SCR domains of CR2. In certain embodiments, the CR2 portion comprises the first four N-terminal SCR domains of CR2. In certain embodiments, the CR2 portion comprises (and in some embodiments consists of or consists essentially of) at least

the first two N-terminal SCR domains of CR2, including for example at least any of the first 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 SCR domains of CR2.

[0132] Cleavage of C3 results initially in the generation and deposition of C3b on the activating cell surface. The C3b fragment is involved in the generation of enzymatic complexes that amplify the complement cascade. On a cell surface, C3b is rapidly converted to inactive iC3b, particularly when deposited on a host surface containing regulators of complement activation (i.e., most host tissue). Even in the absence of membrane-bound complement regulators, substantial levels of iC3b are formed because of the action of serum factor H and serum factor I. iC3b is subsequently digested to the membrane-bound fragments C3dg and then C3d by factor I and other proteases and cofactors, but this process is relatively slow. Thus, the C3 ligands for CR2 are relatively long lived once they are generated and are present in high concentrations at sites of complement activation.

Antibodies to C3 and C3 Fragments. ***Antibodies to C3 and C3 Fragments.***

[0133] In place of a targeting moiety derived from CR2, the targeting moiety may comprise antibodies which bind to C3 or C3 fragments such as C3b, iC3b, C3d and C3dg, or an antigen-binding fragment thereof. Antibodies that bind to C3 and to cleavage fragments C3b and C3d, are known. For example, see US Patent 6,572,856, Taylor; Tosic et al., J. Immunological Methods, 120:241-249 (1989); Sokoloff et al., Cancer Immunology and Immunotherapy, 49:551-562 (2000); Mastellos et al., Molecular Immunology, 40:1213-1221 (2004); Dilillo et al., Molecular Immunology, 43:1010-1019 (2006); Campagne, US 2009/0081211; Etemad-Gilbertson et al., US 2009/0175875; Aguado et al., J. Clin. Invest., 76:1418-1426 (1985). The disclosure of these documents is hereby incorporated herein by reference. Such antibodies, and functional fragments thereof, may be useful in the present invention as the targeting moiety for directing therapeutic fragments to tissue experiencing activated complement activity, and thus expressing C3 or its fragments. Functional fragments of antibodies may include, for example, single-chain variable fragments (scFvs) which preferably comprise of V_H and V_L domains, optionally joined by a flexible peptide linker. Such scFvs usually retain the specificity of the parental antibody and bind the target antigen in a monovalent manner. Conversion of antibodies to scFvs is well known process (see Nat. Biotechnol. 23:1126 (2005); Biomol Eng. 24:201 (2007); J Immunol Methods., 168:149 (1994); Arch Virol. 148:497 (2003). ScFvs can be constructed by either de novo gene synthesis, overlap extension polymerase chain reaction (PCR) or sequential ligation of

the individual heavy (V_H) and light (V_L) chain variable gene segments. Sequential cloning of the individual V_H and V_L genes into a vector containing the synthetic linker sequence (e.g., $(Gly_4Ser)_3$) can be performed as well. The sequence can be either V_H–linker-V_L or V_L–linker-V_H.

[0134] In particularly preferred embodiments, the targeting moiety may comprise an antibody which binds to C3d, such as those produced by: (1) hybridoma cell line 3d-9a/25, deposited on May 26, 2010, and designated as ATCC Patent Deposit PTA-10998; (2) hybridoma cell line 3d-8b/2, deposited on May 26, 2010, and designated as ATCC Patent Deposit PTA-10999; (3) hybridoma cell line 3d-29/5/2, deposited on May 26, 2010, and designated as ATCC Patent Deposit PTA-11000; (4) hybridoma cell line 3d-10/14/1, deposited on June 2, 2010, and designated as ATCC Patent Deposit PTA-11010; (5) hybridoma cell line 3d-11/14, deposited on June 2, 2010, and designated as ATCC Patent Deposit PTA-11011; (6) hybridoma cell line 3d-15A9, deposited on June 2, 2010, and designated as ATCC Patent Deposit PTA-11012; (7) hybridoma cell line 3d-3/28/4, deposited on June 9, 2010, and designated as ATCC Patent Deposit PTA-11025; (8) hybridoma cell line 3d-16/3/3, deposited on June 9, 2010, and designated as ATCC Patent Deposit PTA-11026; and (9) hybridoma cell line 3d-31/A6/9, deposited on June 9, 2010, and designated as ATCC Patent Deposit PTA-11027. Those antibodies are described in more detail in the US Provisional Patent Application Serial No. 61/357,499, filed on June 22, 2010, entitled "Antibodies to the C3d Fragment of Complement Component 3 ," which is incorporated herein by reference in its entirety. The complete nucleotide and amino acid sequences of the monoclonal antibodies produced by those hybridoma cell lines can easily be determined with standard methods such as, for example, the polymerase chain reaction (PCR) and automated sequencing.

Conversion of anti-C3d monoclonal antibodies into single-chain variable fragments

[0135] (scFvs). Conversion of antibodies to scFvs is well known process (see Nat. Biotechnol. 23:1126 (2005); Biomol Eng. 24:201 (2007); J Immunol Methods., 168:149 (1994); Arch Virol. 148:497 (2003). ScFvs can be constructed by either de novo gene synthesis, overlap extension polymerase chain reaction (PCR) or sequential ligation of the individual heavy (V_H) and light (V_L) chain variable gene segments. Sequential cloning of the individual V_H and V_L genes into a vector containing the synthetic linker sequence (e.g.,

$(Gly_4Ser)_3$) can be performed as well. The sequence can be either V_H–linker-V_L or V_L–linker-V_H. Engineered anti-C3d-scFvs can be linked to complement inhibitors such as CFI. Single chain Fvs of the present invention preferably comprise of V_H and V_L domains joined by flexible peptide linker that prevents the dissociation. AntiC3d monoclonal antibody can be converted into single-chain variable fragments in an equivalent manner as described above. The scFvs usually retain the specificity of the parental antibody and bind the target antigen in a monovalent manner.

[0136] A series of linkers can be used to identify the optimal distance between antiC3d-scFv and CFI-related targeting moiety, ranging from no linker to linker of 40 amino acids, and the sequence chosen for the linker can be for example (GlyGlyGlyGlySer)n where n = 0-8. The construction of the antiC3d-scFv linked to CFI-related moiety can be done by de novo gene synthesis, overlap PCR, sequential cloning or ligation. The N-terminal moiety of the final construct can be either CFI-related moiety or antiC3d-scFv.

Factor H and FH Fragments

[0137] Factor H has at least three distinct binding domains for C3b, which are located within SCRs 1-4; SCRs 5-8, and SCRs 19-20. Each site of Factor H binds to a distinct region within the C3b protein: N terminal sites bind to native C3b; the second site, located in the middle region of factor H, binds to the C3c fragment; and the site located within SCR19-20 binds to the C3d region. Factor H also contains binding sites for heparin, which are located within SCR 7, SCRs 5-12 and SCRs 19-20 of Factor H and overlap with those of the C3b binding sites. In some embodiments, the targeting moiety comprises a non-complement regulatory fragments of Factor H that retain the ability to bind to one or more fragments of C3, including C3b, iC3b and C3d or other tissue-associated fragments of C3. In some embodiments, the target moiety comprises SCR domains 5-8 and SCR domains 19-20 of factor H.

[0138] As used herein, the term "complement factor H," "factor H," or "FH" refers to complement factor H, a single polypeptide chain plasma glycoprotein, including homologs thereof. The protein is composed of 20 conserved short consensus repeat (SCR) domains of approximately 60 amino acids, arranged in a continuous fashion like a string of beads, separated by short linker sequences of 2-6 amino acids each. Factor H binds to C3b, accelerates the decay of the alternative pathway C3-convertase (C3bBb), and acts as a cofactor for the proteolytic inactivation of C3b. In the presence of factor H, proteolysis by

factor I results in the cleavage and inactivation of C3b. Factor H has at least three distinct binding domains for C3b, which are located within SCRs 1-4, SCRs 5-8, and SCRs 19-20. Each site of factor H binds to a distinct region within the C3b protein: the N-terminal sites bind to native C3b; the second site, located in the middle region of factor H, binds to the C3c fragment and the site located within SCR19 and 20 binds to the C3d region. In addition, factor H also contains binding sites for heparin, which are located within SCR 7, SCRs 5-12, and SCR 20 of factor H and overlap with those of the C3b binding sites. Structural and functional analyses have shown that the domains for the complement inhibitory activity of factor H are located within the first four N-terminal SCR domains.

Antibodies to Inflammatory Neoepitopes.

[0139] In other preferred embodiments, the targeting moiety may comprise an antibody which binds to an inflammatory neoepitope, such as annexin IV, annexin A2, phospholipids, such as cardiolipin, and citrulline-modified protein. Suitable antibodies may include, for example, antibody B4, which binds to annexin IV; antibody C2, which binds to cardiolipin; and antibody D5, which binds to citrulline-modified protein (See Holers et al., WO 2007/112403; Thurman and Holers, WO 2010/034015); See also, Allison, US Patents 6,962,903; 7,407,475 (Annexin V); 7,635,676; 7,635,678; 7,635,679; 7,635,680; and 7,645,739; proximal tubule targeting moiety, see Quigg et al., US 2005/0265995. As described above, functional fragments of antibodies such as ScFvs can be used as the targeting moiety. The complete nucleotide and amino acid sequences of the monoclonal antibodies produced by those hybridoma cell lines can easily be determined with standard methods such as, for example, the polymerase chain reaction (PCR) and automated sequencing.

Other Targeting Moieties.

[0140] The present invention also contemplates that other targeting moieties may be used. The targeting moiety should ideally bind to one or more of the fragments of C3 that bind to cells in areas of complement activation. This also includes targeting moieties that bind to one or more neoepitopes that are expressed on cells in areas of complement activation.

INHIBITION OF COMPLEMENT ALTERNATIVE PATHWAY

[0141] The compositions described herein in some embodiments comprise an active agent moiety that significantly or selectively inhibits the complement alternative pathway. By "significantly inhibiting the complement alternative pathway" it is meant that the active agent inhibits the complement alternative pathway with a potency or percent inhibition that measures at least 10%, preferably 20%, 30% or 40%, and more preferably at least about 50%, 60%, 80% or 90% of alternative complement activity in the absence of said active agent moiety. By "selectively inhibits the complement alternative pathway" it is meant that the active agent inhibits the complement alternative pathway with a potency or percent inhibition which measures at least two, three, four, five or ten times greater than that of the classical or lectin complement pathways. More preferably, the active agent will selectively inhibit the complement alternative pathway at a potency or percent inhibition of at least one, two or three orders of magnitude greater than that the classical or lectin complement pathways.

[0142] In preferred embodiments, active agent moiety comprises an inhibitor of the complement alternative pathway, or a biologically active fragment thereof, selected from the group consisting of Factor H protein, Crry, Decay Accelerating Factor (DAF), MCP, CR1, or biologically active fragments thereof. In some embodiments, the active moiety is an inhibitor of the amplification loop of the alternative complement pathway and will prevent the formation and activity of the amplification C3 convertase, as well as the downstream C5 convertase. In some embodiments, the active moiety will be able to inactivate the initiation C3 convertase once it is formed on the red blood cell surface, and may partially prevent formation of the initiation C3 convertase in the fluid phase.

[0143] In a particularly preferred embodiment, the active agent moiety comprises Factor H protein or a biologically active fragment thereof, which retains the ability to inhibit the complement alternative pathway. Suitable Factor H protein an biologically active fragments thereof include the first four N-terminal short consensus sequences (SCRs of Factor H, and are described in US 2008/0221011, Gilkeson et al, the disclosure of which is hereby specifically incorporated herein by reference.

[0144] Other complement inhibitors, and fragments thereof, which may be useful as the active agent moiety in the present invention are described, for example, in Tomlinson and Holers, US 2008/0267980. Suitable complement inhibitors, for example, may include

complement receptor 1 (CR1); MCP; Crry; or DAF. The disclosure of Tomlinson and Holers is hereby specifically incorporated herein by reference. Other complement inhibitors, which may be useful as the active agent moiety include compstatin, see Janssen et al., J. Biol. Chem. 282:29241-7 (2007). In preferred embodiments, the complement inhibitor is an inhibitor of the amplification loop of the alternative complement pathway.

[0145] In certain embodiments, the active moiety comprises two or more complement inhibitors, for example, two or more biologically active fragments of factor H as in TT31; or an active fragment of factor H in combination with an active fragment of complement receptor 1, MCP; Crry; complement factor I; CD59 or DAF. In such embodiments, the active moiety may be provided in multiple forms, for example, when the active moiety comprises a combination of Factor H and CD59, the composition may include two or more of the following: CR2-FH-CD59; CR2-CD59-FH; antiC3d-FH-CD59 and antiC3d-CD59-FH.

[0146] As used herein, the term "biologically active" fragment of a complement inhibitor refers a fragment of the complement inhibitor which retains some or all of the inhibitory activity of the full-length complement inhibitory protein. For example, "biologically active" fragments of factor H include, but are not limited to, factor H fragments comprising SCRs 1-4, SCRs 1-5, SCRs 1-8, SCRs 1-18, SCRs 19-20, or any homolog of a naturally-occurring factor H or fragment thereof, as described in detail below. In certain embodiments, the biologically active fragment of factor H has one or more of the following properties: (1) binding to C-reactive protein (CRP), (2) binding to C3b, (3) binding to heparin, (4) binding to sialic acid, (5) binding to endothelial cell surfaces, (6) binding to cellular integrin receptor, (7) binding to pathogens, (8) C3b co-factor activity, (9) C3b decay-acceleration activity, and (10) inhibiting the alternative complement pathway.

[0147] It is contemplated that variants and modifications of the complement inhibitors described above may be used as the active agent moiety in certain embodiments of the present invention. For example, through deletion analysis, it may be possible to identify smaller fragments of some of the above complement inhibitors, which comprise the minimal sequence elements required for complement inhibition. In other embodiments, the complement inhibitors, or the minimal sequence elements thereof required for complement inhibition, may be modified in order to increase half-life, stability or potency of the active agent moiety as an inhibitor. For example, the active agent moiety may comprise the complement inhibitor, or active fragment thereof, tethered to a protein or non-protein scaffold

which is intended to maintain the active agent moiety in a conformation capable of complement inhibition, while reducing susceptibility to proteases or otherwise extending the half-life, stability or potency of the active agent moiety.

[0148] The following description refers to methods and compositions in which the targeted therapeutic agent is a fusion protein comprising a targeting moiety derived from CR2 and an active agent derived from FH (CR2-FH fusion protein). This description is non-limiting, and one skilled in the art will be able to practice the invention with respect to other embodiments of the invention, including the alternative targeting moieties and alternative active moieties that are mentioned herein.

[0149] Also contemplated are inhibitors that inhibit the complement alternative pathway indirectly. For example, in some embodiments, the inhibitor inhibits two or more complement pathways. In some embodiments, the inhibitor inhibits the lectin complement pathway (for example, in some embodiments, the inhibitor is an anti-MASP antibody). In some embodiments, the inhibitor inhibits the classical pathway (for example, in some embodiments, the inhibitor is CR1). Other complement inhibitors are also contemplated.

[0150] In some embodiments, the targeted construct described herein comprises two or more complement inhibitors or fragments thereof. These two or more complement inhibitors or fragments thereof in each construct can be the same or different. The two or more complement inhibitors or fragments thereof in each construct can inhibit the same or different complement pathways.

Compositions:

[0151] The compositions described herein can be administered to an individual via any route, including, but not limited to, intravenous (e.g., by infusion pumps), intraperitoneal, intraocular, intra-arterial, intrapulmonary, oral, inhalation, intravesicular, intramuscular, intra-tracheal, subcutaneous, intraocular, intrathecal, transdermal, transpleural, intraarterial, topical, inhalational (e.g., as mists of sprays), mucosal (such as via nasal mucosa), subcutaneous, transdermal, gastrointestinal, intraarticular, intracistemal, intraventricular, rectal (i.e., via suppository), vaginal (i.e., via pessary), intracranial, intraurethral, intrahepatic, and intratumoral. In some embodiments, the compositions are administered systemically (for example by intravenous injection). In some embodiments, the compositions are administered locally (for example by intraarterial or intraocular injection).

[0152] In some embodiments, the compositions are administered intravascularly, such as intravenously or intraarterially. In some embodiments (for example for the treatment of renal diseases), the compositions are administered directly into arteries (such as renal arteries). In preferred embodiments, the compositions are administered subcutaneously.

[0153] The optimal effective amount of the compositions can be determined empirically and will depend on the type and severity of the disease, route of administration, disease progression and health, mass and body area of the individual. Such determinations are within the skill of one in the art. The effective amount can also be determined based on in vitro complement activation assays. Examples of dosages of CR2-FH molecules which can be used for methods described herein include, but are not limited to, an effective amount within the dosage range of any of about 0.01 ug/kg to about 300 mg/kg, or within about 0.1 ug/kg to about 40 mg/kg, or with about 1 ug/kg to about 20 mg/kg, or within about 1 ug/kg to about 10 mg/kg, or within about 0.1 mg/kg to about 100 mg/kg, or within about 0.1 mg/kg to 50 mg/kg or within about 0.1 mg/kg to about 25 mg/kg, or within about 0.1 mg/kg to about 10 mg/kg. In some embodiments, the effective amount is about 0.1 mg/kg to about 10 mg/kg. In some embodiments, the effective amount is about 0.1 mg/kg to about 20 mg/ kg. In some embodiments, the effective amount is about any one of 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 mg/kg. For example, when administered subcutaneously, the composition may be administered at low microgram ranges, including for example about 0.1 ug/kg or less, about 0.05 ug/kg or less, or 0.01 ug/kg or less. In some embodiments, the amount of CR2-FH administered to an individual is about 10 ug to about 500 mg per dose, including for example any of about 10 ug to about 50 ug, about 50 ug to about 100 ug, about 100 ug to about 200 ug, about 200 ug to about 300 ug, about 300 ug to about 500 ug, about 500 ug to about 1 mg, about 1 mg to about 10 mg, about 10 mg to about 50 mg, about 50 mg to about 100 mg, about 100 mg to about 200 mg, about 200 mg to about 300 mg, about 300 mg to about 400 mg, about 400 mg to about 500 mg, about 500 mg to about 600 mg, about 600 mg to about 700 mg, about 700 mg to about 800 mg, about 800 mg to about 900 mg, or about 900 mg to about 1000 mg per dose.

[0154] The CR2-FH compositions may be administered in a single daily dose, or the total daily dose may be administered in divided dosages of two, three, or four times daily. The compositions can also be administered less frequently than daily, for example, six times a week, five times a week, four times a week, three times a week, twice a week, once a week,

once every two weeks, once every three weeks, once a month, once every two months, once every three months, or once every six months. The compositions may also be administered in a sustained release formulation, such as in an implant which gradually releases the composition for use over a period of time, and which allows for the composition to be administered less frequently, such as once a month, once every 2-6 months, once every year, or even a single administration. The sustained release devices (such as pellets, nanoparticles, microparticles, nanospheres, microspheres, and the like) may be administered by injection or surgical implanted in various locations in the body.

[0155] In some embodiments, the composition (such as TT30) is provided in water or saline at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/ml.

[0156] In certain embodiments of the invention, the dosing of CR2-FH fusion protein is titrated such that the dose is sufficient to reduce or prevent red blood cell lysis, and may fully or partially inhibit or block the formation and activity of the amplification C3 convertase (C3bBb) on the red blood cell surface; but at a low enough concentration such that C3 coating of abnormal cells is still observed systemically, such that the C3 is able to form initiation C3 convertase (C3iBb) in the fluid phase.

Gene Therapy

[0157] The CR2-FH molecules can also be delivered by expression of the CR2-FH fusion protein in vivo, which is often referred to as "gene therapy." For example, cells may be engineered with a polynucleotide (DNA or RNA) encoding for the fusion protein ex vivo, the engineered cells are then provided to an individual to be treated with the fusion protein. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding for the fusion protein of the present invention. Local delivery of the fusion proteins of the present invention using gene therapy may provide the therapeutic agent to a localized target area.

[0158] Methods of gene delivery are known in the art. These methods include, but are not limited to, direct DNA transfer, see, e.g., Wolff et al. (1990) Science 247: 1465-1468; 2) Liposome-mediated DNA transfer, see, e.g., Caplen et al. (1995) Nature Med. 3:39-46; Crystal (1995) Nature Med. 1:15-17; Gao and Huang (1991) Biochem. Biophys. Res. Comm. 179:280-285; 3) Retrovirus-mediated DNA transfer, see, e.g., Kay et al. (1993) Science 262:117-119; Anderson (1992) Science 256:808-813; 4) DNA Virus-mediated DNA transfer.

Such DNA viruses include adenoviruses (preferably Ad2 or Ad5 based vectors), herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., Ali et al. (1994) Gene Therapy 1:367-384; U.S. Pat. No. 4,797,368, incorporated herein by reference, and U.S. Pat. No. 5,139,941.

[0159] Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Mouse Leukemia Virus, spleen necrosis virus, retroviruses such as Rotis Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Mouse Leukemia Virus.

[0160] Adenoviruses have the advantage that they have a broad host range, can infect quiescent or terminally differentiated cells, such as neurons or hepatocytes, and appear essentially non-oncogenic. See, e.g., Ali et al. (1994), supra, p. 367. Adenoviruses do not appear to integrate into the host genome. Because they exist extrachromosomally, the risk of insertional mutagenesis is greatly reduced. Ali et al. (1994), supra, p. 373.

[0161] Adeno-associated viruses exhibit similar advantages as adenoviral-based vectors. However, AAVs exhibit site-specific integration on human chromosome 19 (Ali et al. (1994), supra, p. 377).

[0162] The gene therapy vectors may include one or more promoters. In some embodiments, the vector has a promoter that drives expression in multiple cell types. In some embodiments, the vector has a promoter that drives expression in specific cell types (such as cells of retina or cells in the kidney). Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CVM) promoter described in Miller et al. (1989) Biotechniques 7(9):980-990, or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and .beta.-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

[0163] The nucleic acid sequence encoding a CR2-FH fusion protein is preferably under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoA1 promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the .beta.-actin promoter; and human growth hormone promoter.

[0164] Retroviral plasmid vectors can be employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which maybe transfected are described in Miller (1990) Human Gene Therapy 1:5-14. The vectors may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host. The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

[0165] In some embodiments, the complement activation is inhibited by contacting a body fluid with a composition comprising a CR2-FH molecule ex vivo under conditions that permit the CR2-FH molecule to function to inhibit complement activation. Suitable body fluids include those that can be returned to the individual, such as blood, plasma, or lymph. Affinity adsorption apheresis is described generally in Nilsson et al. (1988) Blood 58(1):38-44; Christie et al. (1993) Transfusion 33:234-242; Richter et al. (1997) ASAIO J. 43(1):53-59; Suzuki et al. (1994) Autoimmunity 19: 105-112; U.S. Pat. No. 5,733,254; Richter et al. (1993) Metabol. Clin. Exp. 42:888-894; and Wallukat et al. (1996) Int'l J. Card. 54:1910195.

[0166] Accordingly, the invention includes methods of treating one or more diseases described herein in an individual comprising treating the individual's blood extracoporeally (i.e., outside the body or ex vivo) with a composition comprising a CR2-FH molecule under conditions that permit the molecule to function to inhibit complement activation, and returning the blood to the individual.

Unit Dosages, Articles of Manufacture, and Kit

[0167] Also provided are unit dosage forms of CR2-FH molecule compositions, each dosage containing from about 0.01 mg to about 50 mg, including for example any of about 0.1 mg to about 50 mg, about 1 mg to about 50 mg, about 5 mg to about 40 mg, about 10 mg to about 20 mg, or about 15 mg of the CR2-FH molecule. In some embodiments, the unit dosage forms of CR2-FH molecule composition comprises about any of 0.01 mg-0.1 mg, 0.1 mg-0.2 mg, 0.2 mg-0.25 mg, 0.25 mg-0.3 mg, 0.3 mg-0.35 mg, 0.35 mg-0.4 mg, 0.4 mg-0.5 mg, 0.5 mg-1.0 mg, 5.0 mg-15 mg, 10 mg-20 mg, 20 mg-50 mg, 50 mg-80 mg, 80 mg-100 mg, 100 mg-150 mg, 150 mg-200 mg, 200 mg-250 mg, 250 mg-300 mg, 300 mg-400 mg, or 400 mg-500 mg CR2-FH molecule. In some embodiments, the unit dosage form comprises about 0.25 mg CH2-FH molecule. In other embodiments, the unit dosage form comprises about 10 mg CH2-FH molecule. The term "unit dosage form" refers to a physically discrete unit suitable as unitatry dosages for an individual, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical carrier, diluent, or excipient. These unit dosage forms can be stored in a suitable packaging in single or multiple unit dosages and may also be further sterilized and sealed.

[0168] In some embodiments, the composition (such as TT30) is provided in water or saline at a concentration of about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mg/ml.

[0169] Also provided are articles of manufacture comprising the compositions described herein in suitable packaging. Suitable packaging for compositions (such as ophthalmic compositions) described herein are known in the art, and include, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

[0170] The present invention also provides kits comprising compositions (or unit dosages forms and/or articles of manufacture) described herein and may further comprise

instruction(s) on methods of using the composition, such as uses described herein. The kits described herein may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein.

[0171] The compositions and formulations of the present invention are useful for the treatment of conditions associated with complement activation, preferably those which involve the complement alternative pathway, which is largely unaffected by terminal complement inhibitors [e.g., inhibitors of steps of the complement pathway subsequent to the activation of C3].

[0172] In the foregoing specification and in the examples below, the invention has been described with specific embodiments thereof. However, it will be evident to those skilled in the art that various modifications and changes may be made thereto without departing from the broader scope of the invention.

[0173] All publications that are cited herein are hereby specifically incorporated herein by reference into the disclosure for the teachings for which they are cited.

EXAMPLES

EXAMPLE 1. Demonstration of Possible Mechanism of Protection of Red Blood Cells from Hemolytic Lysis in Paroxysmal Nocturnal Hemoglobinuria (PNH).

[0174] This example uses TT30 (SEQ ID NO:3) and PNH as an example to demonstrate a possible mechanism under which TT30 can protect red blood cells from hemolytic lysis.

[0175] PNH RBCs lack the ability to control C3 convertase via CD55 and C5 convertase-mediated formation of the membrane attack complex (MAC) via CD59. In the absence of local complement alternative pathway (CAP) control (see Figure 20), spontaneous tickover generates fluid-phase C3(H_2O) that associates with factor B (fB) and properdin (P) in the presence of factor D (fD) to form the fluid-phase *initiation* C3 convertase, which cleaves C3 to release C3a and deposit covalently-bound C3b on the RBC surface. Association of C3b with fB and P in the presence of fD generates the surface-bound *amplifying* C3 convertase. The CAP amplification loop generates additional C3b and C3 convertase, and by adding C3b to C3 convertase, forms surface-bound C5 convertase. C5 convertase cleaves C5 to release

C5a and deposit C5b, leading to rapid addition of C6, C7, C8 and poly-C9 to form the MAC, resulting in lysis and release of hemoglobin (Hgb) and lactate dehydrogenase (LDH).

[0176] The primary negative regulator of spontaneous CAP initiation is fH. In the fluid phase, fH blocks the association of C3(H_2O) with fB. On the cell surface, fH also disrupts the amplifying C3 convertase and, in association with the protease Factor I (fI), converts C3b to inactive iC3b on the cell surface, which is subsequently converted to C3dg by fI and complement receptor 1 (CR1), and then to C3d by poorly characterized serum proteases. fH also disrupts the C5 convertase. These fH activities occur on normal and PNH RBCs, but contribute only partially to the complete control of complement activation that is necessary on RBCs. Most importantly, on normal RBCs (see Figure 21), C3 convertase undergoes accelerated decay due to CD55 (DAF), while MAC formation is disrupted by CD59 at the C9 binding steps. Thus, in the presence of normal protective mechanisms, spontaneous CAP activation is held in check. While fH activity is not compromised in PNH, in the absence of CD55 and CD59 function, fH cannot control CAP activation sufficiently on the RBC surface (see Figure 20), and hemolysis results.

[0177] One way to protect PNH RBCs from intravascular hemolysis (IVH) is to prevent formation of the MAC, which can be accomplished by blocking C5 participation with an anti-C5 monoclonal antibody, such as eculizumab (see Figure 22). In this setting, fH function is unchanged. However, fH alone cannot prevent all amplifying C3 convertase function, so that PNH RBCs gradually become "coated" with C3 fragments. Risitano et al., Blood, 113: 4094-100 (2009). This leads to removal of these C3 fragment-coated PNH RBCs within the liver and the spleen, a process known as extravascular hemolysis (EVH). In addition to accumulating C3 fragments, continuous activity of the amplifying C3 convertase will likely result in increased formation of C3 and C5 convertases on the PNH RBC surface. This condition would render PNH RBCs vulnerable to lysis if unblocked C5 were to become available, and may explain why eculizumab is not able to prevent hemolysis of PNH RBCs in vitro. Risitano et al., Blood, 114:71 (2009; Abstract No. 158; Accessed at: http://ash.confex.com/ash/2009/webprogram/Paper19102.html).

[0178] Without being bound by theory, it is hypothesized that TT30 (targeted fH) will bind to C3 fragments deposited on the RBC surface via spontaneous tickover, and will prevent formation of the C3 and C5 convertases and the MAC, thus preventing IVH. Prevention of hemolysis should result in a decrease in serum LDH concentrations and an increase in

hematocrit. In addition, TT30 may to prevent the accumulation of C3 fragments associated with EVH (Figure 23).

EXAMPLE 2. In Vitro Model of Complement-Mediated Hemolysis of PNH RBCs; Effect of Different Complement Inhibitors

[0179] PNH is a hematological disorder characterized by complement (C)-mediated intravascular hemolysis (IVH) due to a lack of C inhibitors CD55 and CD59 on affected red blood cells (RBCs). Normal, spontaneous activation of the complement alternative pathway, when uncontrolled by CD55, may lead to deposition of C3 on PNH RBCs which is not detectable in untreated PNH, generation of C3 and C5 convertases and, due to lack of CD59, to formation of the membrane attack complex (MAC) and lysis.

[0180] TT30 is a 65 kDa recombinant chimeric human fusion protein of the present invention. TT30 comprises the iC3b/C3d-binding region of C receptor 2 and the functional domains of the CAP regulator factor H (fH), which acts like CD55 to block CAP C3 convertase formation and activity on the surface of RBCs. RBCs were obtained from 5 PNH subjects (2 untreated and 3 on Ecu). Sera were obtained from the same subjects and from ABO-matched healthy subjects. Sera from PNH subjects on Ecu were collected immediately after Ecu infusion, at an estimated peak concentration of ~200 μg/mL.

[0181] The Ham acid hemolysis test is a diagnostic method to assess, in vitro, the increased susceptibility of PNH RBCs to IVH upon Complement activation after a brief incubation in acidified serum. Ham and Dingle, J. Clin. Invest., 18:657 (1939). The main principle of the Ham Test is to challenge PNH RBCs (which are, by definition, sensitive to complement activation due to the lack of CD55 and CD59) in fresh normal human sera, after in vitro activation of the complement cascade by acidification. As part of the present invention, the present inventors developed a Ham Test modified to delay hemolysis and allow serial evaluations to assess the susceptibility of PNH RBCs to the subsequent hemolysis (resulting from terminal-complement activation) and to pre-hemolytic complement activation (as assessed by initial C3 deposition in double color [C3-FITC vs CD59-PE] flow cytometry). Hemolysis was measured by classical spectrometry of the supernatant and also quantified by flow cytometry of RBCs (Ferreira and Pangburn, Blood 2007). Thus, the modified Ham Test allowed the evaluation of the effects of various C regulators on hemolysis of PNH RBCs, as well as on the initial C3 activation and deposition through the CAP.

[0182] C3-bound RBCs have been shown to develop during treatment with the terminal complement inhibitor eculizumab (Ecu) in PNH and are a possible cause of extravascular hemolysis (EVH) in these subjects (Risitano et al, Blood 2009); in this modified Ham test, C3 deposition was quantified by double color flow cytometry. The complement inhibitor TT30 (Taligen Therapeutics, Cambridge, MA) was evaluated. TT30 is a targeted inhibitor of C3 convertase, comprising a targeting moiety isolated from CR2 and a complement inhibiting moiety isolated from Factor H.

[0183] RBCs were obtained were obtained from PNH subjects both untreated and on eculizumab. Sera were obtained from ABO-matched healthy individuals. In brief, fresh normal sera were supplemented with MgCl, and pre-incubated with the complement inhibitor (TT30). After 15 minutes, HCl was added to acidify the sera and activate the complement cascade. PNH were added (1:20 of a 50% suspension) to sera, and incubated at 37° C. Hemolysis and C3 deposition were measured at different intervals, to assess the effect of the complement inhibitor. Details of materials and experimental methods are described below.

[0184] In this modified Ham test, delayed lysis of RBCs was observed that originated from untreated PNH subjects; lysis was partial after 1h (50-70%), and became almost complete at 72h. Results were expressed as survival of initial PNH RBCs: after 1, 6, 24 and 72h, survival was ~65, 40, 20 and <10%, respectively. Hemolysis was observed at the same rate in PNH RBCs from subjects receiving Ecu, when they were incubated with ABO-matched sera. TT30 was able to significantly inhibit hemolysis of RBCs from untreated PNH subjects. TT30 demonstrated concentration and time dependent inhibition of hemolysis. TT30 concentrations of 3-100nM (1000nM = 65 μg/ml) did not improve PNH RBC survival even at 1h. In contrast, 300nM TT30 resulted in temporary inhibition (surviving PNH RBCs ~70%) at 1h, which was not sustained (surviving PNH RBCs were ~50 and 20% at 6 and 24h). Higher concentration of TT30 (3000nM, equal to 195 μg/mL) resulted in complete inhibition of hemolysis as long as at 24h, though hemolysis was evident with longer incubation (surviving PNH RBCs were ~90, 90, 90 and 50% at 1, 6, 24 and 72h, respectively).

[0185] When washed RBCs from PNH subjects on Ecu were utilized, TT30 resulted in inhibition of hemolysis as on untreated PNH RBCs. Finally, the modified Ham test to investigate the effect of complement inhibitors on C3 activation and deposition on PNH RBCs. Consistent with the mechanism of local RBC surface inhibition of C3 convertase activity, TT30 exposition did not result in any C3 deposition on PNH RBCs, throughout a

120h incubation. In addition, when PNH RBCs from PNH subjects on Ecu were utilized, TT30 did not allow further C3 binding on PNH RBCs, even if pre-existing C3+ RBCs remained unchanged.

[0186] In this model, TT30 demonstrated concentration and time dependent inhibition of hemolysis. TT30 concentrations of 3-100nM (1000nM = 65 μg/ml) did not improve PNH RBC survival even at 1h. In contrast, 300nM TT30 resulted in an substantial inhibition (surviving PNH RBCs ~70%) at 1h, which was not sustained longer (surviving PNH RBCs were ~50 and 20% at 6 and 24h). Higher concentrations of TT30 (3000nM, equal to 195 μg/mL) resulted in complete inhibition of hemolysis as long as at 24h, though hemolysis was evident with longer incubation (surviving PNH RBCs were ~90, 90, 90 and 50% at 1, 6, 24 and 72h, respectively). A modified Ham test was conducted to investigate the effect of C inhibitors on C3 activation and deposition on PNH RBCs. Consistent with the mechanism of local RBC surface inhibition of C3 convertase activity, exposure to TT30 did not result in any C3 deposition on PNH RBCs, throughout a 120h incubation. Our data show that modulation of the CAP using TT30 inhibits hemolysis of PNH RBCs in vitro. TT30 also inhibits the C3 activation and deposition on surviving PNH RBCs, which has been recently described as a primary cause of residual hemolysis and anemia in PNH subjects receiving the terminal complement inhibitor eculizumab. These findings provide the rationale for a potential new mechanism for treating both IVH and EVH associated with PNH by targeting and inhibiting the CAP.

[0187] In conclusion, the "modified Ham test" described herein was developed to assess in vitro the efficacy of C inhibitors on PNH RBCs. Data from this test show that modulation of the CAP using TT30 inhibit hemolysis of PNH RBCs *in vitro*. However, unlike Ecu, TT30 also inhibits the C3 activation and deposition on surviving PNH RBCs. These findings provide evidence of efficacy for a potential new mechanism for treating both IVH and EVH associated with PNH by targeting and inhibiting the CAP.

Materials and experimental methods.

[0188] Fresh sera was obtained from ABO-matched donors. Fresh red blood cells were prepared from PNH subjects, by washing thrice by NaCl 0.9%; use a 50% or 25% resuspension for experiments (1:20 and 1:10 to serum, respectively). A 50 % RBC resuspension should be about $5x10^6$ RBCs/μL.

[0189] The following solutions and reagents were prepared:

[0190] $MgCl_2$ (hexahydratate, MW 203) prepare a 100mM stocking solution (2.03 g in distilled water 10 mL); prepare a 30mM working solution (1:3.3 from stocking solution), to be used 1:20 to serum in final experiments.

[0191] HCl Start from available 37% solution (=12M); prepare a 1M = 1N solution (8.1 mL in 100 mL of distilled water); prepare 0.4 or 0.2 working solution (to be used 1:20 and 1:10 to serum, respectively).

[0192] TT30 (MW 65 kDa) - Start from 118 μM solution (7.71 mg/mL); prepare two working solutions; 6 μM (1:20 from stocking solution); 0.6 μM (1:10 from working solution A). Use the appropriate amount according to dilution curve (final range 3-3000nM).

[0193] Preparations were made according to TABLE 1. 500 μL (10 parts; 1 part equal to 50 μL) of serum (or water, as appropriate) was added to all tubes. 25 μL (0.5 parts) of 30 mM MgCl solution was added in the appropriate tubes. The appropriate concentration of inhibitor, as indicated in **TABLE 1**, was added and incubated for 15 minutes. HCl was added to acidify the serum.

TABLE 1

TUBES AND CONTENTS

	CONDITIONS			INHIBITOR
1	**Water**	PNH RBCs		
2	**NaCl 0.9%**	PNH RBCs		
3	**Serum**	PNH RBCs	MgCl	
4	**Acidified Serum**	PNH RBCs	MgCl	
5	**Acidified Serum**	PNH RBCs	MgCl	TT30 3000 nM (15 μL of 120 μM)
6	**Acidified Serum**	PNH RBCs	MgCl	TT30 1000 nM (5 μL of 120 μM)
7	**Acidified Serum**	PNH RBCs	MgCl	TT30 300 nM (30 μL of 6 μM)
8	**Acidified Serum**	PNH RBCs	MgCl	TT30 100 nM (10 μL of 6 μM)
9	**Acidified Serum**	PNH RBCs	MgCl	TT30 30 nM (30 μL of 0.6 μM)
10	**Acidified Serum**	PNH RBCs	MgCl	TT30 10 nM (10 μL of 0.6 μM)
11	**Acidified Serum**	PNH RBCs	MgCl	TT30 3 nM (3 μL of 0.6 μM)

[0194] 50 μL (1 part) of 0.2 M HCl were added to each tube. RBC resuspension was added to all tubes, as 25 μL (0.5 parts) of 50% RBC suspension, about $5x10^6$/uL; 25uL of 50% RBC suspension for each tube) was used. The tubes were incubated at 37°C (for at least 72 h).

Check for hemolysis and C3 coating (at 1h, 6h, 24h and 72h): RBC pellet by CD59/C3 flow cytometry staining.

[0195] 1 μL of RBC pellet (about 10^7 RBCs) was diluted 1:1000; 50uL of a 10^4/uL* RBC resuspension in NaCl was incubated with 1 uL of the anti-C3 polyclonal antibody and 5 uL of the anti-CD59 monoclonal antibody. Samples were incubated 1h at room temperature; and analyzed by flow cytometry after addition of 250-500 uL of NaCl, without additional washing (Risitano et al, Blood 113:4094-4100 (2009)).

[0196] Survival of PNH RBCs was calculated as follows: (Ferreira and Pangburn, Blood, 110:2190-2192 (2007)): % survival = (% PNH post/% N post) x (% N pre/% PNH pre)

[0197] Lysis of PNH RBCs was calculated as follows: % lysis = 100 – (% survival).

[0198] EXAMPLE 2A: In Vitro Model of Complement-Mediated Hemolysis of PNH RBCs; Effect of Different Complement Inhibitors

[0199] An in vitro model was developed to allow evaluation of the comparative efficacy of TT30 and eculizumab. This in vitro model is a modified Ham test, in which PNH RBCs are exposed to ABO-matched acidified normal serum (ANS), which results in spontaneous CAP activation (Pascariello et al., *European Hematologic Association (EHA)*, Barcelona, June 10-13, (2010)). When PNH RBCs from untreated patients were incubated with ANS for 24 hours, 74±16% of the PNH RBCs were lysed, with RBC ghosts staining for the presence of C3 fragments (C3frag). In contrast, incubation with 1 or 3 μM (65 or 195 μg/ml) TT30 resulted in hemolysis of only 14±26% or 5±7% of the PNH RBCs and surviving PNH RBCs were C3frag-negative on their surface. Equimolar concentrations of human fH produced much less inhibition of hemolysis (about 50% lysis), supporting the notion that TT30 is cell-targeted. The targeted fH supplementation by TT30 was confirmed by demonstration of bound TT30 on PNH RBC surface, using an anti-fH mAb by flow cytometry. Thus, TT30 prevented hemolysis representative of that observed with IVH, as well as C3frag accumulation as occurs with EVH in eculizumab-treated PNH patients.

[0200] The efficacy of TT30 has been directly compared to that of eculizumab in this model (Risitano et al., *Biologics: Targets & Therapy*, 2: 205-22 (2008), Risitano et al., *Blood*, 113: 4094-100 (2009)). PNH RBCs were obtained from 5 PNH patients (2 untreated and 3 on eculizumab); sera were obtained from the same patients and from ABO-matched healthy

subjects. Sera from PNH patients on eculizumab were collected immediately after eculizumab infusion, at an estimated peak concentration of ~200 μg/mL. TT30 was spiked into serum to final concentrations ranging from 0.195 to 195 μg/mL (0.003 to 3 μM). PNH RBCs were incubated in sera, with or without eculizumab or TT30, and evaluated at various timepoints.

[0201] After 1 hour, up to ~70% of PNH RBCs were lysed by ABO-matched serum. Eculizumab significantly reduced hemolysis (to only ~25% at 1 hour), but could not provide complete protection and was associated with the progressive accumulation of C3frag+ PNH RBCs (Figure 31). In PNH patients, the recommended minimum plasma eculizumab concentration to be maintained for prevention of hemolysis is 35 μg/mL (Risitano et al., *Biologics: Targets & Therapy*, 2: 205-22 (2008)). Therefore, this in vitro hemolysis assay may overestimate the effective concentrations for prevention of hemolysis in PNH.

[0202] TT30 prevented hemolysis of PNH RBCs in a concentration-dependent manner. Complete (~100%) inhibition of hemolysis was achieved at 65 μg/mL. At 195 μg/mL, TT30 completely prevented C3 fragment accumulation and hemolysis through 24 hours (Figure 31). The effect of TT30 on PNH RBC survival across a range of concentrations was evaluated at each timepoint (Figure 32) and IC_{10} and IC_{90} values were calculated (Table 1A). After incubation for 1 hour, the IC_{10} and IC_{90} values for inhibition of CAP-mediated hemolysis of PNH RBCs were 4.3 and 87.9 μg/mL, respectively.

TABLE 1A

IC_{10} and IC_{90} Values for TT30 Inhibition of CAP-mediated Hemolysis of $CD59^-$ PNH RBCs in an In Vitro Hemolysis Assay

Time (hr)	IC_{10} (μg/mL)	IC_{90} (μg/mL)
1	4.3	87.9
3	5.9	57.5
6	11.4	30.3
24	16.5	55.9

IC_{10} and IC_{90} values calculated using data illustrated in Figure 32.

[0203] In addition, when C3frag+ PNH RBCs from PNH patients on eculizumab were utilized, TT30 did not allow further C3frag accumulation and TT30 could be demonstrated to be bound to the surface of these protected PNH RBCs. These results suggest that modulation of the CAP with TT30 is more effective than inhibition of C5 with eculizumab at preventing hemolysis of PNH RBCs, and that, unlike eculizumab, TT30 also inhibits the C3 activation and deposition on surviving PNH RBCs. It is thus hypothesized that the in vitro hemolysis of RBCs from PNH patients on eculizumab despite the presence of protective concentrations of eculizumab may be related to increased susceptibility to lysis of C3frag+ RBCs. An alternative explanation might be that, in addition to being coated with C3frag, the PNH RBCs become coated by C3 and C5 convertases and that some small amount of C5 is released by eculizumab and cleaved by the C5 convertases, leading to MAC formation and lysis.

EXAMPLE 3: Biological Mechanism of Residual Anemia in PNH Subjects Treated with Anti-C5 Antibodies.

[0204] The following experiment was conducted to investigate the biological mechanisms underlying residual anemia in some PNH subjects on eculizumab, looking for a possible role of the early steps of the complement cascade. The results have been published in Risitano et al., Blood, 113:4094-4100 (2009), the disclosure of which is hereby incorporated herein by reference.

[0205] A total of 56 PNH subjects were examined. Of these, 41 had previously received eculizumab treatment; and 15 did not receive eculizumab treatment. All subjects receiving eculizamab were treated according to a standard procedure: (900 mg every 14±2 days, after a loading phase of 600 mg every 7±1 days for 4 doses). Thirteen subjects were analyzed before

and during treatment. Ten healthy volunteers were examined as negative controls; and 5 subjects with cold agglutinin disease (CAD) were examined as positive controls.

[0206] As shown in TABLE 2**,** decoration of red blood cells with C3d was observed in 8 out of 8 subjects after treatment with anti-C5 antibody when evaluated in a Direct Antiglobulin Test. Further, as shown in FIGURE 4, while untreated subjects did not exhibit C3 fragment binding within PNH red blood cells, subjects treated with anti-C5 antibody showed broad heterogeneity with respect to the percent age of C3+ red blood cells within the PNH population. As shown in FIGURE 2A, C3 fragment binding appears a few weeks after starting anti-C5 antibody treatment and remains largely stable over a long term period of two years.

TABLE 2

C3 BINDING ON RBCs IN PNH SUBJECTS ON ANTI-C5 ANTIBODY

(DIRECT ANTIGLOBULIN TEST)

Subject	Pre-Treatment	During Treatment
1	-	+
2	-	+
3	-	+
4	-	+/-
5	-	+/-
6	+ (IgG) *	+/-
7	-	+
8	-	+/-

* = Polytransfused; ANA + ve
+/- = Mixed Fields

[0207] For the purpose of the study, hematological improvement in subjects having taken Eculizamab was classified according to the categories listed in TABLE 3:

TABLE 3

CLINICAL RESPONSE TO ANTI-C5 ANTIBODY

CLASS	CRITERIA	NUMBER
Optimal Responders	Transfusion independence, Hb stably >11	N = 15 (37%)
Major Responders	Transfusion independence, Hb stably >8	N = 18 (44%)
Partial Responders	Reduction >50% of transfusion need	N = 5 (12%)

Minor Responders	Transfusion need reduced <50% or unchanged*	N = 3 (7%)

* = ***likely due to associated aplastic anemia***

[0208] As shown in FIGURE 6, C3 binding on PNH RBCs exhibited a strong correlation with hematological response. Subjects achieving the best hematological response had lower percentages of C3-bound red blood cells.

[0209] Supported by the data in this application, including *in vivo* RBC survival, the inventors theorized that this hemolysis in subjects treated with anti-C5 antibody is the result of C3-mediated extravascular hemolysis (EVH). This C3 binding is restricted to C59 negative red blood cells. The C3 binding is largely due to C3 convertase activation, an early phase of the complement cascade, which is not affected by anti-C5 antibody and remains uncontrolled given the lack of CD55 on PNH cells. The extent of C3 binding is higher in subjects not achieving an optimal hematological response and correlates with measures of ongoing hemolysis, such as reticulocyte count. Some paradigmatic subjects with a high percentage of C3-coated RBCs showed decreased RBC half-life by in vivo 51-Cr survival study, with excess counts on spleen and liver

[0210] The presence of C3-mediated residual extravascular hemolysis is consistent with the observation of persistent reticulocytosis and raised unconjugated bilirubin in most PNH subjects on eculizumab (with normal LDH). Low-level extravascular hemolysis may be the rule rather than the exception in PNH subjects treated with anti-C5 antibody. It is not clear whether this is activated by anti-C5 antibody, or if it existed prior to treatment. However, prior to treatment, this phenomenon was subclinical and difficult to detect in the absence of C5-blockade. The reasons why such novel mechanism of disease has variable clinical consequences in different subjects are not fully understood, and are currently under investigation.

[0211] The percentage of C3+ binding was found to increase with the absolute reticulocyte count (ARC) during treatment with terminal complement inhibitor. FIGURE 7 illustrates the correlation of C3 binding on PNH RBCs with measures of ongoing hemolysis, such as ARC, but not with LDH. This correlation is consistent with the hypothesis that the C3+ binding relates to continued extravascular hemolysis in subjects treated with terminal complement inhibitors.

[0212] Several subjects were studied for survival of red blood cells in the spleen and liver during treatment with anti-C5 antibody. As exemplified in FIGURE 8, increased entrapment of red blood cells in both spleen and liver was detected in all three subjects studied.

[0213] RBCs from subjects with PNH were then treated with varying doses of TT30, a potent inhibitor of C3 convertase. As demonstrated in FIGURES 9 through 16, PNH RBC survival increased with TT30 treatment in a dose dependent fashion. It was found that TT30 strongly inhibited hemolysis of PNH RBCs in acidified serum. The protection was dose and time dependent. TT30 inhibited C3 binding on PNH RBCs taken from subjects who had been previously untreated, as well as PNH RBCs taken from subjects who had been previously treated with the terminal complement inhibitor eculizumab.

[0214] Figures 17 through 19 illustrate the results of the modified Ham test, in which red blood cells from subjects who had not been previously treated (Figure 17 and Figure 18), and from a subject who had previously been treated with the terminal complement inhibitor eculizumab (Figure 19), were placed in acidified serum and subjected to various treatments over a 24 hour period, including (a) Acidified serum (AcS) without additional treatment; (b): AcS + 3000 nM TT30. As can be seen from these figures, surviving C3-positive cells were seen in subjects who had been pretreated with eculizumab. However, subjects who had not been treated with eculizumab did not exhibit C3-positive RBCs.

EXAMPLE 4: Treatment of Subjects Exhibiting Complement-Mediated Hemolytic Disorders with Targeted CAP Inhibitor TT30.

[0215] Subjects exhibiting a complement-mediated hemolytic disorder, such as PNH, who have not previously been treated are dosed with TT30. Treatment lasts for a duration of 3 months, during which LDH, Hemoglobin gain and C3 binding was monitored at regular intervals.

EXAMPLE 5: Treatment of Residual Anemia in PNH Subjects Previously Treated with Anti-C5 Antibodies.

[0216] Subjects exhibiting PNH who have previously been treated with anti-C5 antibody, but who exhibit at least one characteristic of complement-mediated extravascular hemolysis are dosed with TT30. Treatment with both anti-C5 antibody and TT30 lasts for a duration of 3 months, during which LDH, Hemoglobin gain and C3 binding are monitored at regular intervals.

EXAMPLE 6: Treatment of Subjects Exhibiting Complement-Mediated Hemolytic Disorders with Other Complement Alternative Pathway Inhibitors.

[0217] Subjects exhibiting a complement-mediated hemolytic disorder, such as PNH, are dosed with at least one targeted inhibitor of the complement alternative pathway, from the following:

(a) TT30 (CR2-targeted Factor H);
(b) TT32 (CR2-targeted CR1)
(c) Anti-Factor B antibody; (for example, TA106);
(d) TT31 (CR2-targeted double-Factor H);
(e) DAF
(f) Anti-properdin antibody;
(g) Anti-Factor D antibody;
(h) Complement Factor I;
(i) Compstatin;
(j) CR2-targeted Anti-Factor B antibody;
(k) CR2-targeted DAF;
(l) CR2-targeted Anti-Properdin antibody

(m) CR2-targeted Anti-Factor D antibody;

(n) CR2-targeted Complement Factor I;

(o) CR2-targeted Compstatin;.

(p) MCP;

(q) CR2-targeted MCP

(r) Factor H

(s) CR1

(t) MAP1;

(u) CR2-targeted MAP1;

(v) Anti-MASP1 antibody;

(w) CR2-targeted Anti-MASP1 antibody.

[0218] Treatment with the above complement inhibitors may last for a duration of 3 months, during which LDH, Hemoglobin gain and C3 binding are monitored at regular intervals in order to determine the subjects' response to each of the complement alternative pathway inhibitors.

EXAMPLE 8: Binding of TT30 to Asthmatic Human and Cynomolgus Monkey Lungs.

[0219] Asthmatic human and cynomolgus lung tissue samples were prepared and were stained with TT30 and with Anti-iC3b monoclonal antibody, respectively. Both TT30 and Anti-iC3b monoclonal antibody showed similar staining patterns, as shown in Figure 24. Later experiments demonstrated that TT30 staining of asthmatic lung tissue can be blocked by anti-C3b monoclonal antibody. Additional experiments demonstrated that TT30 staining of asthmatic lung tissue can also be blocked with anti-CR2 monoclonal antibodies. These results indicate that TT30 may be used as an immunostaining reagent. These results further support a conclusion that TT30 binds to asthmatic lung tissue via binding of the CR2 portion of TT30 to C3 fragments present on the tissue.

EXAMPLE 9: Rabbit Red Blood Cells (RBC) Hemolysis Assay

[0220] An assay was developed at Taligen Therapeutics to measure CAP-mediated hemolysis of rabbit RBCs by human serum. This assay measures the release of hemoglobin from rabbit RBCs lysed upon exposure to human serum. Water and EDTA were used as positive and negative controls for the assay, respectively. The reported values for serum CAP-mediated

hemolysis of rabbit RBCs are expressed as percent (%) of lysis observed with water (100%). This assay can be used for the purpose of assessing TT30 activity, i.e., the extent to which TT30 inhibits CAP-mediated hemolysis of rabbit RBCs. As serum C3 is activated and C3 activation fragments (iC3b, C3dg and C3d) are deposited on rabbit RBCs, TT30 binds to these fragments via CR2 SCR1-4 and inhibits CAP-mediated C5b-9 formation via fH SCR 1-5. In the presence of TT30, serum CAP activity will be reduced (inhibited) in a concentration-dependent manner. TT30 activity can therefore be expressed as the relative (%) inhibition of CAP-mediated rabbit RBC hemolysis, as compared to a serum sample without TT30 (e.g., baseline samples).

[0221] Rabbit RBC (Bioreclamation, Liverpool, NY) were washed, adjusted to 2.9 x 10^9 erythrocytes/mL and incubated with human complement-preserved serum containing serial dilutions of TT30 under experimental conditions promoting CAP activity (MgEGTA) and subsequent hemolysis. After 30 minutes at 37^0C, 25mM EDTA was added to stop the reaction, followed by centrifugation and removal of the supernatant to a new plate that was read at 415 nm. Percent lysis was calculated as $(A415_{ser\ x}- A415_{ser\ x\ bkgd})/(A415_{water})*100$. RBCs protected from hemolysis assay were stained with anti-C3 fragment antibodies and TT30-detection reagents as described under *Flow Cytometry.*

[0222] Figure 25 illustrates that TT30 exhibited a concentration-dependent inhibition of RBC lysis. At a concentration of 0.46 uM, TT30 exhibits greater than 80% inhibition of hemolysis of RBCs in this assay.

Flow Cytometry

[0223] RBCs protected from hemolysis were pooled, centrifuged to remove residual serum and resuspended in PBS/0.1%BSA for flow cytometric staining. RBC were stained with biotinylated monoclonal anti-human C3d (Quidel Corp., San Diego, CA) for 30 minutes at ambient temperature, followed by incubation with streptavidin-conjugated APC (BD Biosciences, San Jose, CA) and FITC-conjugated HB5 (Santa Cruz Biotechnology, Santa Cruz, CA) for additional 30 minutes at ambient temperature. Isotype-matched controls were from BD Biosciences. Cells were analyzed on Accuri® C6 cytometer (Accuri Cytometers Inc., Ann Arbor, MI).

[0224] Figure 26 illustrates that human serum effectively lyses rabbit RBCs in the absence of EDTA (bottom row). Cell counts indicate that approximately 95% of cells detected stain positively for the presence of C3d fragments.

[0225] Figure 27 illustrates that, with addition of increasing concentrations of TT30, at a TT30 concentration of 0.46 uM, TT30 bound to the cells is detected in approximately 70% of the stained cells (upper right quadrant). At a TT30 concentration of 1.4 uM, which is shown to inhibit hemolysis, approximately 90-95% of detected cells stain positively for the presence of C3 fragments. Since the RBCs have not been lysed, this most likely indicates the presence of inactive C3 fragments. Very little TT30 is observed, indicating that TT30 is no longer bound to the cells, but is most likely in the fluid phase. At a TT30 concentration of 3.7 uM, no C3 fragments or TT30 is detected on the RBC surface. This indicates that the concentration of TT30 is most likely present in an amount sufficient to prevent binding of C3 fragments to the RBCs, most likely by TT30 binding of C3 fragments in the fluid phase.

[0226] Figures 28 through 30 illustrate that the TT30 which is detected on the surface of 70% of RBCs at T=0, with a TT30 concentration of 0.46 uM remains present at 2 hours after addition of TT30 (67.5%). Significant numbers of RBCs continue to stain positively for TT30 at T= 4 hours (54.7%) and T= 24 hours (44.4%).

Sequences

[0227] SEQ ID NO: 1 [complete amino acid sequence of human complement receptor 2 (CR2)]:

MGAAGLLGVF LALVAPGVLG ISCGSPPPIL NGRISYYSTP IAVGTVIRYS
CSGTFRLIGE KSLLCITKDK VDGTWDKPAP KCEYFNKYSS CPEPIVPGGY
KIRGSTPYRH GDSVTFACKT NFSMNGNKSV WCQANNMWGP TRLPTCVSVF
PLECPALPMI HNGHHTSENV GSIAPGLSVT YSCESGYLLV GEKIINCLSS
GKWSAVPPTC EEARCKSLGR FPNGKVKEPP ILRVGVTANF FCDEGYRLQG
PPSSRCVIAG QGVAWTKMPV CEEIFCPSPP PILNGRHIGN SLANVSYGSI
VTYTCDPDPE EGVNFILIGE STLRCTVDSQ KTGTWSGPAP RCELSTSAVQ
CPHPQILRGR MVSGQKDRYT YNDTVIFACM FGFTLKGSKQ IRCNAQGTWE
PSAPVCEKEC QAPPNILNGQ KEDRHMVRFD PGTSIKYSCN PGYVLVGEES
IQCTSEGVWT PPVPQCKVAA CEATGRQLLT KPQHQFVRPD VNSSCGEGYK
LSGSVYQECQ GTIPWFMEIR LCKEITCPPP PVIYNGAHTG SSLEDFPYGT

TVTYTCNPGP ERGVEFSLIG ESTIRCTSND QERGTWSGPA PLCKLSLLAV
QCSHVHIANG YKISGKEAPY FYNDTVTFKC YSGFTLKGSS QIRCKRDNTW
DPEIPVCEKG CQPPPGLHHG RHTGGNTVFF VSGMTVDYTC DPGYLLVGNK
SIHCMPSGNW SPSAPRCEET CQHVRQSLQE LPAGSRVELV NTSCQDGYQL
TGHAYQMCQD AENGIWFKKI PLCKVIHCHP PPVIVNGKHT GMMAENFLYG
NEVSYECDQG FYLLGEKNCS AEVILKAWIL ERAFPQCLRS LCPNPEVKHG
YKLNKTHSAY SHNDIVYVDC NPGFIMNGSR VIRCHTDNTW VPGVPTCIKK
AFIGCPPPPK TPNGNHTGGN IARFSPGMSI LYSCDQGYLV VGEPLLLCTH
EGTWSQPAPH CKEVNCSSPA DMDGIQKGLE PRKMYQYGAV VTLECEDGYM
LEGSPQSQCQ SDHQWNPPLA VCRSRSLAPV LCGIAAGLIL LTFLIVITLY
VISKHRERNY YTDTSQKEAF HLEAREVYSV DPYNPAS

[0228] SEQ ID NO: 2 [amino acid sequence of short consensus repeat (SCR) domains 1 and 2 of human CR2]:

ISCGSPPPIL NGRISYYSTP IAVGTVIRYS CSGTFRLIGE KSLLCITKDK
VDGTWDKPAP KCEYFNKYSS CPEPIVPGGY KIRGSTPYRH GDSVTFACKT
NFSMNGNKSV WCQANNMWGP TRLPTCVS

CLAIMS

1. A method of treating a subject having a complement-mediated hemolytic disorder affecting blood cells, the method comprising:

administering an effective amount of a composition that inhibits activation of the complement alternative pathway,

wherein the composition inhibits activation of complement component C3 (C3) and opsonization of red blood cells by fragments of C3.

2. A method of treating complement-mediated hemolysis in a subject, the method comprising:

administering an effective amount of a composition that inhibits activation of the complement alternative pathway,

wherein the composition maintains normal serum levels of lactate dehydrogenase and hemoglobin.

3. The method of claim 1 or 2, wherein the composition selectively inhibits the complement alternative pathway.

4. The method of claim 2, further comprising administering a terminal complement inhibitor to the subject.

5. The method of any one of claims 1-4, wherein the subject has paroxysmal nocturnal hemoglobinuria (PNH) and the subject's red blood cells are opsonized by fragments of C3 in the absence of the composition.

6. The method of any one of claims 1-5, wherein the composition that inhibits the activity of the complement alternative pathway comprises a fusion protein comprising:

a complement receptor 2 (CR2) protein or biologically active fragment thereof; and

a factor H (fH) protein or biologically active fragment thereof.

7. The method of claim 6, wherein the fusion protein comprises the first four amino-terminal short consensus repeat (SCR) domains of CR2 fused to the first five amino-terminal SCR domains of fH.

8. The method of any one of claims 1-7, wherein the individual has previously been treated with a terminal complement inhibitor.

9. The method of claim 8, wherein the individual was not responsive, partially responsive, or has progressed on the treatment of the terminal complement inhibitor.

10. The method of any one of claims 1-9, wherein the terminal complement inhibitor inhibits cleavage of complement protein C5 (C5).

11. The method of claim 10, wherein the terminal complement inhibitor is a humanized anti-C5 antibody or antigen-binding fragment thereof.

12. The method of claim 11, wherein the terminal complement inhibitor is eculizumab.

13. The method of any one of claims 1-12, wherein the subject has one or more of the following characteristics:

i. the subject exhibits signs or symptoms continued loss of red blood cells by ongoing or intermittent intravascular hemolysis and/or extravascular hemolysis;

j. the subject has red blood cells opsonized by fragments of C3;

k. the subject requires periodic blood transfusions;

l. the subject has low normal or below normal levels of hemoglobin;

m. the subject has low normal or below normal levels of platelets;

n. the subject has high normal or above normal reticulocytes;

o. the subject has high normal or above normal bilirubin; or

p. the subject has iron overload or is at risk of iron overload.

14 The method of claim 13, wherein the subject requires periodic blood transfusions.

15. The method of claim 13, wherein the subject is thereby rendered transfusion independent.

16. The method of claim 13, wherein the subject has below normal levels of hemoglobin.

17. The method of any one of claims 1-16, wherein the composition increases the survival of red blood cells in the subject.

18. The method of any one of claims 1-17, wherein the complement-mediated hemolytic disorder is sickle cell anemia.