Complement inhibitors to treat IgM-mediated autoimmune hemolysis

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ABSTRACT

Complement activation in autoimmune hemolytic anemia may exacerbate extravascular hemolysis and may occasionally result in intravascular hemolysis. IgM autoantibodies as characteristically found in cold autoantibody autoimmune hemolytic anemia, in cold agglutinin disease but also in a considerable percentage of patients with warm autoantibodies are very likely to activate complement *in vivo*. Therapy of IgM-mediated autoimmune hemolytic anemia mainly aims to decrease autoantibody production. However, most of these treatments require time to become effective and will not stop immediate ongoing complement-mediated hemolysis nor prevent hemolysis of transfused red blood cells. Therefore pharmacological inhibition of the complement system might be a suitable approach to halt or at least attenuate ongoing hemolysis and improve the recovery of red blood cell transfusion in autoimmune hemolytic anemia. In recent years, several complement inhibitors have become available in the clinic, some of them with proven efficacy in autoimmune hemolytic anemia. In the present review, we give a short introduction on the pathogenesis of autoimmune hemolytic anemia, followed by an overview on the complement system with a special focus on its regulation. Finally, we will discuss complement inhibitors with regard to their potential efficacy to halt or attenuate hemolysis in complement-mediated autoimmune hemolytic anemia.

Introduction

Autoimmune hemolytic anemia (AIHA) is a rare disease with an estimated incidence of 17 per 100,000 per year, which is characterized by the formation of autoantibodies (auto-Abs) directed to red blood cells (RBC) with or without complement activation resulting in shortened lifespan of RBCs in the circulation.[1-3] Based on the optimal binding temperature, AIHA is classified into warm autoantibody AIHA (WA-AIHA), cold autoantibody AIHA (CA-AIHA), and mixed forms, respectively.[1-3] The diagnosis of AIHA is based on the presence of hemolytic anemia with a positive direct antiglobulin test (DAT) for IgG and/or complement C3d. The degree of anemia at presentation correlates with the severity of AIHA and with the probability of relapse.[4] The lowest hemoglobin levels have been observed in patients suffering from warm AIHA with IgG and C3d positivity and mixed forms of AIHA (e.g. co-existence of warm and cold autoantibodies, respectively). In these forms of AIHA, complement is activated as evidenced by the positive DAT for C3d. Atypical AIHA which is, at least in part, caused by warm IgM, is also associated with a more severe disease course. In a significant percentage of patients suffering from these forms of warm AIHA, IgM reactive at 37°C (warm IgM) can be detected.[4] In an older study, 13% of all patients had detectable monophasic "hemolysins", which mainly consist of warm IgM.[4] This is in accordance with our own data from patients suffering from AIHA with a DAT positive for IgG and C3d. A majority of these patients also have evidence for the presence of RBC specific IgM, which escapes routine diagnostic testing (Meulenbroek *et al.*, accepted for publication). Given the fact that warm IgM reacts at 37°C, these auto-Abs can efficiently activate complement *in vivo* resulting in complement-mediated destruction of RBCs. Taken together, these data show that the presence of IgM with a broad temperature amplitude and/or optimal binding temperature above 30°C induce complement activation *in vivo* and are associated with a more severe course of AIHA.

Treatment of AIHA mainly aims to abrogate RBC auto-Ab production by B cells as well as to inhibit clearance of RBC in the spleen. In WA-AIHA, the first-line treatment is steroids, and as second-line treatments, splenectomy or rituximab (anti-CD 20) turned out to be effective.[5] Established effective therapy in CA-AIHA consists of rituximab optionally combined with fludarabine.[6] Needless to say that treatment of the underlying disease is essential.[3] All these therapeutic approaches need time to become effective. However, in patients presenting with acute symptomatic AIHA or experiencing an exacerbation of AIHA, the primary goal of treatment is to halt acute hemolysis. In addition, restoration of oxygen carrier in symptomatic anemia is mandatory. Yet autoantibodies will react with donor cells as well, resulting in an inadequate recovery of RBC transfusion. In addition, RBC transfusion may exacerbate hemolysis with the potential risk to develop hyperhemolysis. In addition, there is a significant risk to develop RBC alloantibodies. In IgM-mediated AIHA, complement-mediated RBC destruction significantly contributes to the severity of acute hemolysis, to the exacerbation of chronic AIHA, and to the decreased recovery of RBC transfusion. Therefore, treatment with complement inhibitors may halt or at least attenuate acute complement-mediated hemolysis in these patients and may improve recovery of RBC transfusion. In this article we will give an overview of the physiology and pathophysiology of the complement system and its role in AIHA. Then we will discuss the mechanism of action and the efficacy of complement inhibitors in the treatment of acute AIHA.

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Complement system

The complement system is an evolutionary highly conserved cascade system that makes up part of the innate immune system.[7-9] Complement activation can occur *via* three distinct pathways (classical pathway (CP), lectin pathway (LP) and alternative pathway (AP) that converge at the level of C3 cleavage and eventually lead to a common terminal pathway (TP) (Figure 1A).

The AP can be initiated by spontaneous hydrolysis of the central complement component into C3b(H_2O). C3b(H_2O) is an acceptor for the next AP protein Factor B (FB) which is then cleaved by the serine protease factor D (FD), resulting

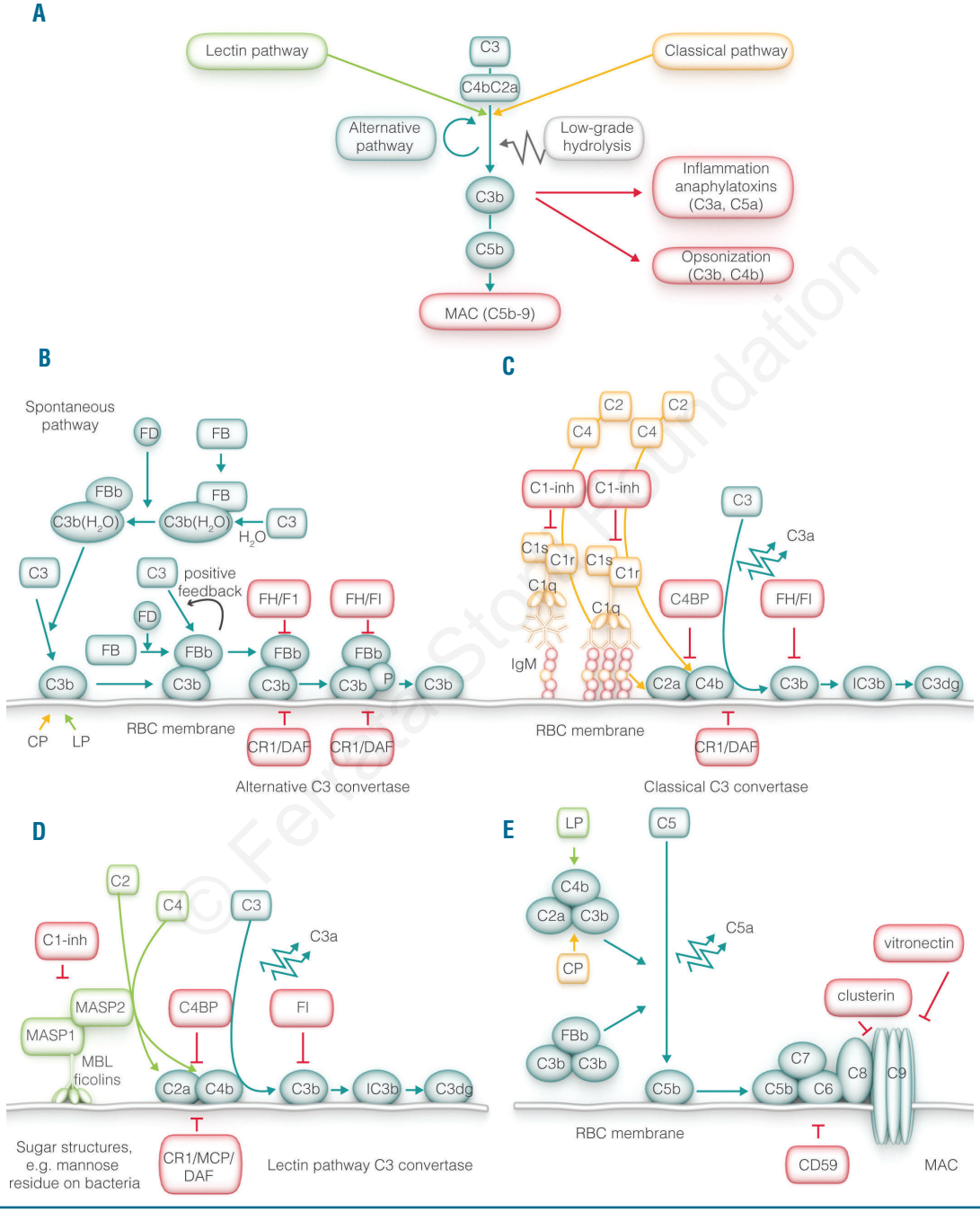

Figure 1. Overview of the complement system. (A) Overview of the complement system including the main activation pathways. (B) The alternative pathway is initiated by spontaneous low-grade conversion of C3 into active C3 (C3b), which together with activated factor B (Bb) forms the alternative C3 convertase which can induce additional C3 cleavage in a positive feedback loop. (C) The classical pathway is activated by antibodies [one IgM molecule, multiple (preferably 6) IgG molecules] leading to the formation of the classical C3 convertase (C2aC4b) by the activation C2 and C4 by C1s/C1r. (D) The lectin pathway is initiated by binding of MBL (or ficolins) to sugar structures followed by activation of C2 and C4 by MASP1/MASP2, leading to the formation of lectin C3 convertase (C2aC4b). (E) C3-activation by the classical, lectin or alternative C3 convertase results in the formation of the C5 convertase. C5 convertase subsequently activates C5 resulting in the formation of the membrane attack complex (MAC). C: complement factor; MAC: membrane attack complex; MBL: mannan binding lectin; MASP: MBL–associated serine protease; P: properdin; C1-inh: C1-inhibitor; FI: factor I; CR1: complement receptor 1; MCP: membrane co-factor protein; DAF: decay accelerating factor; C4BP: C4-binding protein; FH: factor H.

in the fluid phase C3 convertase (C3b(H_2O)Bb), that can cleave multiple C3 molecules into C3b and C3a. C3b binds to nucleophilic targets on cell membranes[10] and C3a acts as a pro-inflammatory anaphylatoxin (Figure 1B). Low-level activation of C3 can significantly be accelerated through a positive feedback loop resulting in the formation of additional alternative C3 convertases on the surface (C3bBb) that are stabilized by properdin (P) and eventually give rise to the formation of a C5 convertase (C3bBbC3b), which subsequently cleaves C5 into C5b and C5a.[10] C5b attaches to the surface and subsequently binds to C6, C7 and C8 to form the C5bC8 complex allowing polymerization of C9 to form the membrane attack complex (MAC), which inserts into target membranes and induces cell lysis (Figure 1A and E).[11,12] Next to lysis by the MAC, cleavage of both C3 and C5 results in the generation of pro-inflammatory anaphylatoxins (C3a, C5a) that attract and activate leukocytes[13] and C3b opsonization of the target surface facilitates uptake by phagocytic cells in the liver and spleen.

During evolution complement activation became more specific by the development of recognition molecules. The CP is initiated by binding of C1q to the Fc-part of IgM or IgG complexed with their target antigens. IgM is most efficient in complement activation, due to its polymeric nature. Human IgG activates complement in the order IgG3>IgG1>IgG2, whereas IgG4 does not activate complement at all.[14] As the affinity of C1q for a single IgG Fc tail is very low, C1q needs multiple Fc tails in close proximity for efficient binding and subsequent complement activation. Recently, Diebolder *et al.* described a novel concept.[15] They observed that IgG antibodies that are bound to a cellular surface are organized in a hexameric structure by non-covalent Fc interactions, thereby increasing the avidity of C1q and allowing optimal complement activation. Potentially, this phenomenon also plays a role in IgG binding to RBCs in AIHA, thereby influencing the strength of subsequent classical pathway activation. Upon binding of C1q to its ligand, the associated serine proteases C1r and C1s are activated and these subsequently cleave C4 and C2 into C4b and C2a together forming a C3 convertase (C4bC2a) on the surface[10,16] (Figure 1C). The LP is triggered by mannan binding lectin (MBL) or ficolins recognizing polysaccharides of various microbes,[17] subsequently activating MBL-associated-serine-protease-1 (MASP-1) and MASP-2.[18,19] MASP-2 cleaves both C4 and C2, resulting in the formation of a similar C3 convertase (C4bC2a) (Figure 1D).

Host cells are well protected by several membrane-bound and soluble complement-regulating proteins to minimize damage to healthy host tissue. The most important plasmatic inhibitors of the AP are factor H (FH) and factor I (FI)[10] (Figures 1B and 2). FH induces the dissociation of C3 convertase and has co-factor activity in the proteolytic degradation of C3b by FI. C3b is proteolytically degraded stepwise by FI to inactivated C3b (iC3b) and subsequently to C3d. Next to its function in fluid phase, FH can exert its protective effects on cellular surfaces by binding to polyanionic residues on the cell surface.[20] The CP and LP are controlled by 2 plasmatic inhibitors: C1-inhibitor (C1-inh) and C4-binding protein (C4BP). C1-inh decreases C2 and C4 activation and hence C3 convertase formation by the inhibition

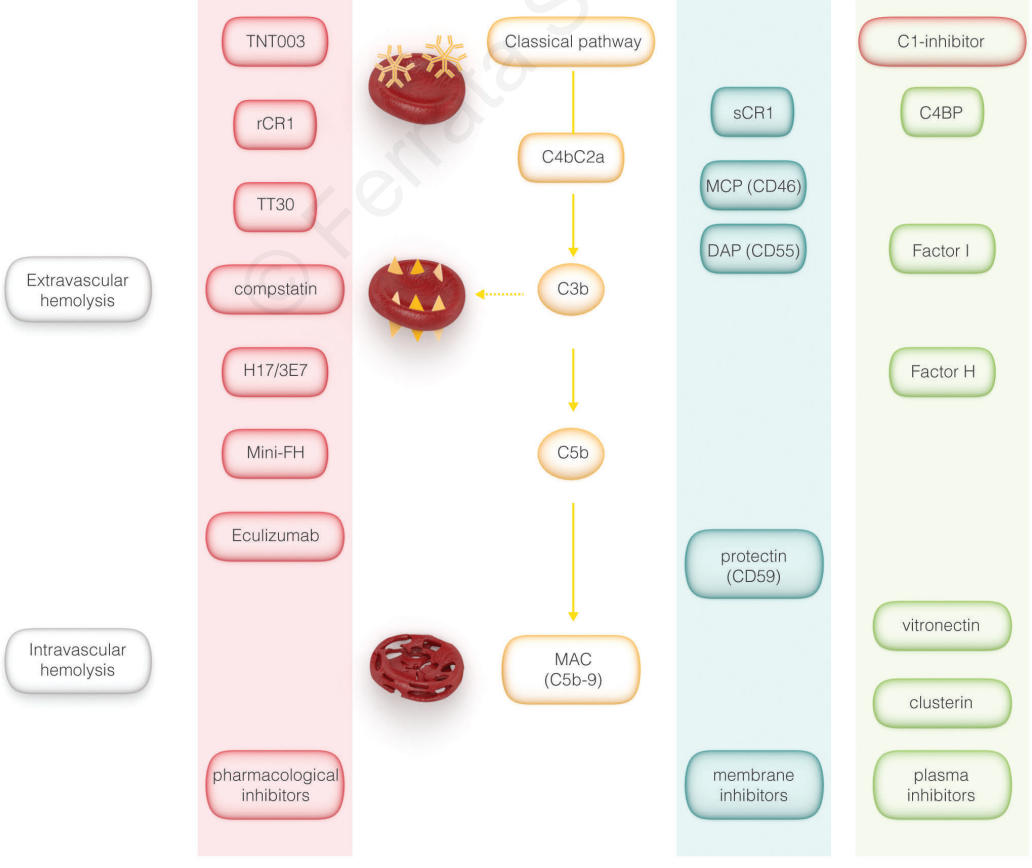

Figure 2. Regulation of the complement system by endogenous and pharmacological inhibitors. Plasmatic (green), membrane-bound (purple) and pharmaceutical (red) complement inhibitors. IgM is shown as pentamers on the red blood cells. C3b deposition is depicted as triangles on the red blood cell. Hemolysis by the MAC is depicted as red pores on the red blood cell membrane and a disintegrated membrane (so called red blood cell "ghost"). C4BP: C4b binding protein; MCP: membrane co-factor protein; DAF: decay accelerating factor; (s)CR1: soluble complement receptor 1; for other details see text.

of C1r/C1s as well as MASP1/MASP2[21,22] (Figure 1C and D and Figure 2). C4bp decays C3 convertase by binding C4b and may serve as co-factor for FI-mediated cleavage of C4b[10] (Figure 1C and D and Figure 2). Membrane co-factor protein (MCP, CD46) serves as a co-factor for FI in the inactivation of surfaced-bound C4b and C3b MCP is expressed on nucleated cells, but is lacking on red blood cells. Glycophosphatidylinositol (GPI)-linked decay accelerating factor (DAF, CD55) prevents the formation of C3-convertase (C4bC2a, C3bBb) and GPI-linked CD59 interferes with the polymerization of C9 and MAC formation on cells (Figure 1E and D and Figure 2). Fluid phase regulators of the MAC are vitronectin and clusterin. Complement receptor 1 (CR1, CD35) binds to C4b and C3b, thereby exerting decay-accelerating properties by separating the active subunits. In addition, CR1 serves as a co-factor for the inactivation of C4b and C3b by FI.[10]

Complement-mediated hemolysis in IGM-mediated AIHA

Complement–mediated hemolysis occurs in both WA- and CA-AIHA. In CA-AIHA, autoantibodies to RBCs are nearly exclusively of IgM isotype.[2,23] Although WA-AIHA is generally considered to be caused by IgG, in a significant percentage (up to 13%) IgM seems to contribute to hemolysis.[4,23] A significant proportion of our own patients suffering from AIHA with a DAT positive for IgG and C3d also have IgM in their serum, which is not measurable with the routine techniques (Meulenbroek *et al.*, accepted for publication). IgM auto-Abs can efficiently activate complement *via* the CP leading to the formation of the CP C3 convertase (C4bC2a) on the surface of RBCs.[10,24] IgM circulates as a planar pentameric and hexameric structure, the latter characterized by absence of a J-chain, both forms being unable to bind C1q in fluid phase. Upon antigen binding, IgM undergoes a conformational change providing access of C1q to the Fc parts of IgM.[25,26] Hexameric IgM was reported to activate the CP and hence induce lysis of RBCs more efficiently as compared to pentameric IgM.[27,28] Hexameric IgM was found to be present in significant amounts in patients with cold agglutinin disease (CAD). IgM antibodies, which can agglutinate RBC at low temperature (4°C), are called cold-agglutinins. Polyclonal cold agglutinins can be found in healthy subjects.[29] In contrast, monoclonal cold agglutinins with high thermal amplitude are characteristic for CAD.[30] CAD is characterized by IgM-mediated agglutination of RBCs in the microcirculation exposed to lower temperatures, e.g. fingers, toes, etc., which becomes clinically apparent as acrocyanosis.[31] Depending on the strength of the cold agglutinin titer (which goes in parallel with the density of cold-agglutinin deposition on RBCs) and the width of the thermal amplitude cold-agglutinins can also induce complement activation.[30,32] This is illustrated by the fact that C3 deposition on RBC membranes increases and C1s levels in plasma decrease with increasing titers of cold agglutinins.[33] A considerable percentage of AIHA patients have IgM, which does not behave as a classical cold agglutinin antibody, but has characteristics of a classical warm autoantibody (classified as "mixed" and "atypical" forms or "hemolysins"). These IgM autoantibodies reactive at 37°C or at least over 36°C are potent complement activators and

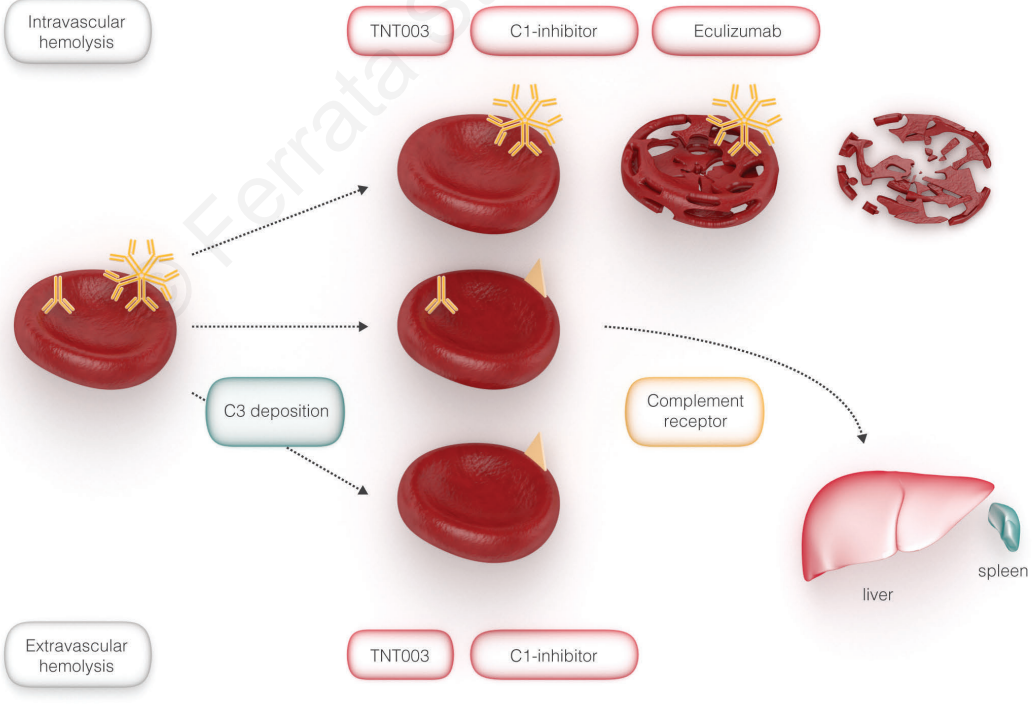

Figure 3. Inhibition of intra- and extravascular hemolysis in AIHA by complement inhibitors. Complement-mediated removal of RBCs can occur intravascular or extravascular by complement-receptor mediated phagocytosis, mainly in the liver and to some extent also in the spleen. C3b deposition is depicted as triangles on the red blood cell. C5b deposition is depicted as circles on the red blood cell. Hemolysis by the MAC is depicted as a disintegrated membrane. Eculizumab inhibits intravascular hemolysis, whereas TNT003 and C1-inhibitor are able to inhibit both intravascular- and extravascular hemolysis (adapted from Zeerleder [3]).

may cause acute hemolysis. Activation of complement by IgM auto-Abs induces cleavage of C3 by means of C3 convertase of the classical pathway leading to the deposition of C3b on the surface of RBCs, which is subsequently proteolytically cleaved by FI to iC3b, and eventually C3d. Complement receptor-mediated phagocytosis of RBC opsonized with C3 results in extravascular hemolysis and the amount of C3 deposition was reported to be predictive for hemolysis.[34] In the presence of strong complement activation, e.g. by IgM, complement activation may proceed to the formation of C5 convertase and insertion of MAC into the RBC membrane, resulting in intravascular hemolysis[3] (*see above*).

Complement-mediated hemolysis induces a severe systemic inflammation, as evidenced by clinical symptoms such as fever and chills, and, in extreme cases, hypotension and shock. Complement activation leads to the generation of vasoactive and chemotactically active C3a and C5a.[13] In systemic inflammatory diseases with a comparable complement activation to AIHA, C3a and C5a concentration significantly correlated with disease severity and fatality.[21,35]

Upon extravascular lysis, RBC are removed by orchestrated phagocytosis *via* Fc-gamma receptors and/or complement receptors. Degradation of RBC cell constituents in macrophages results in the breakdown of heme consisting in a protoporphyrin ring and ferrous iron by means of heme oxygenase into carbon monoxide, billiverdin and ferric iron, the latter consequently bound to ferritin.[36] Upon intravascular lysis, hemoglobin and heme are released into the circulation. The plasma proteins haptoglobin (Hp) and hemopexin (Hpx) neutralize free hemoglobin and heme efficiently.[36] However, due to rapid consumption of both Hp and Hpx, free hemoglobin and heme may escape neutralization and subsequently contribute to systemic inflammation. Heme–bound and free iron in the form of ferrous iron become oxidized resulting in the generation of highly cytotoxic reactive oxygen species.[37] In addition, free heme may activate the complement system *via* the alternative pathway, thereby promoting C3 deposition on bystander RBCs that may be efficiently cleared. So *via* complement activation, free heme may perpetuate the development of anemia in patients with extravascular hemolysis.[38,39] In contrast, a negative effect of free heme on C1q binding to IgG and IgM has been described.[40,41] It was mentioned that classical pathway activation can be modulated by released heme by inhibiting the binding of C1q to its ligands. However, it still remains to be investigated whether the amount of released heme in AIHA patients with extravascular hemolysis is sufficient to exert the reported effects on the complement system *in vivo*. In addition, most of the studies make use of the hemin and hematin, consisting of protoporphyrin ring containing a ferric iron with either a chloride or hydroxide ligand, respectively, both having a restricted solubility, and which differs from heme consisting of a protoporphrin ring containing ferrous ion. Upon massive RBC lysis release of RBC membrane fragments occurs, which may occasionally result in disseminated intravascular coagulation.[42,43] Altogether, complement inhibition may not only be a suitable approach to halt complement-mediated RBC lysis and to improve RBC survival, but also to limit the potentially fatal pro-inflammatory effects of generated complement activation products and release of RBC constituents, such as heme, Hb and iron, and RBC membrane fragments.

Pharmacological complement inhibitors potentially effective in the treatment of complement-mediated AIHA

To date, several complement inhibitors have been described that interfere with different steps in the activation cascade to limit complement activation.[44] However, only a few have been evaluated for prevention of complement-mediated RBC destruction so far.

Eculizumab is a humanized monoclonal antibody that binds to the C5 component of complement and inhibits terminal complement activation. Treatment of PNH patients with eculizumab significantly improves morbidity and mortality.[45] Eculizumab has been occasionally administrated to AIHA patients that were refractory to rituximab treatment.[46] Two case reports described patients with CAD who were successfully treated with eculizumab.[46,47] In both cases, hemolysis improved significantly and clinical symptoms resolved. In AIHA, complement activation is mainly driven by IgM-mediated classical pathway activation, eventually leading to C3 opsonization and only rarely resulting in MAC formation.[48] Eculizumab will, therefore, only be effective in AIHA patients with intravascular hemolysis because anti-C5 acts downstream C3 deposition and hence does not influence complement receptor-mediated uptake of C3 opsonized RBCs by phagocytic cells in the liver and spleen (Figure 2). This is also illustrated by the fact that during treatment with eculizumab increased C3 deposition on RBCs can be observed in a significant percentage of PNH patients.[49,50] Moreover, long-term C5 blockade renders patients more susceptible for bacterial infections, such as meningococcal infection. AIHA patients receive immunosuppressive treatment (e.g. prednisolone, rituximab), which increases the risk of infections and may potentially mitigate the efficacy of prophylactic measures, such as vaccination against capsuled bacteria.[51]

Therapeutic intervention at the level of C3 activation, or even more upstream in the classical pathway activation is another attractive approach to treat AIHA patients during the time window period that immunosuppressive therapy is initiated to treat the underlying B-cell-mediated auto-Ab production. In recent years, several inhibitors have been reported to efficiently inhibit C3. The Parker group reported monoclonal antibody H17/3E7 directed to C3b/C3bi, which efficiently inhibited hemolysis of RBCs from PNH patients.[52] Monoclonal H17/3E7 binds to C3(H_2O) and C3b, thereby preventing C3- as well as C5-convertase formation resulting in efficient inhibition of the alternative pathway leaving classical pathway activation unaffected. Therefore, H17/3E7 efficiently inhibits both, hemolysis and C3 deposition on PNH RBCs *in vitro*. Theoretically, H17/3E7 might also be efficient to inhibit auto-Ab-mediated hemolysis *in vitro*, by blocking the amplification loop[52] (Figure 2).

Another elegant approach to inhibit complement activation on the level at C3 has been reported by Fridkis-Hareli and co-workers.[53] This group describes a fusion protein (TT30) linking the first 4 short consensus repeats (SCR) of human complement receptor 2 (CR2) with SCR1-5 of FH. The CR2 part of this fusion protein serves as recognition subunit for C3 degradation products (e.g. iC3b, C3dg) targeting the protein to the location of complement activation.[54] The FH SCR1-5 domains of the fusion protein efficiently prevent C3 convertase formation of the alternative pathway by its decay accelerating activity.[52,53] The efficacy of TT30 to inhibit alternative pathway activation at the site of complement activation has been demonstrated *in*

vitro in a hemolysis assay using rabbit RBCs.[53] Risitano and co-workers reported TT30 to efficiently inhibit C3 deposition on and hemolysis of PNH RBCs.[55] Therefore, TT30 might be a suitable therapeutic, not only to inhibit intravascular hemolysis, but also extravascular hemolysis in PNH patients (Figure 2). In analogy to H17/3E7, TT30 is not effective in the inhibition of classical pathway activation. However, it might prevent intra- and extravascular hemolysis in AIHA by the limitation of the amplification loop and hence C3 deposition. Another drug under development that specifically targets surface AP activation is mini-FH, which is an engineered complement inhibitor consisting of SCR1-4 linked to SCR19-20 of FH. Mini-FH has high affinity for C3b and C3d and showed better inhibitory function *in vitro* compared to native FH.[56,57] In addition, as with eculizumab, safety issues still have to be defined with regard to infectious complications. A more specific approach to inhibit complement activation via the classical pathway on the level of C3 has been described by Yazdanbaksh and co-workers.[58] They showed that recombinant soluble CR1 (sCR1) by specifically binding C4b and C3b efficiently inhibits both the classical as well as the alternative pathway C3 convertase. In addition, sCR1 serves as a co-factor for factor I-mediated inactivation of C4b and C3b, respectively. Soluble CR1 effectively reduces complement-mediated RBC destruction both *in vitro* and in a mouse model of immune mediated transfusion reaction.[58] In this antibody-mediated transfusion reaction model, a decrease in C3 sensitization of the transfused RBCs was correlated with prolonged survival of the transfused RBCs. sCR1 has been studied in several human clinical trials with possible favorable outcomes.[59,60]

Another complement inhibitory drug targeting C3 is the cyclic peptide compstatin. Compstatin binds native C3 and prevents its cleavage by C3 convertases (Figure 2). Therefore, compstatin is an ideal compound to inhibit classical, lectin as well as AP activation.[61] It has been tested in several animal models and shown to be an effective complement inhibitor.[62,63] Recently, Risitano and co-workers elegantly demonstrated that pegylated compstatin not only efficiently prevents lysis of PNH-RBCs, but also C3 deposition.[64] Currently, compstatin is being tested in a human clinical trial for age-related macular degeneration (AMD) and in pre-clinical studies for several other inflammatory diseases.[44] However, to our knowledge, its ability to inhibit complement-mediated immune hemolysis *in vivo* has still not been tested. Blocking C3 activation by a compound such as compstatin might be the best way to totally shut off the complement cascade, as both the release of the pro-inflammatory C3a and opsonization by C3b are blocked, and in addition, the membrane attack complex does not form.[61] Therefore, short-term blockade of C3 activation may be helpful to inhibit intra- and extravascular hemolysis in AIHA. However, by shutting down the three activation pathways of the complement system, patients may become vulnerable to infectious complications. This risk may significantly increase in the present patient population due to concomitant immunosuppressive treatment. Besides efficacy, clinical studies have to proof safety of compstatin with regard to the susceptibility for infections.

Since complement activation in AIHA is mainly driven by IgM-mediated CP activation, eventually leading to C3 opsonization, and in rare cases, MAC formation, selective inhibition of the CP upstream the C3 convertase may be an attractive therapeutic approach (Figures 2 and 4).[2,3] By selective inhibition of the CP, the other arms of complement activation, and thus the ability to fight infections, remain intact. Recently two studies have been published in which inhibitors of the CP were shown to inhibit *in vitro* complement activation on antibody-sensitized RBCs.[33,65] In the first study, supraphysiological levels of an endogenous plasma inhibitor of the CP, C1-inh, were used.[65] Red blood cells that were *in vitro* opsonized with patient antibodies were protected from C3 deposition and hemolysis upon co-incubation with high levels of C1-inh. In this paper, one anecdotal AIHA patient was treated with high doses of plasma-purified C1-inh to improve transfusion efficiency. Hemolysis appeared to be inhibited and RBC survival increased after administration of C1-inh in this particular patient.[65] Although high levels were required to inhibit auto-Ab-induced complement-mediated RBC destruction, C1-inh might be an attractive approach to improve transfusion efficacy during hemolytic crises because of its excellent safety profile.[22,66] Plasma-purified C1-inh has been used for more than 30 years to treat patients suffering from hereditary angioedema (HAE). Moreover, C1-inh has been efficiently used in studies in patients suffering from sepsis and ischemia reperfusion injury.[22,66] We are currently investigating the efficacy of C1-inh to improve recovery of RBC transfusion in patients suffering from complement-mediated autoimmune RBC destruction in an open label study.

Another elegant approach to inhibit the activation of the classical pathway of complement in AIHA has been reported by Shi and co-workers by using an antibody (TNT003) targeting the serine protease C1s.[33] TNT003 efficiently inhibits dose-dependent *in vitro* hemolysis induced by auto-Abs from patients suffering from CAD. Both complement deposition on the RBC surface and complement-mediated phagocytosis by macrophages were inhibited by TNT003. TNT003 also efficiently reduced the generation of pro-inflammatory and vasoactive anaphylatoxins.[33] Moreover, TNT003 efficiently inhibited cold-agglutinin-mediated RBC hemolysis. In a humanized form, TNT003 might be a suitable inhibitor to block complement-mediated RBC destruction in AIHA.[33] Recently, TNT003 was successfully tested in an *in vitro* model for antibody-mediated graft rejection (AMR) showing its potency to reduce antibody-mediated classical pathway activation.[67] In addition, plasma-derived C1-inh is currently being tested in clinical trials for its efficacy in preventing AMR in kidney transplantation (*clincialtrials.gov identifier: 01147302 and 01134510*). Both inhibitors, C1-inh and TNT003, seem to be suitable to efficiently inhibit complement–deposition as well as insertion of the MAC into RBCs, and hence complement-mediated extra- and intravascular hemolysis, respectively.[33,65] A major advantage of this approach is that classical pathway inhibition leaves the alternate complement pathway activation unaffected reducing the risk of potential fatal bacterial infections in these patients who are extremely susceptible to infectious complications due to concomitant therapy and/or possible underlying disease. It must be noted though, that long-term inhibition of the classical pathway of complement potentially increases the risk for development of autoimmune disorders, as deficiencies in early classical pathway components are predisposing for systemic lupus erythematosus (SLE).[68] Whether long-term classical pathway-targeted therapy will induce lupus-like or other autoimmune complications remains to be investigated. In conclusion, complement inhibition at the very beginning of the classical pathway of complement activation is an effective potential

therapy to inhibit intra- and extra vascular hemolysis in AIHA patients with complement-activating antibodies.

In summary, complement-mediated acute and chronic breakdown of RBC in AIHA may be potentially fatal and poses a major challenge for clinicians. Targeted therapy to inhibit complement activation might, therefore, be a suitable approach. There are several complement inhibitors available, which are potentially effective *in vivo*. Complement inhibition at the level of C5 may prevent MAC formation and hence intravascular hemolysis, but will leave C3 deposition and, therefore, extravascular hemolysis, unaffected.[47,69] Complement inhibition at the C3 level is an alternative approach. Compstatin might be a valuable candidate leading to the inhibition of all three complement activation pathways, but may carry the risk of infectious complications.[61,64] In contrast, other inhibitors (e.g. TT30 and monoclonal H17/3E7) only affect alternative pathway activation, but leave activation of the classical complement pathway unaffected.[52] Therefore, a strategy targeting the furthest upstream in the classical complement pathway by means of, for example, C1-inh or TNT003 is most suitable, since it attenuates complement deposition and MAC complex formation, and hence inhibits both, intra- and extravascular hemolysis[33,65] (Figures 2 and 3).

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