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Mutations of early proteins of the complement cascade – functional characteristics, potential applications

Mutacje wczesnych białek kaskady dopełniacza – charakterystyka funkcjonalna, potencjał aplikacyjny

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Streszczenie

Układ dopełniacza składający się z kilkudziesięciu białek surowicy, stanowi element układu odporności nieswoistej i jedną z pierwszych linii obrony organizmu przed drobnoustrojami. Jego aktywacja może odbywać się poprzez drogę klasyczną, lektynową oraz drogę alternatywną. Mutacje w ścieżce alternatywnej są dobrze poznanym czynnikiem etiologicznym wielu schorzeń autoimmunologicznych (np. mutacje w czynniku B). Dla porównania, wiedza na temat mutacji w składnikach drogi klasycznej/lektynowej i ich roli w chorobach autoimmunologicznych jest bardzo ograniczona. **Celem realizowanej przeze mnie pracy doktorskiej była analiza funkcjonalna oraz potencjalne zastosowanie mutantów wczesnych białek kaskady dopełniacza.** Wyniki uzyskane w ramach projektu pokazują nowatorskie zastosowanie mutantów czynnika B oraz białka C2, będących składowymi kluczowych kompleksów enzymatycznych drogi alternatywnej oraz klasycznej układu dopełniacza. Co więcej, wyniki te stanowią istotny wkład w poszerzenie wiedzy w zakresie roli układu dopełniacza w rozwoju schorzeń autoimmunologicznych. W projekcie udało mi się wykazać, że mutant czynnika B K323E może stanowić standard w teście wykrywającym obecność przeciwciał patogennych, będących czynnikiem chorobotwórczym glomerulopatii C3. Dodatkowo, zaproponowana przez mnie zmutowana wersja czynnika B może także z powodzeniem pełnić rolę kontroli pozytywnej, do badania przesiewowego na obecność innych czynników, w tym mutacji, wpływających na zmianę aktywności układu dopełniacza. W ramach realizacji projektu dokonałam szerokiej analizy funkcjonalnej mutantów białka C2, otrzymanych na podstawie mutacji zidentyfikowanych u pacjentów ze schorzeniami nefrologicznymi. Spośród przeanalizowanych mutacji jedna (S250C) prowadziła do powstania białka tworzącego nadaktywną i bardziej stabilną konwertazę klasyczną, a efekt ten był bardziej widoczny w obecności inhibitora CD55. W dalszej części projektu opisałam kolejny przypadek pacjenta z mutacją S250C w białku C2 oraz zidentyfikowałam kolejną substytucję R249C prowadzącą do fenotypu „gain-of-function", wskazując na podatność tego regionu białka na mutacje o charakterze nadania funkcji. Ponadto, badania uzupełniłam o propozycję mechanizmu udziału białek z w/w substytucjami w uszkodzeniu śródbłonka naczyniowego nerek w rozwoju glomerulopatii. Znaczący wzrost depozycji C3b na powierzchni komórek śródbłonka nerek po inkubacji z białkiem C-reaktywnym oraz poszczególnymi mutantami białka C2 współgra z obrazem klinicznym, gdzie do manifestacji choroby dochodzi często po przebyciu infekcji. Warto podkreślić, że do tego momentu brak było danych literaturowych na temat naturalnie występujących mutacji w białku C2, a wykonane analizy mogą przyczynić się do lepszego poznania etiologii schorzeń autoimmunologicznych, do poprawy oceny ryzyka, a także do możliwości skonstruowania efektywnej terapii. W kolejnej części projektu udało mi się pokazać, jak przekształcić czynnik potencjalnie patogenny w użyteczne narzędzie o potencjale terapeutycznym. Udowodniłam, że na poziomie *in vitro* mutanty białka C2, które tworzą konwertazę klasyczną o wydłużonym czasie półtrwania, mogą zwiększać efekt cytotoksyczny przeciwciał anty-CD20 na pierwotnych komórkach białaczkowych. Przeciwciała te należą do terapeutyków silnie aktywujących układ dopełniacza i są rutynowo stosowane w leczeniu przewlekłej białaczki limfocytowej oraz chłoniaków nieziarniczych. Moje badania pokazały, że takie białko C2 może nie tylko przyczynić się do obniżenia niezbędnej dawki wspomnianego przeciwciała i w konsekwencji zapobiec wykształceniu oporności, ale także stanowić uniwersalny czynnik wspomagający działanie przeciwciał pierwotnie nieindukujących kaskady dopełniacza. W przypadku pozytywnych wyników przyszłych badań *in vivo* na modelu zwierzęcym, mutanty białka C2 mogą stać się przedmiotem kolejnych badań o charakterze przedklinicznym.

Abstract

The complement system, consisting of dozens of serum proteins, is a part of our innate immunity and constitutes the first-line defence of the organism against invading pathogens. Its activation can occur via the classical, lectin, or alternative pathway. Mutations in the alternative pathway (e.g., mutations in factor B) are well-known etiological factors of several autoimmune diseases. In contrast, knowledge about mutations in components of the classical/lectin pathway and their role in autoimmune diseases is very limited. My Ph.D. project aimed to functionally analyse and test the potential application of mutants of the early proteins of the complement cascade. The obtained results present a novel application of factor B and C2 mutants, which are components of key enzymatic complexes (convertases) of the alternative and classical pathways of the complement system. Moreover, my results make an important contribution to knowledge about the role of the complement system in the development of autoimmune diseases. In my project, I was able to demonstrate that factor B K323E mutant can be applied as a standard in the assay detecting the pathogenic antibodies, the causative agent of C3 glomerulopathy. In addition, the proposed factor B variant can also serve as a positive control to screen for other factors, including mutations, that alter the activity of the complement system. As part of the project, I performed an extensive functional analysis of C2 variants designed on the basis of mutations identified in patients with nephrological pathologies. Of the mutations analysed, one (S250C) resulted in protein forming a hyperactive and more stable classical convertase, and this effect was more pronounced in the presence of the CD55 inhibitor. Later in the project, I described another case of a patient with C2 mutation S250C and identified another R249C substitution leading to a gain-of-function (GOF) phenotype, indicating the presence of gain-of-function mutational hotspot. Furthermore, I completed the study by proposing a mechanism of how such GOF C2 variants can be involved in the damage of glomerular vascular endothelium during the development of glomerulopathy. The significant increase in C3b deposition on the surface of glomerular endothelial cells after incubation with C-reactive protein and C2 mutant is in agreement with the clinical picture, where the post-infection manifestation of the disease occurs. Importantly, to that moment there was no clinical data on naturally occurring mutations in the C2 protein, thus obtained results may contribute to a better understanding of the etiology of autoimmune diseases, to improved risk assessment, and to the possibility of constructing effective therapy. The next part of the project revealed how to transform a potentially pathogenic factor into a useful tool with therapeutic potential. I confirmed that C2 mutants able to form a classical convertase of the prolonged half-life can enhance the cytotoxic effect of anti-CD20 antibodies on primary leukaemia cells. These antibodies are potent activators of the complement system and are routinely used in the treatment of chronic lymphocytic leukaemia and non-Hodgkin's lymphoma. My studies indicate that such C2 protein not only has the potential to reduce the therapeutic dose of anti-CD20 antibodies and consequently prevent the development of resistance but may also act as a universal supporter of antibodies primarily not inducing the complement cascade. In case of positive results from future *in vivo* studies on an animal model, C2 mutant may become the subject of studies of preclinical character.

Overall objectives

The complement system that consists of dozens of serum and membrane-bound proteins is a part of our innate immunity and creates first-line defence of the body against pathogens [1]. Its activation can take place via classical, lectin, and alternative pathway. The first two pathways require specific stimulants on the surface of the target cell, e.g. antibodies or specific polysaccharide groups, whereas the alternative pathway is continuously active at a low level and its further propagation is controlled by the inhibitors localized either on the surface of host cells or present in serum [2]. The above-mentioned two patterns of activation determine the risk associated with autoimmune diseases caused by defective regulation of the complement system and its attack on own tissues and organs. Mutations in proteins that form and regulate the alternative pathway are well recognized etiologic and risk factors for diseases such as C3 glomerulopathies (e.g., membranoproliferative glomerulonephritis, MPGN [3, 4]) or atypical haemolytic uremic syndrome (aHUS) [5, 6, 7, 8], which manifest already in children and young adults. In contrast, knowledge about mutations in components of the classical/lectin pathway and their role in autoimmune diseases is very limited. Nevertheless, recent findings suggest that uncontrolled amplification of the classical pathway may be the cause of autoimmune diseases that manifest later in life. In the framework of my Ph.D. project, I conducted research aimed to further elucidate the role of mutations deregulating the classical complement pathway in the pathogenesis of human diseases. I also revealed the potential applicability of rare genetic variants of complement components factor B and C2 in molecular diagnostics and immunotherapy.

Introduction

Irrespectively on their trigger, all three complement pathways converge at the level of C5 cleavage to C5a and C5b. The latter initiates assembly of the Membrane Attack Complex (MAC) from C5b, C6, C7, C8, and C9 proteins, usually termed as late complement components. Conversely, the components that initiate the pathways and form classical and alternative convertases belong to the group of early complement components (mentioned in the title of my thesis). Convertases are labile enzyme complexes that are the nodal points of the whole cascade, responsible for the production of opsonins, anaphylatoxins, and the initiation of the lytic pathway ultimately leading to the MAC formation [9]. These complexes consist of enzymatic subunits of either factor B for alternative pathway (C3bBb, C3bBbC3b) or C2 protein for the classical/lectin (C4bC2a, C4bC2aC3b) convertases (Fig. 1). C3 convertases catalyse the breakdown of C3 to C3a and C3b fragments. C3b can associate with parental convertase and change the substrate specificity of the complex from C3 to C5 proteins, thus forming a C5 convertase. Alternatively, C3b can give rise to the formation of new complexes of the alternative pathway convertase (C3bBb), initiate the formation of a new alternative C3 convertase (C3bBb) that multiplies the C3b pool, thereby taking part in the amplification loop [10].

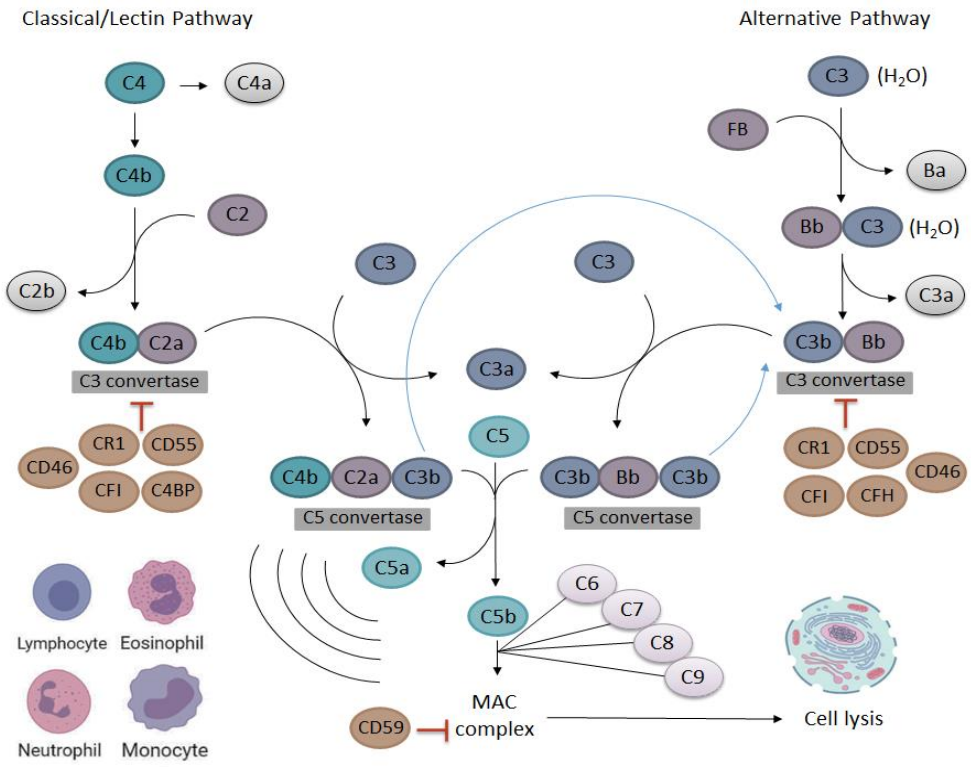

Fig.1 | The simplified scheme of complement system activation. The most important inhibitors are depicted in brown circles. Blue lines indicate an amplification loop. (Based on Chen et al., Nat Rev Nephrol 2017).

Since most of the known inhibitors of the complement system act at the level of convertases, they are thought to be pivotal for the maintenance of physiological balance between intended and excessive (dangerous) complement activation. Indeed, mutations in their components can lead to various autoimmune diseases. In my Ph.D. thesis, I was focused on mutations in proteins that form the enzymatic part of these complexes i.e. C2 and factor B (fB). These proteins are characterized by similar size (764 and 752 amino acids, respectively), sequence identity of 39%, and 50% of sequence similarity [11]. In addition, both proteins exhibit nearly identical structural features that determine how they interact within convertases and with their inhibitors. C2 and fB contain a serine protease activity whose specificity is limited to the major complement protein C3. When C2 and fB bind to their C3 convertase-forming partners (C4b and C3b, respectively), their enzymatic activity is triggered, leading to the conversion of the C3 protein to a C3b fragment and the anaphylatoxin C3a (Fig.1). Later on, when an additional C3b fragment binds parental convertase complex, the same activity of serine protease cleaves C5 into C5b fragment and C5a anaphylatoxin. Under physiological conditions, the convertases retain their activity for a short time (less than 1 minute), after which they are inactivated by dissociation and/or proteolysis with the participation of inhibitors. Once Bb or C2a are separated from the complex, they cannot reassemble to form an active convertase.

Mutations in factor B are the subject of numerous publications, where most of them concern gain-of-function (GOF) mutations [5, 12]. These alterations can lead to inhibitor resistance or a higher affinity of the convertase components for each other, thus facilitating the formation and increasing the half-life of those enzymatic complexes. Each case leads to an increased C3 cleavage rate, which may translate into a more efficient lysis of the target cell. The identified mutations have been described as a causative factor in many cases of autoimmune diseases [6, 13], and therefore functional and genetic analysis of factor B are included in the standard diagnostics of patients with suspected defective

complement regulation. At present, genetic tests are not routinely performed for any of the classical pathway components, although few reports postulated uncontrolled amplification of the classical pathway as the only identified potentially pathogenic event in patients . **Therefore, in my thesis, I hypothesized that mutations in C2 protein can be a yet undiscovered pathogenic factor of complement-related disorders.** Identification of such mutations and their detailed functional analysis would contribute to a better understanding of the etiology of autoimmune diseases, improve risk assessment, and bring the possibility to designing an effective targeted therapy.

Another factor that may affect the half-life of the alternative pathway C3 convertase is C3 nephritic factor (C3NeF) autoantibodies. These autoantibodies have been identified as one of pathogenic factors among patients with C3 glomerulopathy [14]. Their presence leads to stabilization of C3 convertase, increased production and deposition of C3b on the surface of renal endothelial cells. The resulting prolonged inflammation of the glomeruli in most cases leads to loss of function of both kidneys. Detection of C3NeF is a challenge of today's diagnostics due to the lack of a reproducible, reliable method and adequate standardization. There are many methods for the detection of C3NeF, but most are based on the analysis of convertase stabilization, which is formed on artificial surfaces (plastic plate, Biacore chip surface) and created from purified proteins [15] [16]. Such experimental setup increases the risk of artifacts, due to the non-physiological conditions of convertase formation and the absence of other serum components capable of affecting the complement system. Moreover, reference samples containing C3NeF analysed in an independent quality control test (5th External Quality Assessment 2015) conducted by the International Complement Society, were correctly identified by only 50% of the participating laboratories [17]. Being aware of the above-mentioned problems, we propose a new concept for measuring convertase activity. The method is based on erythrocyte lysis and the use of a C5 protein inhibitor. The use of rabbit erythrocytes, on which the human alternative pathway is spontaneously activated, or sheep erythrocytes, activated with antibodies, enables the analysis of the convertase activity of a given pathway of the complement system. A schematic representation of the method is shown in Fig. 2. The test is carried out in the presence of all serum components, and therefore shows only those effects that are physiologically relevant instead of those that can be observed only in a system composed of purified components. However, this and other methods require the use of a reliable standard that assures lot-to-lot consistency. So far, the widely accepted practice was the application of purified antibody fraction or serum collected from individuals previously diagnosed as C3NeF-positive. However, collecting serum from patients with chronic and severe diseases, in whom the level of C3NeF production may vary over time and with the treatment administered, generates not only ethical issues but also a potential problem with reproducibility. **I made a hypothesis that the C3NeF activity is functionally mimicked by certain mutations in complement genes found in patients with glomerulopathies.** Therefore I verified the use of a known mutant of factor B that forms the alternative convertase with an extended half-life and thus resembles convertase activity assay profile characteristic for the samples containing pathogenic C3NeF antibodies. Application of such recombinant protein would obviate the need to use patient-derived material as a positive control. In addition, the proposed standard can be produced in unlimited quantities and would allow a reliable comparison of results obtained in thousands of independent measurements.

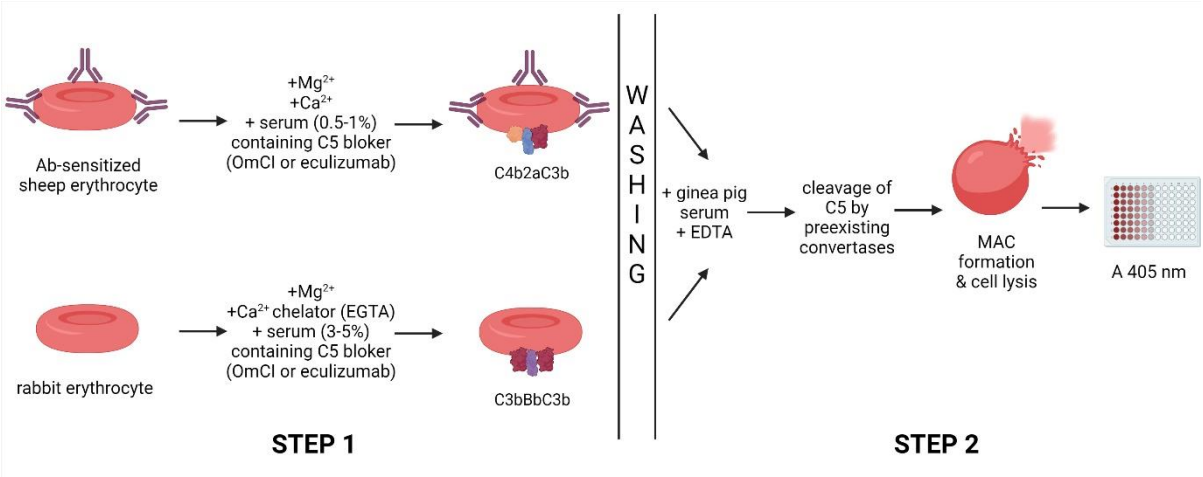

Fig. 2| Two-step convertase activity assay. (Based on Blom AM et al., Clin Exp Immunol 2014).

Another aspect of my Ph.D. thesis that involves the applicability of mutations of early complement components is the use of hyperactive variants of C2. Such C2 mutants may not only be an element critical for the pathomechanism of diseases but also an element that could be used in immunotherapy carried out with anti-cancer antibodies. **In my thesis, I propose the possibility of original applications of GOF mutants of the C2 protein.** Overexpression of complement inhibitors by cancer cells is considered one of the main reasons for their resistance to therapies using complement activating antibodies [18, 19]. This problem has been particularly well described on a model of human chronic lymphocytic leukaemia (CLL) and anti-CD20 antibodies [20]. Inhibition of the classical pathway by complement inhibitors localized on tumour cells not only limits the efficiency of antibody effector mechanisms such as MAC-induced osmotic lysis but also leads to unproductive consumption of early components of the cascade, as demonstrated in an animal model [21, 22]. The first to be consumed is the C2 protein, whose concentration is the lowest of all other so-called early components of the classical pathway. This results in suboptimal antibody cytotoxicity during the first round of immunotherapy, as well as the ineffectiveness of subsequent rounds of treatment. The main reason is the renewal of the leukemic cell repopulating peripheral blood from reservoirs of a lesser exposure to the immunotherapeutic (e.g. bone marrow, lymph nodes) combined with a low rate of recovery of the C2 protein pool [23]. Based on numerous GOF mutations in factor B *Kuttner-Kondo et al*. attempted to construct a panel of C2 mutants, which phenotypically reflected already characterized mutations in fB [24]. The introduction of mutations in four out of five corresponding sites in C2 resulted in a similar phenotype of classical complement convertase, thus confirming that at least some well-defined GOF mutations in factor B may be translated into its functional analogue. I hypothesize that the use of a C2 protein insensitive to complement inhibitors may significantly increase the sensitivity of tumour cells to immunotherapy and possibly will enable the reduction of the doses of antibodies used. In turn, such minimizing of antibody dose will prevent massive depletion of the pool of early complement proteins and on the other hand, it will eliminate one of the basic defence mechanisms of tumour cells. If the planned *in vivo* experiments using the lymphoma rat model give positive results, GOF mutants of C2 protein may become the subject of further preclinical and clinical studies.

Based on available data regarding mutations in early complement proteins and the importance of complement activation as an effector mechanism of several therapeutics, my PhD project proposed three objectives described in listed publications and manuscripts :

1. Identification and functional analyses of gain-of-function mutations of complement C2 protein found in patients suffering from various autoimmune diseases.

Publication: Urban A, Volokhina E, Felberg A, Stasiłojć G, Blom AM, Jongerius I, van den Heuvel L, Thiel M, Ołdziej S, Arjona E, de Córdoba SR, Okrój M. Gain-of-function mutation in complement C2 protein identified in a patient with aHUS. J Allergy Clin Immunol. 2020 Oct;146(4):916-919.e11

Stabilization of complement classical pathway convertases may play an important role in autoimmune events. However, studies on GOF mutations in classical pathway components were not performed although data on similar mutations in analogous proteins of alternative pathway exist. Due to the complete lack of information on naturally occurring GOF mutations in the C2 protein, this part of the project aimed to screen patients with glomerulopathies for alterations in the C2 gene. The identification of such mutations was made possible through collaboration with Prof. de Córdoba of the Centro de Investigaciones Biológicas in Madrid, whose team was devoted to identifying and analysing mutations in patients with rare autoimmune diseases. Professor de Córdoba provided us with sequences of eight mutations identified in the C2 gene leading to single amino acid substitutions - R129H, R243C, S250C, E318D, K415N, D417H, D511N, S574P. Based on this information, I expressed in a eukaryotic system and purified recombinant mutants of the C2 protein and wild-type (WT) protein. As a positive control in all experiments, I used a GOF mutant of the C2 protein (Y347A) derived from *in silico* predictions. The resulting proteins were functionally analysed to determine the phenotypic effect of the respective mutation. Previously described convertase activity assay performed on sheep erythrocytes, allowed me to evaluate the ability of the obtained proteins to form a functional classical convertase. The results showed that only one variant, R243C, was unable to form a functional convertase, whereas Y347A, formed a convertase with an extended half-life, compared to plasma-purified C2. The remaining C2 variants retained varying degrees of activity. Sheep erythrocytes are good, although the not ideal, model for the analysis of convertase activity. The lack of human complement inhibitors and the presence of their sheep homologs may lead to an underestimation of some of the important effects of mutations. Haemolytic assay on human erythrocytes revealed gain-of-function character of variant S250C, which was confirmed by its ability to form the classical convertase of prolonged half-life in the functional assay. To obtain a complete view of the effect of each mutation on C2 function, complement-dependent cytotoxicity was analysed using anti-CD20 monoclonal antibodies and two human cell lines with different CD20/complement inhibitor ratios and thus different sensitivities to the antibodies. A broad range of functional studies proved that S250C mutant forms a convertase characterized by slower dissociation upon addition of CD55 inhibitor.

Manuscript: Urban A *, Kowalska D*, Stasiłojć G, Kuźniewska A, Skrobińska A, Arjona E, Castellote Alonso E, Ángeles Fenollosa Segarra M, Jorgenius I, Spaapen R, Satchell S, Ołdziej S , de Córdoba SR, Okrój M. Gain-of-function mutations R249C and S250C in complement C2 protein increase C3b deposition on glomerular endothelial cells in the presence of C-reactive protein.

** - equally contributed as the first authors*

Continuing the collaboration with prof. de Cordoba we obtained clinical data of another patient harboring S250C mutation together with the sequence of newly identified variant R249C in a patient with C3 glomerulopathy. Obtained data confirmed that C2 mutant R249C forms hyperactive convertase of prolonged half-life and is less susceptible to CD55-mediated decay. This led to the conclusion that this region in C2 protein is a GOF mutational hotspot and that such mutations can play

an important role in the pathogenesis of autoimmune events. We decided to unveil the potential mechanism of how GOF C2 mutants can be the drivers of nephrotic disorders leading to damage of the endothelium. The available clinical data of these patients revealed no detection of autoantibodies in the first patient and retrospectively identified (several years after diagnosis) anti-nuclear antibodies (ANA) in the other patient. Nonetheless, the actual trigger of the putatively deregulated classical pathway remains elusive. Therefore, I tested C-reactive protein-mediated activation in the presence of both C2 mutants on the surface of the glomerular endothelial cells. CRP is an acute-phase protein that elevates its concentration in plasma upon infection and has been described as the activator of the classical pathway that may interact with endothelial cells [25]. Our results demonstrated increased C3b deposition on both glomerular endothelial cells and ELISA-plate when incubated with serum supplemented with CRP and each of C2 mutants. This effect was further confirmed by flow cytometry.

2. Construction of a panel of single and multiple GOF mutants of C2 protein (based on results obtained in pt. 1 and based on available experimental data and molecular modelling) compiled in such a way that the mutations provide insensitivity to complement inhibitors. Then, testing of these mutants *in vitro* on lymphoma cell lines and *in vivo* in a xenograft mouse model for potential improvement of cytotoxic parameters of anti-CD20 therapeutic antibodies.

Manuscript: Urban A , Majeranowski A, Stasiłojć G, Koszałka P, Felberg A, Taszner M, Zaucha JM, Okrój M. Gain-of-function variants of complement C2 support cytocidal activity of anticancer monoclonal antibodies.

The idea of supplementing complement-activating immunotherapeutics with decay-resistant C2 protein is supported by two findings. Firstly, the administration of fresh-frozen plasma into CLL patients improves the effect of rituximab [26]. Secondly, *in vitro* sensitivity of CLL cells is enhanced by the addition of CD55 function-blocking antibody [27]. The concept of this research task is based on the available experimental data on GOF mutations in factor B. We decided to translate known GOF mutations in factor B to C2 protein. On this basis, a panel of single and multiple mutants of the C2 protein was created to obtain the variant providing the greatest resistance to complement inhibitors and the greatest efficiency of the classical convertase. Most of the purified proteins formed the classical convertase of prolonged half-life. Those variants were tested as a supporter of the therapeutic effect of type I anti-CD20 antibodies rituximab and ofatumumab, the potent activators of complement-dependent-cytotoxicity (Fig.3). The results obtained on human lymphoma cell lines of different sensitivity to anti-CD20 mediated lysis and on primary CLL cells showed that some GOF C2 variants significantly increase cancer cell death, however, the most striking effect was observed employing multiple mutants. In addition, the best performing variant was able to spectacularly elevate cell lysis at rituximab concentration as low as 2.5 µg/ml. In this case, the percentage of lysed cells was even higher, than when rituximab was applied alone at saturating concentration (50 µg/ml). Notably, the best performing GOF C2 variant, when added to serum samples collected from CLL patients before the 2nd, 3rd, and 4th infusion of rituximab, was able to support the efficient killing of cancer cells by exploiting the leftovers of the drug accumulated in blood after the previous infusion performed four weeks earlier. When subjected to an increased number of CLL cells up to 1×10^6 per well, the C2 variant was able to improve the CDC of rituximab leading to lysis of 40 to 60% of cells. Trying to mimic the loss of CD20 antigen by CLL cells after the first round of treatment, lymphoma cell line was subjected to siRNA-mediated CD20 knock-down (KD), leading to downregulation of target protein and simultaneous upregulation of CD59 inhibitor. Comparing to parental cells CD20 KD cell line was less susceptible to rituximab induced CDC, however, this effect was abolished when GOF C2 variant was applied. Importantly supplementation with GOF C2 variant was also successful with type II antibody obinutuzumab (potent activator of antibody-dependent cellular cytotoxicity - ADCC and antibody-

dependent phagocytosis - ADP), along with daratumumab and inotuzumab ozogamicin, IgG4 therapeutic mAbs with no recorded ability to induce CDC [28, 29].

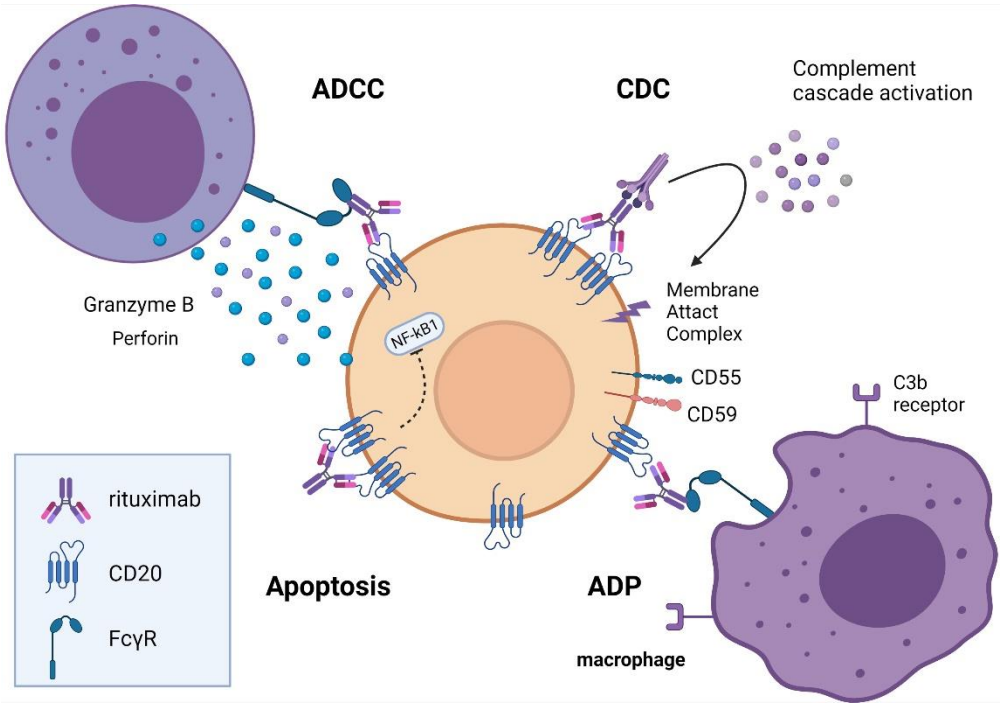

Fig. 3| The effector mechanisms of anti-CD20 antibodies e.g. rituximab. CDC: complement-dependent cytotoxicity, ADCC: antibody-dependant cellular cytotoxicity, ADP: antibody-dependent phagocytosis

3. Application the known GOF factor B mutants as a tool to mimic pathogenic autoantibody C3NeF in a convertase activity assay and evaluate the possibility of using such mutants as a standard in C3NeF diagnostic tests.

Publication: Urban A, Borowska A, Felberg A, van den Heuvel L, Stasiłojć G, Volokhina E, Okrój M. Gain of function mutant of complement factor B K323E mimics pathogenic C3NeF autoantibodies in convertase assays. *Autoimmunity*. 2018 Feb;51(1):18-24.

The key result of the publication is a demonstration of the complement factor B K323E variant's utility as the internal standard in assays detecting C3NeF. The convertase activity curve obtained for the serum supplemented with factor B mutant was similar to the serum supplemented with the purified antibody fraction from a patient with suspected C3 glomerulopathy. Compared to the convertase activity obtained for serum alone or with the addition of control Ig, both of the above samples showed a prolonged convertase half-life. An optional method of assaying convertase activity is to use a whole patient serum instead of purified antibody fractions. In this case, the method serves to screen for the presence of factors affecting convertase activity, and the mutant version of Factor B that we have proposed can also successfully act as a positive control.

Discussion

To date mutations in genes encoding early proteins of the complement system were described in several publications, however most of them concern protein deficiencies or rare genetic variants [30]. Mutations of gain-of-function character in factor B are well known pathogenic factors. Single amino

acid substitutions lead to different phenotypes of the protein e.g. resistance to decay acceleration mediated by Factor H, CD55 and CR1 or increased C3b-binding affinity and C3bBb stability [12], leading to lack of control over the alternative pathway. Several communications reported the presence of autoantibodies increasing the half-life of the classical convertase (C4NeF) in patients with MPGN, C3 glomerulopathy, systemic lupus erythematosus, and infections [31, 32, 33]. Considering these premises that the classical pathway can be involved in the pathogenesis of complement-related disorders, the lack of clinical data about mutations in this part of the complement cascade appears to be surprising. The reason is that standard diagnostics omit proteins of the classical pathway. It seems logical that alternative pathway components are a more obvious choice due to no need for specific stimuli. However, pathogenic autoantibodies or elevated CRP which is known to activate the classical pathway are a common feature of patients with complement-related disorders [34]. My Ph.D. project resulted in a publication presenting the first-ever, naturally occurring mutation in C2 of gain-of-function phenotype in a patient with aHUS. The second manuscript proved that these mutations may be an important pathogenic factor in patients with elevated CRP, and as such leading to autoimmunity. Indeed, many cases of C3 glomerulopathies are reported as postinfectious, thereby making the aforementioned scenario of coinciding CRP and mutations in C2 plausible. Autoantibodies play a pivotal role in the pathogenesis of many autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis or Sjögren's syndrome [35]. Given that several studies link autoantibodies with the complement activation [36] and that components of the classical pathway were never included in genetic screening we cannot exclude the possible involvement of GOF C2 mutations in the manifestation of the aforementioned disorders. Lack of such mutations may also be the factor that prevents the development of the disease in healthy individuals positive for autoantibodies (around 4% in the general population [37]). These findings may draw the attention of both scientists and clinicians not only in the context of diagnostics but also to consider the application of therapeutics specifically blocking the classical pathway of the complement system.

In my thesis, I was able to prove that even though the complement system is an important part of our innate immunity it can act as a double-edged sword. It protects us against invading pathogens but any mutations leading to dysregulation and hyperactivation of the whole cascade can ultimately lead to autoimmune events unless we redefine a molecular target. Activation of the classical pathway is an important effector mechanism of several clinically approved anti-cancer monoclonal antibodies [38]. The first-ever introduced mAb was an anti-CD20 antibody, rituximab, applied for the treatment of B-malignancies. However, the importance of CDC in rituximab activity remains the subject of ongoing debate due to contradictory data from animal models [39]. It resulted in the development of new generations of Fc-glycoengineered antibodies designed to elevate ADCC, e.g. obinutuzumab. Prolonged progression-free survival (PFS) has been presented in clinical trials comparing obinutuzumab and rituximab head-to-head, both in patients with treatment-naïve CLL and untreated symptomatic follicular lymphoma (FL) [40]. However, it was proved to be less effective as single therapy when compared to combination with chemotherapy [41] or selective BCL2 inhibitor venetoclax [42]. Ibrutinib is a first-in-class irreversible inhibitor of Bruton tyrosine kinase that marked the beginning of the era of kinase-targeted drugs and is indicated for the treatment of CLL and small lymphocytic lymphoma [43]. This drug transformed the landscape of CLL treatment, yet several limitations have been identified when applied as a single agent, including low complete remission rates, development of resistance, and uncommon substantial toxicities [44]. Combination of ublituximab, another new type II antibody, and ibrutinib resulted in a statistically higher overall response rate without affecting the safety profile of ibrutinib in patients with relapsed or refractory high-risk CLL [45]. However, the improvements in survival outcomes did not represent as dramatic breakthrough in CLL treatment as did the addition of anti-CD20 antibody to chemoimmunotherapy regimens. These findings underline

the need and room for improvement of existing therapies instead of developing another generation of "high-cost but low-gain" drugs. Most of the newly designed anti-CD20 antibodies rely on increased ADCC and decreased complement effector mechanisms. However, the relatively high number of effector cells required in the tumour microenvironment to develop cytotoxic damage to cancer cells comprise the main limitation of this approach. In my Ph.D. thesis, I described an original approach of how to convert potentially pathogenic factors into universal supporters of complement-mediated immunotherapy. Presented gain-of-function C2 mutant combining single mutation leading to the most potent activity of classical convertase was able not only to improve the activity of anti-CD20 antibodies on both lymphoma cell lines and primary CLL cells but also substantially reduced the threshold of antibody concentration necessary for the efficient killing of those cells. Most importantly it was able to induce significant cell death by therapeutics primarily non-inducing CDC. Although the importance of CDC activation by clinically approved mAbs remains arguable, my results revealed the untapped potential of this effector mechanism that should be further explored. Future *in vivo* experiments testing the application of GOF C2 in laboratory animals would not only provide a better insight into effector mechanisms of these drugs but may become a topic of further studies of more applied or preclinical character. A positive result would validate the concept of turning autoimmunity-related compounds into adjuvants universal for every complement-based immunotherapies. Such adjuvant could enable to lower therapeutic doses of mAbs, which brings not only financial benefits but might reduce unfavourable side effects like cytokine storm, loss of target antigen by tumour cells, and unproductive depletion of early complement components. Very low physiological concentration of C2, comparing to other complement proteins, brings a real chance for effective supplementation.

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References

1. Rus, H., Cudrici, C. & Niculescu, F. The role of the complement system in innate immunity. *Immunol Res* **33**, 103-112 (2005).

2. Merle, N.S., Church, S.E., Fremeaux-Bacchi, V. & Roumenina, L.T. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol* **6**, 262 (2015).

3. Iatropoulos, P. *et al.* Complement gene variants determine the risk of immunoglobulin-associated MPGN and C3 glomerulopathy and predict long-term renal outcome. *Mol Immunol* **71**, 131-142 (2016).

4. Goodship, T.H. Factor H genotype-phenotype correlations: lessons from aHUS, MPGN II, and AMD. *Kidney Int* **70**, 12-13 (2006).

5. Tawadrous, H. *et al.* A novel mutation in the complement factor B gene (CFB) and atypical hemolytic uremic syndrome. *Pediatr Nephrol* **25**, 947-951 (2010).

6. Alfakeeh, K. *et al.* Rare genetic variant in the CFB gene presenting as atypical hemolytic uremic syndrome and immune complex diffuse membranoproliferative glomerulonephritis, with crescents, successfully treated with eculizumab. *Pediatr Nephrol* **32**, 885-891 (2017).

7. Liu, J., Xiao, J., Chen, L. & Peng, Y. Atypical hemolytic uremic syndrome with C3 mutation: A case report and literature review. *Clin Nephrol* **95**, 156-160 (2021).

8. Okabe, M. *et al.* Renal Damage in Recurrent Atypical Hemolytic Uremic Syndrome Associated with C3 p.Ile1157Thr Gene Mutation. *Intern Med* **60**, 917-922 (2021).

9. Sjöberg, A.P., Trouw, L.A. & Blom, A.M. Complement activation and inhibition: a delicate balance. *Trends Immunol* **30**, 83-90 (2009).

10. Ricklin, D., Hajishengallis, G., Yang, K. & Lambris, J.D. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* **11**, 785-797 (2010).

11. Krishnan, V., Xu, Y., Macon, K., Volanakis, J.E. & Narayana, S.V. The structure of C2b, a fragment of complement component C2 produced during C3 convertase formation. *Acta Crystallogr D Biol Crystallogr* **65**, 266-274 (2009).

12. Goicoechea de Jorge, E. *et al.* Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* **104**, 240-245 (2007).

13. Wu, H., Su, S., Li, L. & Zhang, L. Atypical hemolytic uremic syndrome and acute tubular necrosis induced by complement factor B gene (CFB) mutation: A case report. *Medicine (Baltimore)* **100**, e25069 (2021).

14. Leroy, V. *et al.* Membranoproliferative glomerulonephritis with C3NeF and genetic complement dysregulation. *Pediatr Nephrol* **26**, 419-424 (2011).

15. Paixão-Cavalcante, D. *et al.* Sensitive and specific assays for C3 nephritic factors clarify mechanisms underlying complement dysregulation. *Kidney Int* **82**, 1084-1092 (2012).

16. Harris, C.L., Pettigrew, D.M., Lea, S.M. & Morgan, B.P. Decay-accelerating factor must bind both components of the complement alternative pathway C3 convertase to mediate efficient decay. *J Immunol* **178**, 352-359 (2007).

17. Prohászka, Z., Nilsson, B., Frazer-Abel, A. & Kirschfink, M. Complement analysis 2016: Clinical indications, laboratory diagnostics and quality control. *Immunobiology* **221**, 1247-1258 (2016).

18. Fishelson, Z., Donin, N., Zell, S., Schultz, S. & Kirschfink, M. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. *Mol Immunol* **40**, 109-123 (2003).

19. Gancz, D. & Fishelson, Z. Cancer resistance to complement-dependent cytotoxicity (CDC): Problem-oriented research and development. *Mol Immunol* **46**, 2794-2800 (2009).

20. Golay, J. *et al.* Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* **95**, 3900-3908 (2000).

21. Beurskens, F.J. *et al.* Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J Immunol* **188**, 3532-3541 (2012).

22. Middleton, O. *et al.* Complement deficiencies limit CD20 monoclonal antibody treatment efficacy in CLL. *Leukemia* **29**, 107-114 (2015).

23. Baig, N.A. *et al.* Induced resistance to ofatumumab-mediated cell clearance mechanisms, including complement-dependent cytotoxicity, in chronic lymphocytic leukemia. *J Immunol* **192**, 1620-1629 (2014).

24. Kuttner-Kondo, L.A. *et al.* A corresponding tyrosine residue in the C2/factor B type A domain is a hot spot in the decay acceleration of the complement C3 convertases. *J Biol Chem* **278**, 52386-52391 (2003).

25. Sproston, N.R. & Ashworth, J.J. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol* **9**, 754 (2018).

26. Klepfish, A. *et al.* Enhancing the action of rituximab in chronic lymphocytic leukemia by adding fresh frozen plasma: complement/rituximab interactions & clinical results in refractory CLL. *Ann N Y Acad Sci* **1173**, 865-873 (2009).

27. Bologna, L. *et al.* Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy. *J Immunol* **190**, 231-239 (2013).

28. DiJoseph, J.F. *et al.* Antitumor efficacy of a combination of CMC-544 (inotuzumab ozogamicin), a CD22-targeted cytotoxic immunoconjugate of calicheamicin, and rituximab against non-Hodgkin's B-cell lymphoma. *Clin Cancer Res* **12**, 242-249 (2006).

29. Manna, A. *et al.* Targeting CD38 Enhances the Antileukemic Activity of Ibrutinib in Chronic Lymphocytic Leukemia. *Clin Cancer Res* **25**, 3974-3985 (2019).

30. Degn, S.E., Jensenius, J.C. & Thiel, S. Disease-causing mutations in genes of the complement system. *Am J Hum Genet* **88**, 689-705 (2011).

31. Blom, A.M. *et al.* Testing the Activity of Complement Convertases in Serum/Plasma for Diagnosis of C4NeF-Mediated C3 Glomerulonephritis. *J Clin Immunol* **36**, 517-527 (2016).

32. Corvillo, F. *et al.* Nephritic Factors: An Overview of Classification, Diagnostic Tools and Clinical Associations. *Front Immunol* **10**, 886 (2019).

33. Garam, N. *et al.* C4 nephritic factor in patients with immune-complex-mediated membranoproliferative glomerulonephritis and C3-glomerulopathy. *Orphanet J Rare Dis* **14**, 247 (2019).

34. Rubin, R.L., Teodorescu, M., Beutner, E.H. & Plunkett, R.W. Complement-fixing properties of antinuclear antibodies distinguish drug-induced lupus from systemic lupus erythematosus. *Lupus* **13**, 249-256 (2004).

35. Suurmond, J. & Diamond, B. Autoantibodies in systemic autoimmune diseases: specificity and pathogenicity. *J Clin Invest* **125**, 2194-2202 (2015).

36. Elkon, K. & Casali, P. Nature and functions of autoantibodies. *Nat Clin Pract Rheumatol* **4**, 491-498 (2008).

37. Haller-Kikkatalo, K. *et al.* Demographic associations for autoantibodies in disease-free individuals of a European population. *Sci Rep* **7**, 44846 (2017).

38. Stasiłojć, G., Österborg, A., Blom, A.M. & Okrój, M. New perspectives on complement mediated immunotherapy. *Cancer Treat Rev* **45**, 68-75 (2016).

39. Uchida, J. *et al.* The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* **199**, 1659-1669 (2004).

40. Marcus, R. *et al.* Obinutuzumab for the First-Line Treatment of Follicular Lymphoma. *N Engl J Med* **377**, 1331-1344 (2017).

41. Stilgenbauer, S. *et al.* Safety and efficacy of obinutuzumab alone or with chemotherapy in previously untreated or relapsed/refractory chronic lymphocytic leukaemia patients: Final analysis of the Phase IIIb GREEN study. *Br J Haematol* **193**, 325-338 (2021).

42. Al-Sawaf, O. *et al.* Venetoclax plus obinutuzumab versus chlorambucil plus obinutuzumab for previously untreated chronic lymphocytic leukaemia (CLL14): follow-up results from a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol* **21**, 1188-1200 (2020).

43. Timofeeva, N. & Gandhi, V. Ibrutinib combinations in CLL therapy: scientific rationale and clinical results. *Blood Cancer J* **11**, 79 (2021).

44. Strati, P. *et al.* Achieving complete remission in CLL patients treated with ibrutinib: clinical significance and predictive factors. *Blood* **135**, 510-513 (2020).

45. Sharman, J.P. *et al.* Ublituximab plus ibrutinib versus ibrutinib alone for patients with relapsed or refractory high-risk chronic lymphocytic leukaemia (GENUINE): a phase 3, multicentre, open-label, randomised trial. *Lancet Haematol* **8**, e254-e266 (2021).

Letter to the Editor

Gain-of-function mutation in complement C2 protein identified in a patient with aHUS

To the Editor:

The complement system, a basic defense mechanism of innate immunity, protects us from invading pathogens and supports the maintenance of body homeostasis.[1] As proper functioning of complement is based on the interplay between its activators and inhibitors, functional impairment of a particular component often leads to autoimmune or inflammatory diseases. The kidney is an organ that is especially susceptible to complement-mediated damage because of high blood flow combined with a delicate structure of the filtrating barrier. Additionally, glomerular vessels are vulnerable to thrombosis and damage by heme originating from local hemolysis of red blood cells. Complement attack on the kidney's structures can be mediated by abnormalities in the alternative complement pathway (AP).[2] This route is constantly active at a low level and spontaneously deposits C3 activation fragments (C3b) on cell surfaces.[1] Unlike pathogens, self-cells are equipped with a panel of complement inhibitors that block further pathway propagation. Therefore, the loss of control of the AP is a condition permissive for complement-mediated damage. The underlying mechanisms may involve aberrations that render AP convertases insensitive to regulation. Such aberrations include the presence of autoantibodies termed C3NeF, which stabilize AP convertases (key enzymatic complexes that amplify the complement cascade), autoantibodies binding to soluble complement inhibitors such as factor H, inheritance of rare variants of membrane complement inhibitors (eg, CD46) and factor H, or gain-of-function mutations in complement components such as C3 or factor B (FB).[2] Classical complement-mediated renal diseases are C3 glomerulopathies (C3Gs) and atypical hemolytic uremic syndrome (aHUS). There are reports suggesting that next to dysregulation of the AP, abnormalities in the classical pathway/lectin pathway (CP/LP) may also play a role in pathogenesis. Previously, we screened 13 patients with C3G for alterations increasing or prolonging convertase activity and found 1 individual with no acknowledged risk factors but antibodies stabilizing the CP/LP convertases (C4NeF).[3] Another screening of a cohort of 168 patients with C3G revealed 3 patients who were positive only for C4NeF autoantibodies whereas genetic analysis showed no rare or novel variants of complement genes.[4] Because standard diagnostics of complement-mediated renal diseases has thus far been focused on abnormalities in the AP, the possible role of the CP/LP in pathogenesis may be underestimated. Up to now, no gain-of-function variants within CP/LP convertase components have been identified in patients. We decided to search for such naturally occurring mutations in complement C2 protein —a CP/LP analog and paralog of FB. These 2 proteins share a high degree of amino acid similarity, as well as almost identical length and structural organization.[5] Therefore, it is highly probable that they contain similar mutational hotspots, as already demonstrated by translation of gain-of-function mutations in FB to C2.[6] Above all, the fact that pathogenic, naturally occurring gain-of-function mutants of FB have already been characterized encouraged us to look for analogous pathogenic mutations in C2.

DNA samples of 233 patients with aHUS and C3G who were included in the Spanish aHUS/C3G Registry (https://www.aHUSC3G.es) as of August 29, 2016, were analyzed for genetic variants by using a next-generation sequencing panel that included *THBD, DGKE,* and 42 complement genes. Missense mutations in C2 gene were identified in 8 patients (Table I). A complete list of analyzed genes, sequencing and data analysis procedures, diagnostic criteria, and all methods used for further characterization of mutated proteins are provided in this article's Online Repository (available in this article's Online Repository at www.jacionline.org).

Wild-type (WT) C2 and all mutants were expressed in the eukaryotic system as C-terminal His-tagged proteins (see Fig E1, *A* and *B* in this article's Online Repository at www.jacionline.org). Such modification does not affect the function of C2, as evidenced by a comparison of the hemolytic activity of WT and plasma-purified C2 (see Fig E2, *A* and *B* in this article's Online

TABLE I. Detailed characteristics of missense mutations in C2 identified in the cohort of 233 patients

Position on chromosome 6	ID	No. of patients	cDNA substitution	Amino acid substitution	Genotype	Diagnosis of patients with mutation	Risk polymorphism		Other potential pathogenic complement genetic variants	Anti-FH antibodies	C3Nef	Autoantibodies
							MCP	FH				
31896638	rs367996721	1/233	c.G386A	p.R129H	HET	aHUS	HOM	HOM	None	No	—	ANA
31901954	rs370121006	1/233	c. C727T	p.R243C	HET	aHUS	NO	HET	p.K591Sfs*10: MASP1:HET	No	—	ANCA
31901976	rs150827255	1/233	c. C749G	p. S250C	HET	aHUS	HOM	NO	None	—	—	—
31903804	rs9332739	11/233*	c. G954C	p. E318D	All HET	aHUS, C3GN	—	—	—	—	—	—
31910761	—	1/233	c. G1245C	p. K415N	HET	aHUS	NO	HET	None	No	—	No
31910765	rs907804461	1/233	c. G1249C	p. D417H	HET	C3GN	HET	NO	None	No	No	ANA
31911268	rs142802105	1/233	c. G1531A	p. D511N	HET	C3GN	HOM	NO	None	No	No	No
31911573	—	1/233	c. T1720C	p. S574P	HET	C3GN	HET	HET	None	No	No	ANA

ANA, Anti-nuclear antibody; *ANCA*, anti-neutrophil cytoplasm antibody; *FH*, factor H; *HET*, heterozygous; *HOM*, homozygous; *ID,* identifier; *MCP,* membrane cofactor protein/CD46.

*Common polymorphism with a minor allele frequency greater than 0.01. The rest of the sequence variants display a minor allele frequency less than 0.001.

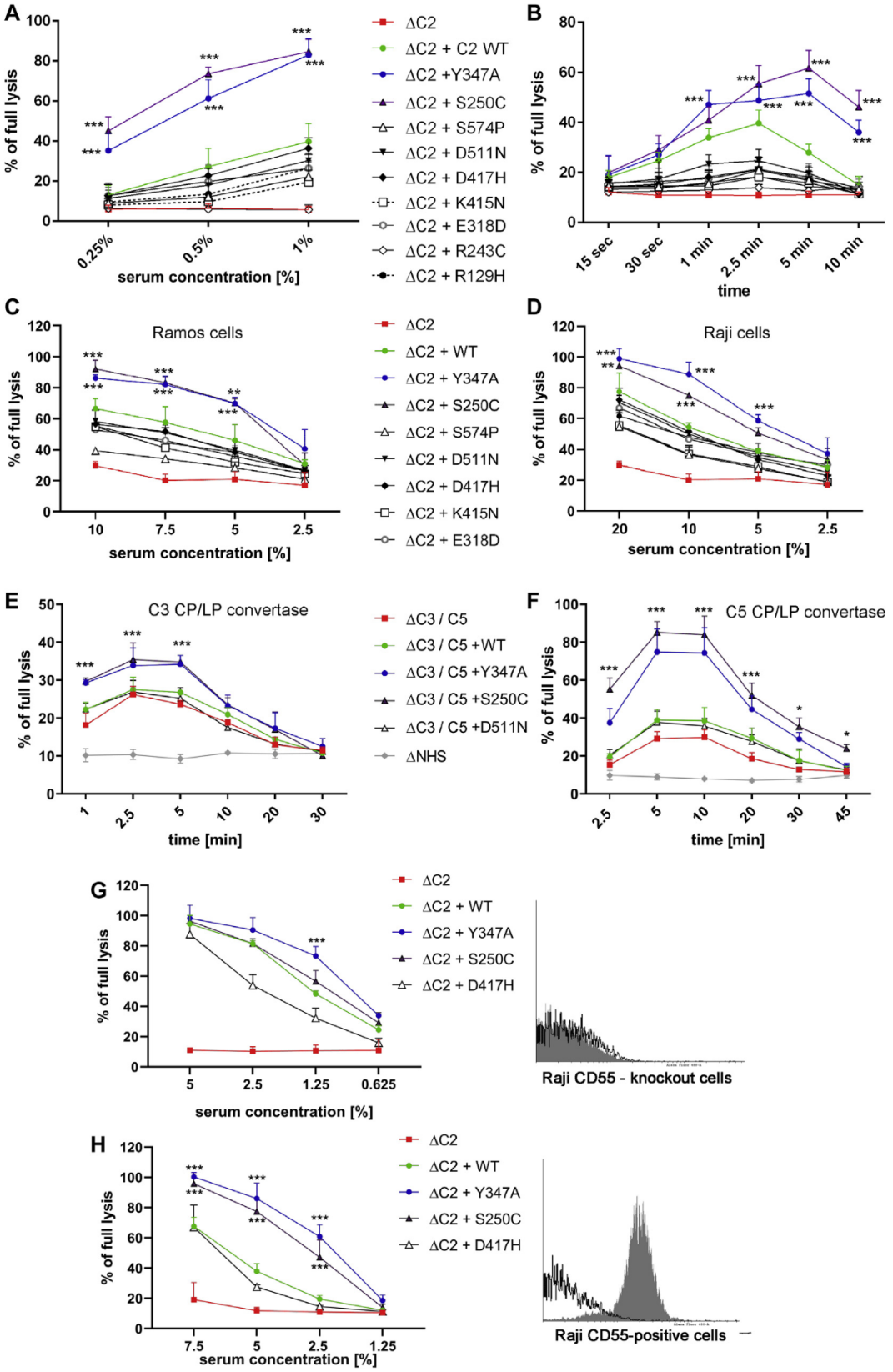

FIG 1. Functional characteristics of recombinant C2 variants. **A,** Hemolytic assay using sensitized human erythrocytes performed in C2-depleted human serum (ΔC2 serum) supplemented with a physiologic concentration of specific C2 variants. **B,** The same model was used for analysis of CP/LP convertase activity. C2 mutants were also tested by using the sensitized human lymphoma cell lines Ramos (**C**) and Raji (**D**)

Repository at www.jacionline.org). The variant Y347A, which was designed *in silico* and experimentally confirmed as resistant to CD55-, CD35- and C4BP-mediated convertase decay,[6] was included as a gain-of-function positive control.

Assays performed on sensitized human erythrocytes in C2-depleted serum showed that the addition of each natural C2 mutant resulted in a lower hemolytic reaction (Fig 1, *A*) and lower CP/LP convertase activity (Fig 1, *B*) than did the addition of WT C2, except in the case of the variant S250C, which performed similarly to Y347A. These results were confirmed in the cytotoxic assay (Fig 1, *C* and *D*) and convertase assay (Fig 1, *E* and *F*) on sensitized human lymphoma cell lines. The gain-of-function S250C mutation did not affect C2 processing to C2a/C2b by C1s enzyme (see Fig E3 in this article's Online Repository at www.jacionline.org). Unlike in experiments on human cells, the activity of the S250C mutant was comparable to that of the WT when tested in the same serum on sensitized sheep erythrocytes (see Fig E4 in this article's Online Repository at www.jacionline.org). This led us to conclude that phenotype is dependent on membrane-bound convertase inhibitors. Further analysis of surface expression of these proteins (see Fig E5 in this article's Online Repository at www.jacionline.org) indicated CD55 as a candidate, as confirmed by complement deposition assay in the presence of CD55 ectodomain (see Fig E6 in this article's Online Repository at www.jacionline.org) and experiments with CD55-KO Raji cells in which the gain-of-function phenotype of the S250C mutant was lost (Fig 1, *G* and *H*). Serine 250 is highly conserved within sequences of C2 and FB (see Table E1 in this article's Online Repository at www.jacionline.org), and according to a model of C2a fragment (see Fig E7 in this article's Online Repository at www.jacionline.org), it is located only 22 Å from the Y347 residue, thus suggesting that these 2 residues together could form a binding site for a single molecule of complement inhibitor.

Genetic screening of patients in whom aHUS and C3G has been diagnosed is usually limited to genes encoding AP elements such as *CFB, CFH, C3, CFI,* and *CD46*. Here, we present the first report of gain-of-function mutation in the CP/LP convertase component found in human disease. Moreover, S250C was the only rare variant in complement genes of a patient with aHUS who also carried the *MCPggaac* haplotype, which decreases transcriptional activity of CD46 promotor region and dramatically increases penetrance of gain-of-function mutation in FB.[7] We propose that excessive activity of the CP/LP convertases together with predisposing haplotypes and environmental factors (eg, infections that increase the level of C-reactive protein) form multiple hits that together precipitate complement-related glomerulopathies. Therefore, routine diagnostics of such patients should not be focused only on the abnormalities in AP. We also underscore that commonly used methods such as hemolysis of sheep erythrocytes may be insufficient for functional analysis of gain-of-function variants of complement proteins, as is likewise evidenced in Felberg et al.[8] The aHUS/C3G-causative nature of 7 C2 variants identified as loss-of-function mutants is less obvious than that of the S250C variant. Low C2 activity may result in higher susceptibility to bacterial infections, which often precede disease episodes.[9] However, the global minor allele frequency of the E318D mutation (a common polymorphism identified as a loss-of-function variant, which was the most frequent in our cohort) is 0.0297 (ClinVar identifier 12130). Because the analogous value in our cohort is lower (0.0236), we consider the representation of this loss-of-function mutation as not exceeding global distribution and probably not pivotal in the disease pathomechanism.

For additional comments on the relevance and limitations of our finding, please see this article's Online Repository.

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in a complement-dependent cytotoxicity assay. Activation of CP in C3-depleted serum (ΔC3) or C5-depleted serum (ΔC5) arrests the complement cascade at the stage of C3 CP/LP convertase or C5 CP/LP convertase, respectively. **E** and **F**, Impact of selected C2 mutants added to such sera on the acticity of C3 CP/LP and C5 CP/LP convertases is shown. Raji CD55-knockout cells (**G**) and control Raji cells (**H**) were sorted to obtain homogenous populations (*islets on the right, with white and gray areas on histograms indicating cells stained with isotype control and anti-CD55 antibody, respectively*), which were used for complement-dependent cytotoxicity assay in ΔC2 serum supplemented with selected C2 mutants. All graphs show the results obtained from 3 independent experiments. Statistical significance of differences between the given C2 variant and the WT was analyzed by using the Dunnett multiple comparison test for nonrepeated measures. $^{**}P < .01$; $^{***}P < .001$.

REFERENCES

1. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010;11:785-97.
2. De Vriese AS, Sethi S, Van Praet J, Nath KA, Fervenza FC. Kidney disease caused by dysregulation of the complement alternative pathway: an etiologic approach. J Am Soc Nephrol 2015;26:2917-29.
3. Blom AM, Corvillo F, Magda M, Stasilojc G, Nozal P, Perez-Valdivia MA, et al. Testing the is of C4NeF-mediated C3 glomerulonephritis. J Clin Immunol 2016; 36:517-27.
4. Zhang Y, Meyer NC, Fervenza FC, Lau W, Keenan A, Cara-Fuentes G, et al. C4 Nephritic factors in C3 glomerulopathy: a case series. Am J Kidney Dis 2017;70: 834-43.
5. Krishnan V, Xu Y, Macon K, Volanakis JE, Narayana SV. The structure of C2b, a fragment of complement component C2 produced during C3 convertase formation. Acta Crystallogr D Biol Crystallogr 2009;65:266-74.
6. Kuttner-Kondo LA, Dybvig MP, Mitchell LM, Muqim N, Atkinson JP, Medof ME, et al. A corresponding tyrosine residue in the C2/factor B type A domain is a hot spot in the decay acceleration of the complement C3 convertases. J Biol Chem 2003;278:52386-91.
7. Goicoechea de Jorge E, Harris CL, Esparza-Gordillo J, Carreras L, Arranz EA, Garrido CA, et al. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. Proc Natl Acad Sci U S A 2007;104: 240-5.
8. Felberg A, Urban A, Borowska A, Stasilojc G, Taszner M, Hellmann A, et al. Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics. Cancer Immunol Immunother 2019;68:587-98.
9. Karpman D, Loos S, Tati R, Arvidsson I. Haemolytic uraemic syndrome. J Intern Med 2017;281:123-48.

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METHODS

Diagnostic criteria of aHUS and C3G

Patients in whom aHUS was diagnosed fulfilled the following criteria: platelet count less than 150×10^9/L or a decrease of more than 25% from baseline values, hemolytic anemia, and serum creatinine level greater than the upper limit of the normal range, together with a negative Coombs test result, normal activity of ADAMTS-13 and a negative Shiga toxin testing result. C3G was diagnosed by renal biopsy, which in many cases included analysis by electron microscopy. DNA from patients was analyzed for genetic variants by using an in-house next-generation sequencing panel including the following 44 genes: *C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C4A, C4BPA, C4BPB, C5, C7, C8A, C8B, C8G, C9, CD46, CD55, CD59, CFB, CFD, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFP, CLU, CR1, CR2, FCN1, FCN2, FCN3, ITGAX, ITGB2, MASP1, MASP2, MBL2, SERPING1, VSIG4*, and *VTN*.

Sequencing procedure and data analysis

Targeted sequences were captured by using the Nextera rapid capture custom Enrichment Kit (Illumina, San Diego, Calif) and sequencing data generated in a Miseq equipment using Miseq reagent kit v2 (300 cycles). Sequence data were analyzed by using Burrows-Wheeler Alignment and Picard software with additional filtering using customs tools. Variant calling was performed both with bcftools and VarScan, and the variant calling files generated were merged into a single file by using custom tools. Common variants with a minor allele frequency value greater than 1% in any population were excluded. To identified novel and/or pathogenic variants, we used different databases (the Exome Aggregation Consortium database, Genome Aggregation Database, 1000 Genomes, National Center for Biotechnology Information Single-Nucleotide Polymorphism Database, aHUS Mutation Database [www.fh-hus.org], or our in-house database). Pathogenicity was established by using multiple functional prediction methods (SIFT, PolyPhen2, etc) included in the ANNOVAR server and the functional data available in our laboratory and in the literature. Variants were categorized as pathogenic, variants of uncertain significance, and benign. The analysis of copy number variations within the *CFH-CFHR* gene region was performed by multiplex ligation-dependent probe amplification with the P236 A1 ARMD mix 1 (MRC-Holland, Amsterdam, The Netherlands).

Cell lines

The human lymphoma cell lines Raji and Ramos (both obtained from the American Type Culture Collection, Manassas, Va) were cultured in RPMI 1640 medium with l-glutamine (Mediatech, New York, NY) supplemented with 10% FBS (PANBiotech, Aidenbach, Germany). Cells were cultivated at 37°C and humidified in a 5% CO_2 atmosphere. Raji cells with CD55 knockout were produced by clustered regularly interspaced short palindromic repeats/Cas9 technology as described in Thielen et al.[E1]

Protein expression, purification, and analysis

Wild-type C2 cDNA sequence (accession number NM_000063) additionally containing 6 histidine codons at the 3′ terminus, as well as sequences for R129H, R243C, S250C, E318D, K415N, D417H, D511N, S574P, and Y347A variants were codon-optimized, synthesized, and cloned into a pCEP4 vector in the framework of GeneArt Gene Synthesis service by Thermo Fisher Scientific (Waltham, Mass). Proteins were expressed in a eukaryotic system and purified as described in Urban et al.[E2] Western blotting was developed with anti-C2 antibody (Complement Technology, Tyler, Tex) diluted 1:1,000, followed by horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody diluted 1:10,000 (Jackson ImmunoResearch, Cambridgeshire, United Kingdom).

Hemolysis-based assay

Hemolytic assays evaluating the activity of recombinant, his-tagged C2 mutants were performed as described previously,[E3] with some modifications. Sheep erythrocytes (Biomaxima, Gdańsk, Poland) were sensitized with amboceptor (Behring Bern, Switzerland) diluted 1:1000 in 1 mL of dextrose-gelatin-Veronal buffer ($DGVB^{+++}$) for 20 minutes at 37°C.[E3] Human erythrocytes were sensitized with anti-human red blood cells antibodies (Rockland, Limerick, Pa) diluted 1:75. Afterward, cells were washed 3 times, pelleted, and resuspended in 1 mL of DGVB++. Ten microliters of these suspensions were overlaid with serial dilutions of C2-depleted serum (ΔC2) (Complement Technology) in DGVB++ supplemented with the particular C2 mutant and incubated for 30 minutes at 37°C. Erythrocytes were centrifuged, and the hemoglobin released to the supernatant was measured at 405 nm in a Synergy H1 microplate reader (Biotek, Winooski, Vt). For the CP/LP convertase activity assay, sensitized erythrocytes were mixed with 2.5 μg/mL of the C5 inhibitor OmCI.[E3] The plate was incubated at 37°C, and 2% of the C2-depleted serum mixed with a particular C2 mutant diluted in DGVB++ was added at the indicated time points. Then, the erythrocytes were washed with EDTA–gelatin-Veronal buffer (GVB),[E3] pelleted, overlaid with EDTA-GVB containing 1:40 dilution of guinea pig serum (Harlan Laboratories, Itingen, Switzerland), and incubated for 30 minutes at 37°C. Lysis was determined by measuring the absorbance at 405 nm. The readout of the sample of erythrocytes mixed with water was considered as 100% (full) lysis.

CDC assay

Complement-dependent cytotoxicity (CDC) was measured by calcein release assay as described in Stasilojc et al,[E4] but ΔC2 serum was used instead of normal human serum. Cell lysis was calculated in reference to the fluorescence readout (490/520 nm) obtained for the supernatant of cells treated with 2% NP40 (full lysis).

CP/LP convertase assays on $CD20^+$ cells

Ofatumumab (100 μg/mL) and 30% of the C5- or C3-depleted serum (Complement Technology) supplemented with 7.5 μg/mL of the analyzed C2 variant were added to calcein-loaded cells at the indicated time points. Complement activation in these conditions was arrested at the stage of C3 CP/LP convertase or C5 CP/LP convertase, respectively. Then, cells were washed with EDTA-GVB buffer, pelleted, and overlaid with EDTA-GVB containing a 1:20 dilution of guinea pig serum followed by a 30-minute incubation (37°C, 600 *g*). Fluorescence readout was performed as already described in the section on CDC assay.

Cleavage of C2 with C1s enzyme

Each C2 mutant was incubated for 2 hours with 4 nM C1s enzyme (Complement Technology) diluted in 5 mM veronal buffer supplemented with 1 mM of Ca^{2+} and Mg^{2+}. Cleavage into C2a and C2b was analyzed by Western blot. C2 polyclonal goat anti-human antibody (No. A212, Complement Technology) diluted 1:1,000 and secondary donkey anti-goat antibody conjugated with HRP (Jackson ImmunoResearch) diluted 1:10,000 were used for detection, followed by 3,3' -diaminobenzidine (Vector Laboratories, Burlingame, Calif) applied for visualization.

C3b microplate deposition assay

ELISA plates were coated with 50 mg/mL of human immunoglobulin solution (Pentaglobin, Biotest) for 1 hour at 37°C and then blocked for 30 minutes with 3% fish gelatin (Sigma). Afterward, the plates were incubated with 0.5% ΔC2 serum and the particular C2 variant was diluted in GVB with or without the addition of soluble ectodomain of CD55 inhibitor (produced as described in Okroj et al[E5]) for 1 hour at 37°C. C3b detection was performed with polyclonal goat-anti human C3 antibody (Complement Technology), followed by rabbit anti-goat antibody conjugated with HRP (Dako, Glostrup, Denmark) diluted 1:10,000 and 1:5,000 in PBS, respectively. The assay was developed by using *o*-phenylenediamine dihydrochloride (Sigma Aldrich, Darmstadt, Germany) according to manufacturer's instructions.

Evaluation of complement regulatory proteins

Expression of CD35, CD46, and CD55 in $CD20^+$ cells and human erythrocytes was assessed by flow cytometry as described in Okroj et al.[E6] Primary antibodies (clone UJ11 for CD35, clone MEM-258 for CD46, and clone HI-55a for CD55) and isotype controls were purchased from Immunotools (Friesoythe, Germany).

Assessment of amino acid conservation in C2 protein

Amino acids sequences of C2 and FB proteins were retrieved from the UniProt[E7] amino acids sequence database searched for the term *complement C2*, and the results were further manually filtered out to obtain only sequences of C2 and FB proteins. For further analysis, 151 sequences were selected. All selected sequences were subjected to multiple sequence alignment by using a multiple alignment using fast Fourier transform algorithm.[E8]

Statistical analysis

Statistical calculations were performed with GraphPad Prism 7 software (GraphPad Software Inc, San Diego, Calif). The Kruskal-Wallis test was applied for column analysis (see Fig E3). The Dunnett multiple comparison test for nonrepeated measures was applied for grouped analysis (see Figs E1, E2, and E4-E7).

RELEVANCE AND LIMITATIONS OF OUR FINDING

Importance of clinical data analysis

The domain structure of FB and complement C2 share a high degree of similarity.[E10] The von Willenbrand type A domain of FB contains several mutational hot spots, alterations of which may provoke a potentially pathogenic gain-of-function phenotype.[E11,E12] We identified 1 rare variant of C2 with mutation at conserved position 250 adjacent to α-helixes forming the von Willenbrand type A domain, which leads to the formation of hyperactive CP/LP convertases. No previous studies have shown the importance of this position for the biologic activity of C2 or FB. Moreover, no prior studies have presented patients with gain-of-function mutations in complement genes coding for CP/LP convertase components. Thus, to the best of our knowledge, this is the first such report confirmed by functional studies. Genetic screening in patients in whom aHUS and C3G have been diagnosed is usually limited to genes encoding AP elements such as FB, CFH, C3, CFI, and CD46,[E13] and on the basis of this scheme, abnormalities in complement are found in 70% of patients with aHUS whereas 30% remain unexplained.[E14] Typically, patients with aHUS are heterozygous in complement mutations, and the same mutation can be carried by healthy relatives. Often, the difference between healthy relatives carrying pathogenic mutations and patients is the presence of predisposing polymorphisms in genes coding complement regulators.[E15-E17] Moreover, aHUS can be preceded by bacterial and viral infections, surgical procedures, use of certain medications, and pregnancy in women.[E18,E19] One of the limitations of our study is the lack of a medical record for the patient with aHUS who was carrying the S250C allele, which was not available. Such detailed clinical data, as well as the family history and a genotype/phenotype analysis of family members, would help to evaluate whether this case is similar to or different from cases of aHUS connected to gain-of-function mutations in FB or C3 proteins. On the other hand, it is highly plausible that overactive CP/LP convertases are responsible for the percentage of aHUS cases previously classified as being of unknown etiology.

Possible stimuli of CP/LP in S250C carriers and potential diagnostic/therapeutic directions

Unlike AP, CP and LP require specific stimuli (eg, antibodies, certain sugar moieties, or C-reactive protein.[E20] Therefore, 2 conditions permissive for CP/LP-mediated autoimmune disease are necessary, namely, loss of proper pathway regulation (eg, formation of decay-resistant CP/LP convertase) and presence of stimuli driving the pathway. Without a detailed clinical report, we can only speculate on a hypothetical explanation of disease development in the patient with the 250C mutation. Nonetheless, infections and presence of autoantibodies (or appearance of antibodies cross-reactive to patient cells and/or tissues) emerge as possible drivers of pathomechanism. In line with reports on C4NeF as the only complement abnormality found in patients with C3G,[E21,E22] we postulate that analysis of CP/LP convertase activity should be included in routine diagnostic procedures for use with patients with aHUS and C3G. Patients with known CP/LP gain-of-function mutations should be closely monitored during infections for first signs of aHUS/C3G so that therapy can be started as early as possible to limit renal damage. Also, therapeutic intervention in patients with gain-of-function mutations in LP/CP may theoretically target pathway stimuli (eg, production of C-reactive protein, source of autoantibodies) and become an alternative for eculizumab (Soliris), which is among the most expensive drugs.

Relevance of loss-of-function mutations in C2

Seven of 233 patients had mutations in C2 resulting in low CP hemolytic activity and no other rare variant in their complement genes (except for the carrier of the R243C mutation, which caused complete loss of CP activity) (Table I, Fig 1, *A* and *B*, and Fig E3). The causative nature of these variants for aHUS/C3G is less obvious than that of S250C. Because the low physiologic concentration of C2 makes it a bottleneck of CP/LP, such patients may have impaired clearance of bacteria. Indeed, the E318D mutation identified in 11 of 233 patients from our cohort was previously associated with an increased rate of pneumonia in patients with trauma.[E23] Infections with non–Shiga toxin-producing *Escherichia coli* (a causative factor of "typical" HUS)[E24] and strains such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa,* or *Bordetella pertussis*[E24-E28] often precede aHUS/CG3 episodes; however, genetic predisposition to infections and connotations of aHUS/C3G were not intensively investigated. Functional impairment of C2 may possibly influence this aspect, but this has to be confirmed by separate epidemiologic studies. Nonetheless, on the one hand, genetic C2 deficiency is among the most common complement deficiencies, with the occurrence of 1:20.000 homozygous carriers[E29]; on the other hand, there is a limited number of reports describing the prevalence of missense C2 mutants and no prior data on their functional characteristics.

Structure-function relationship of S250C mutant

Serine-to-cysteine substitution is considered to be one of the most conservative changes possible between any pair of amino acid residues in proteins, as they differ only at the γ-atom and have similar side-chain volumes.[E30] Therefore, the fact that S250C exchange has a profound impact on the function of C2 is surprising. However, there are studies elaborating on a bigger atomic radius of sulfur in a cysteine than the radius of oxygen in the serine and its impact on hydrogen-bonding propensities, hydrophobicity, and packing, all of which can trigger conformational changes in proteins and affect their biologic functions.[E30,E31] On the other hand, introduction of free cysteine at the surface of the protein can result in the formation of homodimers or heterodimers with other ubiquitous proteins, as well as in the

formation of adducts with low-molecular-weight chemical entities (eg, glutathione). Western blot analysis of S250C mutant under nonreducing conditions showed no obvious bands above the peak of the full-length protein (103 kDa [Fig E1]). Mass spectrometry analysis focused at the area between 102 and 104 kDa (data not shown) revealed the existence of 3 isoforms within the full length WT and S250C proteins (probably because of changes in glycosylation), thus making it difficult to establish a definitive conclusion regarding molecular weight differences.

REFERENCES

E1. Thielen AJF, van Baarsen IM, Jongsma ML, Zeerleder S, Spaapen RM, Wouters D. CRISPR/Cas9 generated human CD46, CD55 and CD59 knockout cell lines as a tool for complement research. J Immunol Methods 2018;456:15-22.
E2. Urban A, Borowska A, Felberg A, van den Heuvel L, Stasilojc G, Volokhina E, et al. Gain of function mutant of complement factor B K323E mimics pathogenic C3NeF autoantibodies in convertase assays. Autoimmunity 2018;51:18-24.
E3. Blom AM, Volokhina EB, Fransson V, Stromberg P, Berghard L, Viktorelius M, et al. A novel method for direct measurement of complement convertases activity in human serum. Clin Exp Immunol 2014;178:142-53.
E4. Stasilojc G, Felberg A, Urban A, Kowalska D, Ma S, Blom AM, et al. Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies. J Immunol Methods 2020;476:112675.
E5. Okroj M, Mark L, Stokowska A, Wong SW, Rose N, Blackbourn DJ, et al. Characterization of the complement inhibitory function of rhesus rhadinovirus complement control protein (RCP). J Biol Chem 2009;284:505-14.
E6. Okroj M, Eriksson I, Osterborg A, Blom AM. Killing of CLL and NHL cells by rituximab and ofatumumab under limited availability of complement. Med Oncol 2013;30:759.
E7. Boutet E, Lieberherr D, Tognolli M, Schneider M, Bansal P, Bridge AJ, et al. UniProtKB/Swiss-Prot, the manually annotated section of the UniProt KnowledgeBase: how to use the entry view. Methods Mol Biol 2016;1374:23-54.
E8. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol 2013;30:772-80.
E9. Milder FJ, Raaijmakers HC, Vandeputte MD, Schouten A, Huizinga EG, Romijn RA, et al. Structure of complement component C2A: implications for convertase formation and substrate binding. Structure 2006;14:1587-97.
E10. Krishnan V, Xu Y, Macon K, Volanakis JE, Narayana SV. The structure of C2b, a fragment of complement component C2 produced during C3 convertase formation. Acta Crystallogr D Biol Crystallogr 2009;65:266-74.
E11. Goicoechea de Jorge E, Harris CL, Esparza-Gordillo J, Carreras L, Arranz EA, Garrido CA, et al. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. Proc Natl Acad Sci U S A 2007;104:240-5.
E12. Marinozzi MC, Vergoz L, Rybkine T, Ngo S, Bettoni S, Pashov A, et al. Complement factor B mutations in atypical hemolytic uremic syndrome-disease-relevant or benign? J Am Soc Nephrol 2014;25:2053-65.
E13. Loirat C, Fremeaux-Bacchi V. Atypical hemolytic uremic syndrome. Orphanet J Rare Dis 2011;6:60.
E14. Joseph C, Gattineni J. Complement disorders and hemolytic uremic syndrome. Curr Opin Pediatr 2013;25:209-15.
E15. Noris M, Caprioli J, Bresin E, Mossali C, Pianetti G, Gamba S, et al. Relative role of genetic complement abnormalities in sporadic and familial aHUS and their impact on clinical phenotype. Clin J Am Soc Nephrol 2010;5:1844-59.
E16. Esparza-Gordillo J, Goicoechea de Jorge E, Buil A, Carreras Berges L, Lopez-Trascasa M, Sanchez-Corral P, et al. Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32. Hum Mol Genet 2005;14:703-12.
E17. Fremeaux-Bacchi V, Kemp EJ, Goodship JA, Dragon-Durey MA, Strain L, Loirat C, et al. The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and membrane cofactor protein: evidence from two independent cohorts. J Med Genet 2005;42:852-6.
E18. Nester CM, Barbour T, de Cordoba SR, Dragon-Durey MA, Fremeaux-Bacchi V, Goodship TH, et al. Atypical aHUS: state of the art. Mol Immunol 2015;67:31-42.
E19. Jokiranta TS. HUS and atypical HUS. Blood 2017;129:2847-56.
E20. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010;11:785-97.
E21. Blom AM, Corvillo F, Magda M, Stasilojc G, Nozal P, Perez-Valdivia MA, et al. Testing the activity of complement convertases in serum/plasma for diagnosis of C4NeF-mediated C3 glomerulonephritis. J Clin Immunol 2016;36:517-27.
E22. Zhang Y, Meyer NC, Fervenza FC, Lau W, Keenan A, Cara-Fuentes G, et al. C4 nephritic factors in C3 glomerulopathy: a case series. Am J Kidney Dis 2017;70:834-43.
E23. Morris JA Jr, Francois C, Olson PK, Cotton BA, Summar M, Jenkins JM, et al. Genetic variation in complement component 2 of the classical complement pathway is associated with increased mortality and infection: a study of 627 patients with trauma. J Trauma 2009;66:1265-70, [discussion: 70-2].
E24. Karpman D, Loos S, Tati R, Arvidsson I. Haemolytic uraemic syndrome. J Intern Med 2017;281:123-48.
E25. Westra D, Wetzels JF, Volokhina EB, van den Heuvel LP, van de Kar NC. A new era in the diagnosis and treatment of atypical haemolytic uraemic syndrome. Neth J Med 2012;70:121-9.
E26. Obando I, Camacho MS, Falcon-Neyra D, Hurtado-Mingo A, Neth O. Atypical hemolytic uremic syndrome associated with Bordetella pertussis infection. Pediatr Infect Dis J 2012;31:1210.
E27. Chaturvedi S, Licht C, Langlois V. Hemolytic uremic syndrome caused by Bordetella pertussis infection. Pediatr Nephrol 2010;25:1361-4.
E28. Pela I, Seracini D, Caprioli A, Castelletti F, Giammanco A. Hemolytic uremic syndrome in an infant following Bordetella pertussis infection. Eur J Clin Microbiol Infect Dis 2006;25:515-7.
E29. Truedsson L. Classical pathway deficiencies - a short analytical review. Mol Immunol 2015;68:14-9.
E30. Santos J, Risso VA, Sica MP, Ermacora MR. Effects of serine-to-cysteine mutations on beta-lactamase folding. Biophys J 2007;93:1707-18.
E31. He JJ, Quiocho FA. A nonconservative serine to cysteine mutation in the sulfate-binding protein, a transport receptor. Science 1991;251:1479-81.

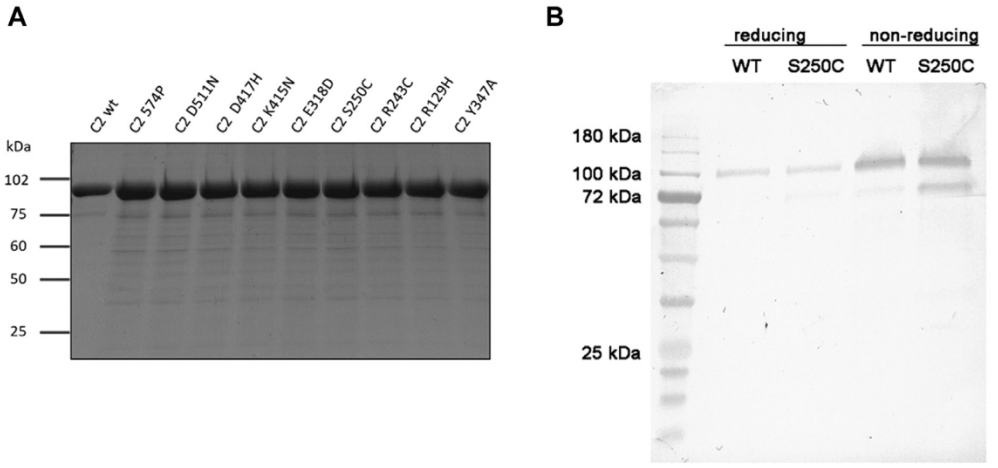

FIG E1. Purification of recombinant C2 variants. **A,** Purified C2 proteins were run on SDS-PAGE under reducing conditions and stained with Coomassie blue. Lanes were purposely overloaded to visualize impurities. **B,** Western blotting of purified WT and S250C C2 proteins under reducing and nonreducing conditions. Western blotting of S250C was performed to visualize any homodimers or heterodimers, which could arise as a result of introduction of cysteine. However, there were no additional bands above 100 kDa.

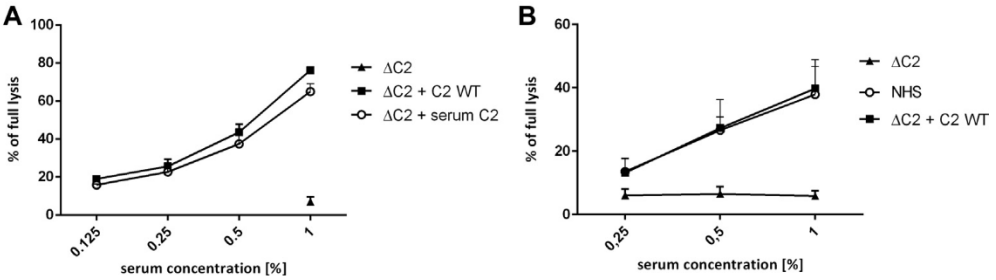

FIG E2. Assessment of his-tag influence on activity of C2 protein. **A,** Comparison of hemolytic activity of recombinant WT C2 protein and serum-purified C2. Proteins were added to C2-depleted serum (ΔC2) at their physiologic concentrations. Assay was performed in sensitized sheep erythrocytes. **B,** Comparison of hemolytic activity of ΔC2 serum reconstituted with recombinant WT C2 and normal human serum (NHS), as performed on sensitized human erythrocytes.

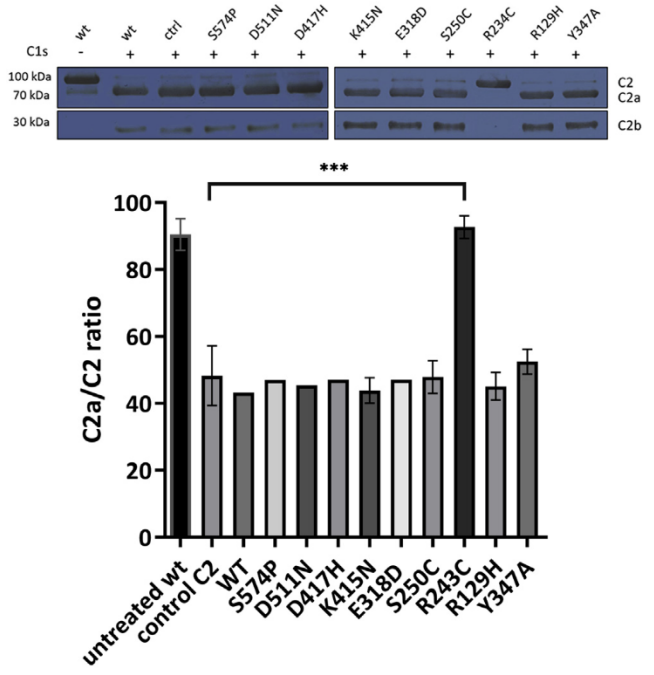

FIG E3. Cleavage of C2 variants by C1s enzyme. Representative Western blot (*top panel*) and associated densitometric analysis (*bottom panel*) present formation of C2a and C2b after a 2-hour incubation with 4 nM C1s of each C2 variant. The graph shows the results from 3 independent experiments. Triple asterisk denotes statistical significance versus plasma-purified C2 at a value of *P* less than .001 according to the Kruskal-Wallis test.

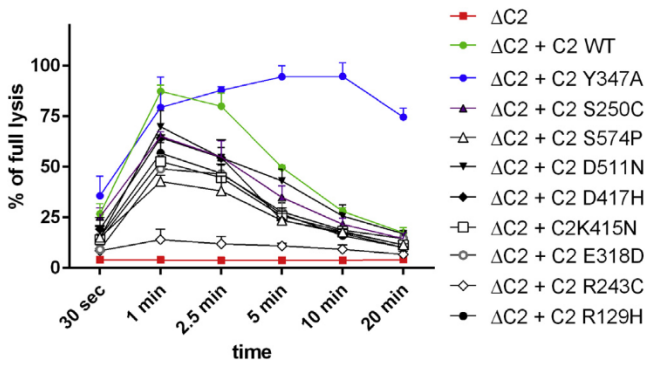

FIG E4. Convertase assay on sensitized sheep erythrocytes. CP/LP convertase activity assay performed on sensitized sheep erythrocytes mixed with ΔC2 serum supplemented with the particular C2 variant.

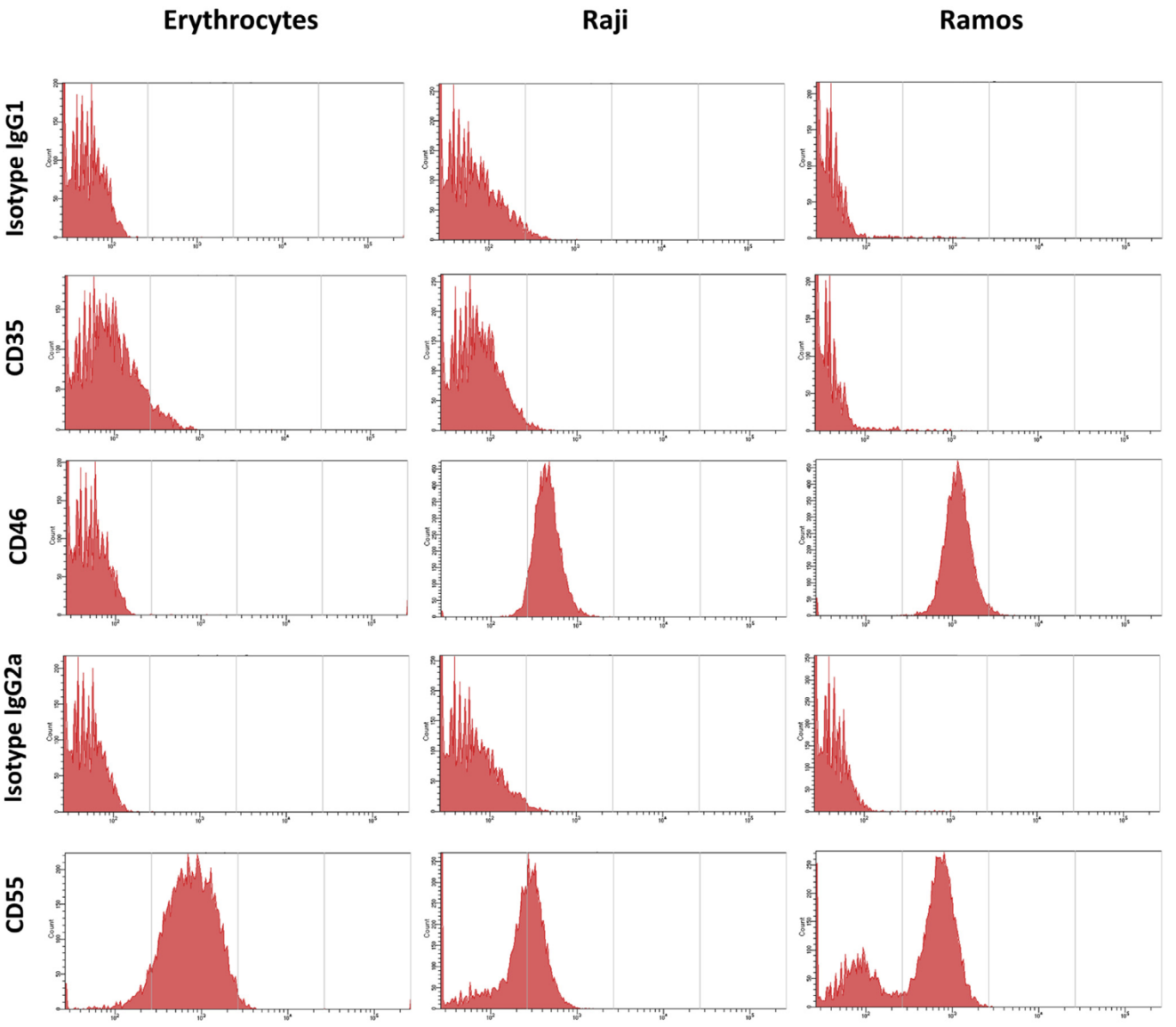

FIG E5. Expression of complement inhibitors on cell surface. Expression of CD35, CD46 (compared with isotype control mouse IgG1), and CD55 (compared with isotype control mouse IgG2a) on human erythrocytes, Raji cells, and Ramos cells.

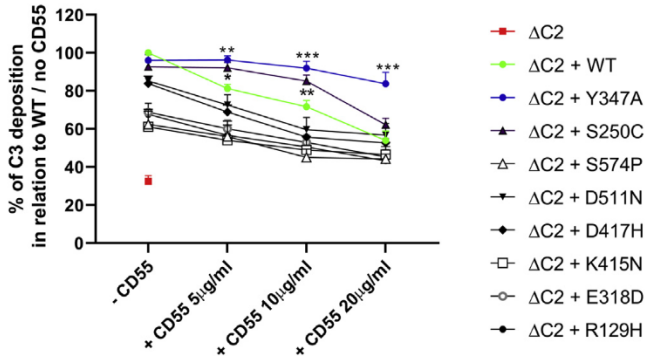

FIG E6. C3b deposition in the presence of soluble ectodomain of CD55. The graph presents C3b production and deposition on a microplate. Next, 0.5% of the ΔC2 serum supplemented with a particular C2 mutant was incubated for 1 hour on a plate coated with aggregated human IgG. Results show the level of C3b deposition relative to WT C2 with no soluble CD55 ectodomain added. Serum alone (ΔC2) was applied as a negative control. Double asterisks and triple asterisks denote statistical significance at *P* values of <.01 and <.0001, respectively, according to the Dunnett multiple comparison test for nonrepeated measures.

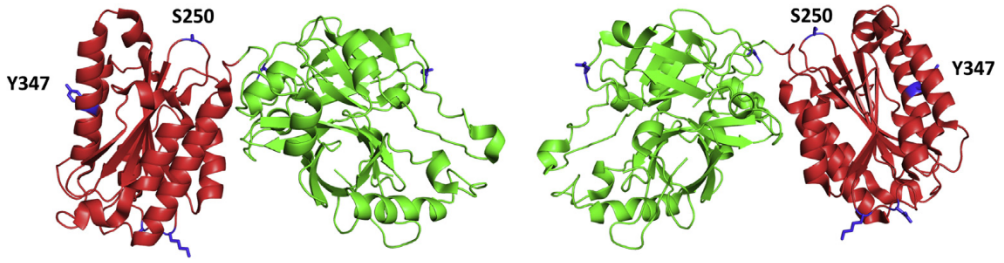

FIG E7. Model of C2a fragment. The structure (2 views) of C2b protein is based on Protein Data Bank record 2I6Q.[E9] The von Willebrand factor A-type domain is shown in red, and the C-terminal trypsin-like serine proteinase domain is shown in green. Positions of the mutated residues are shown in blue; residues S250 and Y347 are labeled.

Gain-of-function mutations R249C and S250C in complement C2 protein increase C3 deposition on glomerular endothelial cells in the presence of C-reactive protein

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The authors declare a potential conflict of interest and state it below

SRdeC performed genetic analyses of aHUS patients for Alexion in the framework of contract and obtained honoraria for lectures/presentations. Other authors have nothing to disclose.

Author contribution statement

Conceptual work: SRdC, MO
Experimental Work and data acquisition: AU, DK, AK, GS, AS, EA, MT, SO,
Preparation of crucial molecular tools / models: IJ, RS, SS
Patients' diagnosis: ECA, MÁFS
Writing and critical review of the manuscript: AU, DK, GS, IJ, RS, SS, SRdC, MO

Keywords

complement system, aHUS, C3 glomerulopathy, Complement C2, Endothelial Cells

Abstract

Word count: 127

Atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G) are rare kidney diseases fueled by the impairment of the alternative complement pathway. We recently described an aHUS patient carrying an exceptional gain-of-function (GoF) mutation (S250C) in the classical complement pathway component C2 leading to the formation of hyperactive classical convertases. We now report the identification of the same mutation and another C2 GoF mutation R249C in two patients with C3G. Both mutations stabilize the classical C3 convertases by a similar mechanism and result in increased deposition of C3 on glomerular endothelial cells exposed to human serum supplemented with C-reactive protein. Our data justify the inclusion of classical pathway genes in the aHUS/C3G genetic analysis and identify antibody-independent triggers of complement dysregulation in carriers of these C2 variants.

Contribution to the field

Several communications reported the presence of autoantibodies increasing the half-life of the classical complement convertase (C4NeF) in patients with MPGN, C3 glomerulopathy, and systemic lupus erythematosus. Considering these premises that the classical pathway can be involved in the pathogenesis of complement-related disorders, the lack of clinical data about mutations in this part of the complement cascade appears to be surprising. The reason is that standard diagnostics was so far focused on the alternative pathway components and omitted proteins of the classical pathway, mostly because the classical pathway needs a specific stimulus, and therefore the direct pathogenic character of mutations leading to the loss of pathway regulation was not considered obvious. Herein, we provide evidence that genetic analyses of the classical pathway components should be included in the routine diagnostics of patients with rare kidney diseases. We also demonstrate a possible mechanism of pathogenesis driven by non-immunoglobulin stimuli like acute-phase proteins.

Funding statement

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Gain-of-function mutations R249C and S250C in complement C2 protein increase C3 deposition on glomerular endothelial cells in the presence of C-reactive protein.

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Abstract

Atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G) are rare kidney diseases fueled by the impairment of the alternative complement pathway. We recently described an aHUS patient carrying an exceptional gain-of-function (GoF) mutation (S250C) in the classical complement pathway component C2 leading to the formation of hyperactive classical convertases. We now report the identification of the same mutation and another C2 GoF mutation R249C in two patients with C3G. Both mutations stabilize the classical C3 convertases by a similar mechanism and result in increased deposition of C3 on glomerular endothelial cells exposed to human serum supplemented with C-reactive protein. Our data justify the inclusion of classical pathway genes in the aHUS/C3G genetic analysis and identify antibody-independent triggers of complement dysregulation in carriers of these C2 variants.

Keywords

Complement system, aHUS, C3 glomerulopathy, complement C2, endothelial cells

INTRODUCTION

The etiology of rare kidney diseases such as C3 glomerulopathy (C3G) and atypical hemolytic uremic syndrome (aHUS) involves dysregulation of the complement system [1]. The common etiologic factor is impairment of proteins engaged in the alternative complement pathway (AP), as this route is constantly active at a low level, and its further propagation depends on endogenous inhibition on self surfaces. Conversely, the classical and lectin complement pathways (CP/LP) need specific stimuli, and therefore loss of pathway regulation may not be sufficient for the occurrence of pathology. Elements of AP but not CP/LP are included in routine genetic diagnostics of glomerulopathies. Previously, our group identified the first-ever gain-of-function (GoF) mutation in the CP/LP component, C2, in an aHUS patient [2]. Substitution of serine 250 to cysteine renders CP/LP convertase insensitive to regulation by CD55 complement inhibitor, which significantly increases generation and deposition of C3 on target cells. Heterozygous S250C mutation in C2 was the only complement pathogenic variant found in this patient, who also carried the homozygous risk polymorphism in the promoter region of the *MCP* gene that encodes membrane-bound complement inhibitor CD46 [2, 3]. However, the mechanism that triggers the pathogenic scenario in the S250C mutation carrier remains unknown. Herein, we report the identification of the S250C mutation and another C2 GoF mutation, R249C, adjacent to the S250C mutation, in two C3G patients. To investigate the role of R249C and S250C C2 proteins in the development of C3G, we have used immortalized glomerular endothelial cell cultures. We show that both GoF C2 variants increase the deposition of C3b in the presence of C-reactive protein (CRP), an acute phase protein that elevates its concentration in plasma up to 1000 times upon infection and/or inflammation [4].

METHODS

Cells

The human lymphoma cell lines Raji and Ramos (both obtained from the American Type Culture Collection, ATCC) were cultured in RPMI 1640 medium with l-glutamine (ATCC) supplemented with 10% FBS (ATCC). Cells were cultivated at 37°C and in humidified 5% CO_2 atmosphere. Raji cells with CD55 knockout were produced by clustered regularly interspaced short palindromic repeats/Cas9 technology as described in [5]. Immortalized human glomerular endothelial cells (iGEnC) [6] were cultured in EGM2-MV medium (Lonza) at 33°C to activate the temperature-sensitive SV40LT transgene. Before the experiments cells were transferred them to 37°C and kept for five days to ensure the transgene is inactive.

Expression and purification of C2 variants.

All C2 variants used in the current study were produced in HEK293 Freestyle cells (ThermoFisher) as described in [7] and [2]. Similarly to WT and S250C variants previously described, cDNA coding R249C sequence was codon-optimized, codons for six histidine residues were added at 3' and all matrice was synthesized in the framework of GeneArt Synthesis® service by ThermoFisher. Conctruct was cloned into pCEP4 vector and transfected using Freestyle Max reagent (ThermoFisher). One microgram of C2 R249C protein was separated in 12% poliacrylamid gel electrophoresis in reducing conditions. Laemmli buffer was used for sample loading and Coomassie Brilliant blue for gel staining.

Patients

Information about rare genetic variants in THBD, DGKE, *C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C4A, C4BPA, C4BPB, C5, C7, C8A, C8B, C8G, C9, CD46, CD55, CD59, CFB, CFD, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFP, CLU, CR1, CR2, FCN1, FCN2, FCN3, ITGAX, ITGB2, MASP1, MASP2, MBL2, SERPING1,*

VSIG4, and *VTN* genes among aHUS and C3G patients were retrieved from the Spanish aHUS/C3G Registry (https://www.aHUSC3G.es). Diagnostics criteria and detailed methodology of next generation sequencing with following data analyses were described in details in [2].

CDC and classical convertase assays

Complement-dependent cytotoxicity (CDC) assay was performed in Raji cells as described in [8]. Briefly, cells were loaded with 1mM calcein-AM (Sigma) for 30 min at 37°C. Then, cells were washed twice with PBS buffer, plated onto V-shape 96-wells plate in the amount 1 x 10^5 cells/well and overlaid with 25 µl of anti-CD20 antibody ofatumumab (50 µg/ml) and 25 µl of ΔC2 serum (final concentrations 5%, 2.5% and 1.25%) supplemented with physiological concentrations of C2 WT, R249C or S250C mutant. After 30 min of incubation at 37°C, fluorescence of calcein released into supernatant was measured with Synergy H1 microplate reader (BioTek). Percentage of cell lysis was calculated in reference to fluorescence level obtained for cells incubated with 30% DMSO (full lysis control).

Ramos cells were used for classical convertase assays, as in [2]. Cells were loaded with calcein-AM as in CDC assay, then treated overlayed with ofatumumab (50 µg/ml) and 10 % C3-depleted (ΔC3) or C5-depleted (ΔC5) serum (Complement Technology) supplemented with physiological concentration of C2 variants (WT, R249C and S250C). Convertase formation was stopped by the addition of EDTA GVB++ buffer at indicated time points (15 s, 30 s, 1 min, 2.5 min, 5 min and 10 min for C3 convertase; 1 min, 2.5 min, 5 min, 10 min and 20 min for C5 convertase). Cells were washed and suspended in 5% guinea pig serum (Harlan Laboratories) in EDTA GVB++ and incubated for 30 min at 37°C with shaking. The readout and calculation of percentage of cell lysis were performed as in CDC assay. Heat-inactivated normal human serum (ΔNHS) served as a negative control.

Fluorescent microscopy and flow cytometry analysis of iGEnCs

iGEnCs were seeded on 24-wells plate until reached the full confluency and incubated with serum-free medium overnight. Staining for C3b was performed as described in [4] with a few changes. Cells were incubated for 30 min at 37°C with 10% NHS and 150 µg/ml of CRP, supplemented with a physiological concentration of a particular C2 variant. Detection of C3b and CD31 was conducted with anti C3c-FITC (Dako)/CD31-PE antibodies (Sigma-Aldrich) diluted 1:200 in 0.1% BSA in PBS for 30 min in 4°C. Cells were washed with PBS and either gently trypsinized to be analyzed by flow cytometry using CytoFLEX (Beckman Coulter) or left on the plate and overlaid with mounting medium with TRITC-Phalloidin (Vectashield) for fluorescent microscopy imaging (Olympus IX83).

C3b deposition measurement by ELISA

ELISA plates (Nunc MaxiSorp ™, ThermoFisher) were coated with 1, 0.75, 0.5 and 0.25 µg/mL of human CRP (Sigma) over night at 4°C and then blocked for 1h with 3% BSA (Sigma). Afterward, the plates were incubated with 0.25% NHS and the particular C2 variant was diluted in GVB buffer and incubated for 30 min at 37°C with mild shaking. C3b protein (Complement Technology) serially diluted in NHS and coated on the plate instead of CRP was used for the preparation of the standard curve. C3b detection was performed with polyclonal goat-anti human C3 antibody (Complement Technology), followed by rabbit anti-goat antibody conjugated with HRP (Dako, Glostrup, Denmark) diluted 1:10,000 and 1:5,000 in PBS, respectively. The assay was developed by using 3,3',5,5'-Tetramethylbenzidine (Sigma Aldrich) according to manufacturer's instructions.

Molecular modeling and bioinformatics analysis.

Three-dimensional model of C2 protein was build using SWISS-MODEL programme [9] using structure of Factor B protein as a template (PDB code 2XWB) [10]. For assessing amino frequency occurrence in position 249 we use the same methods and data set as it is described in reference [2]. Amino acids

sequences of C2 and FB proteins were retrieved from the UniProt amino acids sequence database searched for the term *complement C2*, and the results were further manually filtered out to obtain only sequences of C2 and FB proteins. For further analysis, 151 sequences were selected. All selected sequences were subjected to multiple sequence alignment by using a multiple alignment using fast Fourier transform algorithm.

RESULTS

Clinical synopsis of C3G patients with R249C and S250C mutations and additional genetic data are described in the supplementary material section and in Table S1.

Recombinant, His-tagged wild type (WT), S250C, and R249C C2 variants were purified as shown in [2] and Fig.S1. Functional assays testing the activity of C2 proteins revealed that S250C and R249C exerted significantly higher CDC than WT C2 (Fig.1A). CP/LP C3 convertase formed in the presence of each mutated C2 variant showed prolonged activity compared to the enzyme formed upon serum supplementation with WT protein (Fig.1B) whereas C5 convertase showed either higher amplitude or prolonged activity (Fig.1C). Notably, the GoF character of the R249C mutation was more pronounced than that of S250C. The differences in CDC between the R249C and S250C mutants and the WT protein were eliminated by using Raji cells devoid of CD55 complement inhibitor as a target (Fig.S2), which illustrates that convertase stability is an important factor for the observed phenotype.

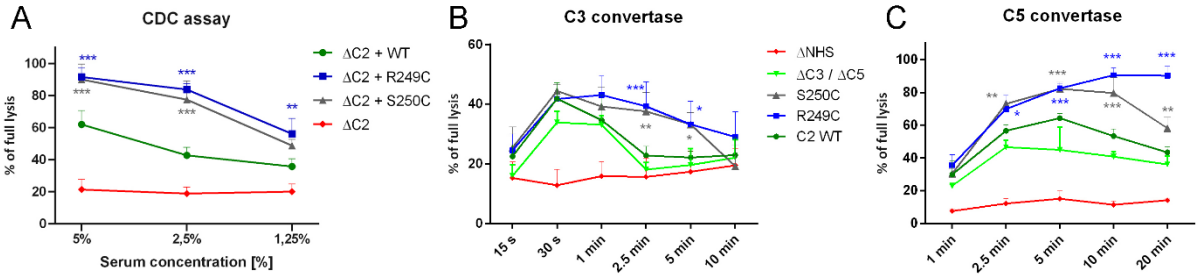


Fig. 1 Functional assays of C2 variants.

A) CDC assay. Calcein-loaded Raji cells sensitized with anti-CD20 mAb (ofatumumab) were suspended in C2-depleted serum (ΔC2) supplemented with physiological concentration of C2 protein. Cells were incubated for 30 min. at 37 °C. Supernatant was collected and fluorescence of released calcein was measured. The readout obtained for cells lysed with 30% DMSO was considered as full lysis and the readout obtained for cells incubated with ΔC2 serum alone indicated background lysis (negative control). B and C) Convertase activity assays for classical C3 convertase (B) and classical C5 convertase (C) were performed on calcein-loaded Ramos cells suspended in 15% of C3- depleted serum (ΔC3) (B) or C5- depleted serum (ΔC5) (C) supplemented with physiological concentration of C2 variants. After indicated time period cells were washed with EDTA-containing buffer to disable further convertase formation and then suspended in 5% guinea pig serum diluted in the same EDTA buffer. The readout was performed as in CDC assay but heat-inactivated serum (ΔNHS) instead of ΔC2 served as a negative control. Data are collected from at least 3 experiments. Symbols *, ** and *** denote statistically significant differences vs. WT supplementation, at p levels of 0.05, 0.01 and 0.001 according to Dunn's multiple comparison test.

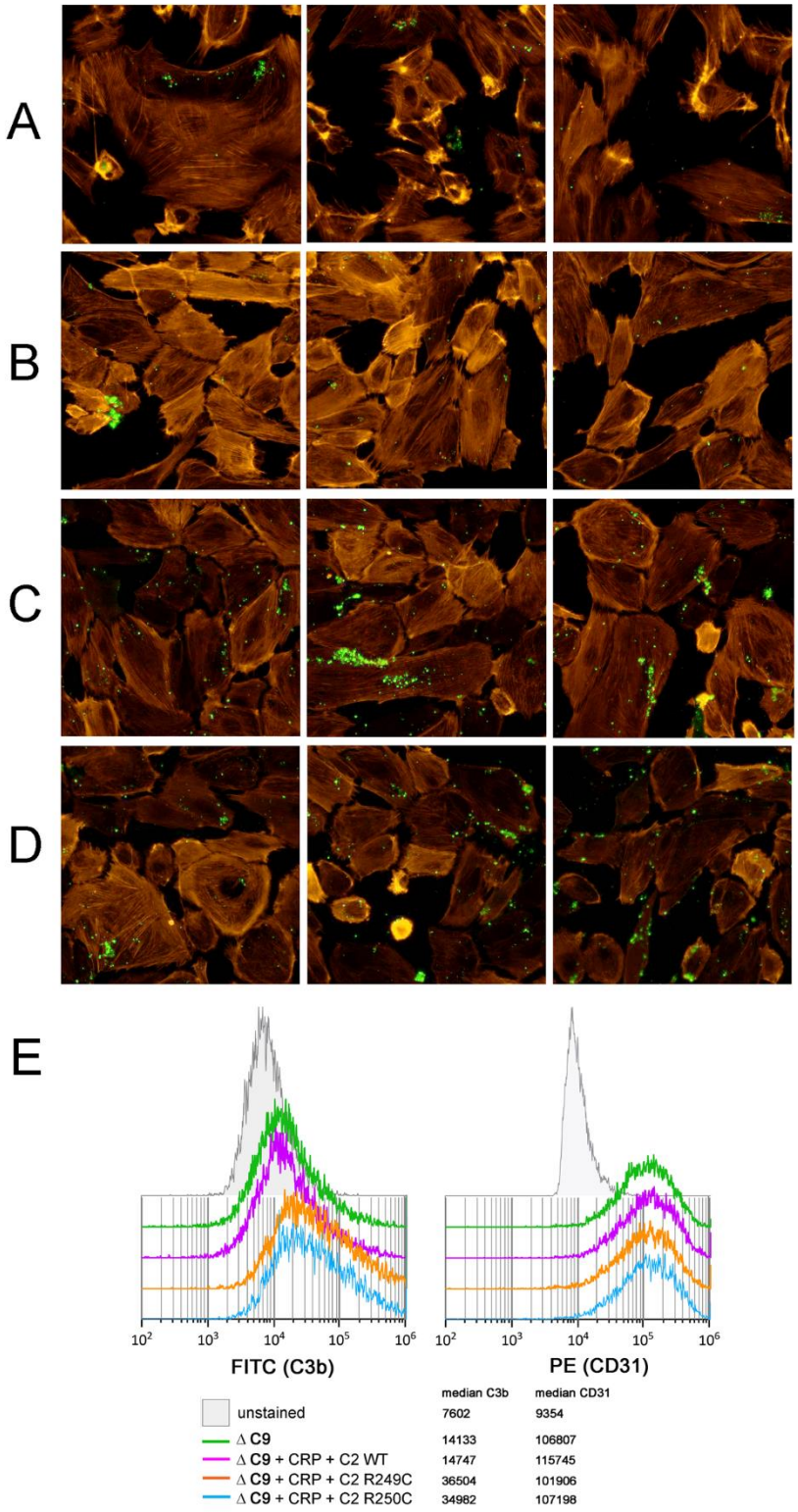


Fig. 2 C3b deposition on glomerular endothelial cells.

Immortalized glomerular endothelial cells were seeded onto glass chambers and exposed to C9-depleted human serum (ΔC9) in order to prevent lysis. Deposition of C3b (green) was analyzed by fluorescent microscopy (A-D) when nothing (A), CRP + WT C2 (B), CRP + R249C variant (C) or CRP + S250C variant (D) was added. A labeled phalloidin (orange) was used as a counterstaining. Three independent images are shown for each condition. E) Alternatively, for quantitative analysis, cells were harvested and examined by flow cytometry for both C3b deposition (left histogram) and CD31 expression (right histogram). Results show ten thousand events, previously gated to eliminate cell doublets and cell debris.

The addition of either S250C or R249C C2 variants, but not of WT C2, to serum supplemented with C-reactive protein (CRP) significantly increased the deposition of C3 on the surface of glomerular endothelial cells. Fluorescent microscopy revealed larger and brighter C3 deposits (Fig.2A-D), which were confirmed quantitatively by flow cytometry (Fig.2E). To find out the mechanistic insight of this phenomenon, we coated ELISA plate with increasing concentrations of CRP and overlaid it with normal human serum +/- C2 WT or R249C or S250C variants. A threshold of CRP coating was observed, beyond which the rate of C3 deposition in GoF C2 variant-containing sera increased significantly higher than in non-supplemented serum or serum supplemented with WT C2 (Fig. S3).

Molecular modeling, based on the available structural data indicates that the C2a domain undergoes spatial reorganization upon dissociation of the C2b domain (Fig. 3), as previously suggested by others [11]. The importance of the region including the mutated residues for the function of C2 protein is emphasized by the strong conservation of residues occurring at positions 250 [2] and 249 (see Table S2). In the structure of the complete C2 protein, the 243-250 region is unstructured and exposed to solvent, possibly to facilitate access of the C1s protease. Upon cleavage, this fragment is re-located inside the von Willebrand factor A domain (VWA) domain of C2a, taking the place previously occupied by the C-terminal helix of the C2b domain (Fig.3).

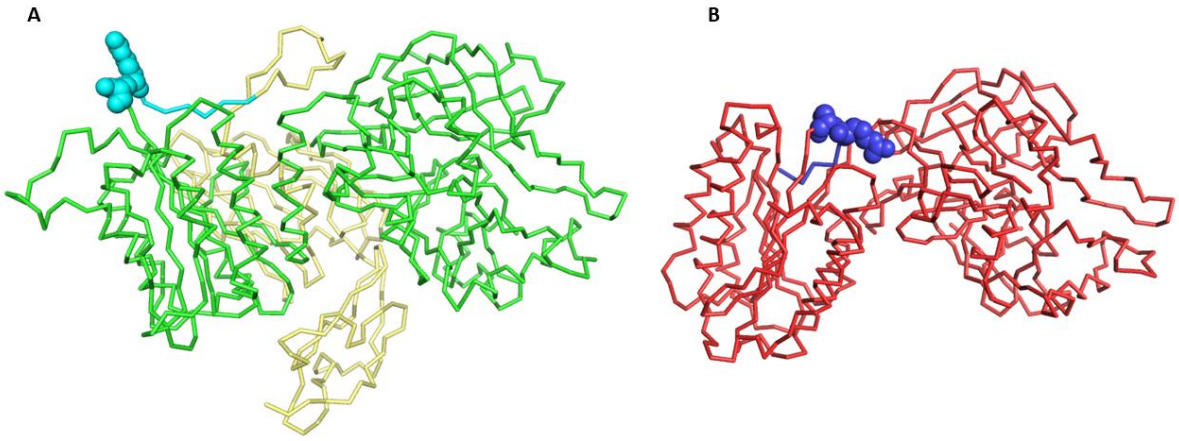


Fig. 3 Molecular modeling of C2 and C2b.
A)Three-dimensional model of full C2 sequence build by homology modeling using Factor B structure (PDB code 2XWB) as a template. C2b domain in yellow, C2a domain in green, residues 243-250 in light blue, residues 249 and 250 are shown in spheres representation. B) Three-dimensional structure of C2a (PDB code 2I6Q). Residues 243-250 in dark blue, residues 249 and 250 are shown in spheres representation. Both structures are shown in the same orientation with VWA domain on the left and SP domain on the right side of each structure, respectively.

DISCUSSION

In contrast to mutations in AP convertase components, the causative role of mutations in their CP/LP counterparts in the course of complement-mediated renal diseases is not straightforward. Similar to the case of the aHUS patient carrying the S250C mutation in C2 [2], there are reports of C4NeF autoantibodies stabilizing CP/LP convertases as a sole, potentially pathogenic factor identified in patients with C3 glomerulopathies [12, 13]. However, the information about specific triggers that could initiate CP or LP (e.g. other autoantibodies, elevated level of pentraxins [14, 15]) was not available in the abovementioned cases. In the current study, we report a new C2 GoF mutation R249C in a C3G without anti-FH, C3NeF, and most common autoantibodies. This finding imposed the question of whether non-Ig stimuli can act as a driver of the excessive glomerular C3 deposition (including the subendothelial

space), which is characteristic in C3G-affected individuals [16], as well as for the C3 deposition in the renal microvascular endothelium cell lining in aHUS patients [17, 18]. We chose to study the impact of CRP, as this acute phase protein may directly interact with endothelial cells [19, 20] and has the ability of direct CP activation by binding C1q [4]. The observation that aHUS is often preceded by bacterial infections [21] and that a substantial percentage of C3G patients presents with high titers of antistreptolysin-O [22] make CRP a strong candidate for being a trigger of complement dysregulation in carriers of C2 GoF mutations. Indeed, our data reveal the possibility that combined action of CRP and GoF C2 mutations results in elevated complement deposition in kidney microvasculature and as such grounds a pathogenic process. Our *in vitro* experiment demonstrated that the amounts of C3 deposited upon CP stimulation by CRP are significantly higher when the convertases are formed by GoF C2 variants.

Here we provided evidence that two C2 mutations associated with aHUS and C3G, which are located in the short motif located between C2a/C2b cleavage site (residue 243) and VWA domain (residues 254-452), result in the formation of the more stable and processive CP/LP convertases. Based on the large numbers of identified GoF variants in AP converstase components (factor B, C3) associated with aHUS and C3G [3, 7, 18, 23] and the substantial similarity between AP and CP/LP convertases components (C2, C4) this should not be surprising, but has only recently being suggested [24]. An important conclusion of our studies is that routine genetic diagnostic in patients with a clinical diagnosis of aHUS and C3G must include the genes encoding the CP/LP components. Assays detecting C4NeF, which are not as common as C3NeF assays, should also be included in the analysis of C3G patients [24].

One of our C3G patients was retrospectively diagnosed with ANA autoantibodies, which could theoretically drive the CP activation. However, we also report that acute phase proteins that initiate CP in an Ig-independent way may trigger complement dysregulation in carriers of the GoF C2 variants. Confirmation of the aforementioned scenario and identification of more mutational hotspots in C2 may bring new directions in the management of such C3G and aHUS cases, e.g. eradication of microbial pathogens or autoreactive B cell clones as well as close monitoring for inflammatory mediators or increased titers of autoantibodies. In the context of a precision medicine, our understanding of the role of CP/LP dysregulation in the etiology of aHUS and C3G would suggest that these cases might be better treated with CP inhibitors, rather than C3 or C5 inhibitors.

Disclosure statement

SRdeC performed genetic analyses of aHUS patients for Alexion in the framework of contract and obtained honoraria for lectures/presentations. Other authors have nothing to disclose.

Acknowledgments

The project was funded by National Science Centre Poland grants no. 2015/18/M/NZ6/00334 and 2018/29/N/NZ6/01413. SRdC was supported by grants from the Spanish Ministerio de Economía y Competitividad-FEDER (PID2019-104912RB-I00) and Autonomous Region of Madrid (S2017/BMD-3673).Computational resources used in this project were provided by the Informatics Center of the Metropolitan Academic Network (IC MAN-TASK) in Gdańsk.

References

1. Wong EKS, Kavanagh D. Diseases of complement dysregulation-an overview. *Semin Immunopathol* 2018; **40:** 49-64.
2. Urban A, Volokhina E, Felberg A*, et al.* Gain-of-function mutation in complement C2 protein identified in a patient with aHUS. *J Allergy Clin Immunol* 2020; **146:** 916-919 e911.
3. Goicoechea de Jorge E, Harris CL, Esparza-Gordillo J*, et al.* Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* 2007; **104:** 240-245.

4. Sproston NR, Ashworth JJ. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol* 2018; **9:** 754.
5. Thielen AJF, van Baarsen IM, Jongsma ML*, et al.* CRISPR/Cas9 generated human CD46, CD55 and CD59 knockout cell lines as a tool for complement research. *J Immunol Methods* 2018; **456:** 15-22.
6. Satchell SC, Tasman CH, Singh A*, et al.* Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney Int* 2006; **69:** 1633-1640.
7. Urban A, Borowska A, Felberg A*, et al.* Gain of function mutant of complement factor B K323E mimics pathogenic C3NeF autoantibodies in convertase assays. *Autoimmunity* 2018; **51:** 18-24.
8. Stasilojc G, Felberg A, Urban A*, et al.* Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies. *J Immunol Methods* 2020; **476:** 112675.
9. Waterhouse A, Bertoni M, Bienert S*, et al.* SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res* 2018; **46:** W296-W303.
10. Forneris F, Ricklin D, Wu J*, et al.* Structures of C3b in complex with factors B and D give insight into complement convertase formation. *Science* 2010; **330:** 1816-1820.
11. Milder FJ, Raaijmakers HC, Vandeputte MD*, et al.* Structure of complement component C2A: implications for convertase formation and substrate binding. *Structure* 2006; **14:** 1587-1597.
12. Blom AM, Corvillo F, Magda M*, et al.* Testing the Activity of Complement Convertases in Serum/Plasma for Diagnosis of C4NeF-Mediated C3 Glomerulonephritis. *J Clin Immunol* 2016; **36:** 517-527.
13. Zhang Y, Meyer NC, Fervenza FC*, et al.* C4 Nephritic Factors in C3 Glomerulopathy: A Case Series. *Am J Kidney Dis* 2017; **70:** 834-843.
14. Ricklin D, Hajishengallis G, Yang K*, et al.* Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010; **11:** 785-797.
15. Haapasalo K, Meri S. Regulation of the Complement System by Pentraxins. *Front Immunol* 2019; **10:** 1750.
16. Cook HT, Pickering MC. Histopathology of MPGN and C3 glomerulopathies. *Nat Rev Nephrol* 2015; **11:** 14-22.
17. Noris M, Galbusera M, Gastoldi S*, et al.* Dynamics of complement activation in aHUS and how to monitor eculizumab therapy. *Blood* 2014; **124:** 1715-1726.
18. Roumenina LT, Jablonski M, Hue C*, et al.* Hyperfunctional C3 convertase leads to complement deposition on endothelial cells and contributes to atypical hemolytic uremic syndrome. *Blood* 2009; **114:** 2837-2845.
19. Stenvinkel P, Lindholm B. C-reactive protein in end-stage renal disease: are there reasons to measure it? *Blood Purif* 2005; **23:** 72-78.
20. Devaraj S, Du Clos TW, Jialal I. Binding and internalization of C-reactive protein by Fcgamma receptors on human aortic endothelial cells mediates biological effects. *Arterioscler Thromb Vasc Biol* 2005; **25:** 1359-1363.
21. Obando I, Camacho MS, Falcon-Neyra D*, et al.* Atypical hemolytic uremic syndrome associated with Bordetella pertussis infection. *Pediatr Infect Dis J* 2012; **31:** 1210.
22. Medjeral-Thomas NR, O'Shaughnessy MM, O'Regan JA*, et al.* C3 glomerulopathy: clinicopathologic features and predictors of outcome. *Clin J Am Soc Nephrol* 2014; **9:** 46-53.
23. de Cordoba SR. Complement genetics and susceptibility to inflammatory disease. Lessons from genotype-phenotype correlations. *Immunobiology* 2016; **221:** 709-714.
24. Nilsson B, Ekdahl KN. Complement diagnostics: concepts, indications, and practical guidelines. *Clin Dev Immunol* 2012; **2012:** 962702.

SUPPLEMENTARY RESULTS

Table S1 Characteristics of patients with identified GoF mutations in C2

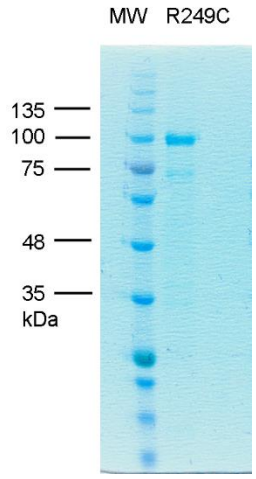
Position on chromosome 6	31901972	31901976	31901976
ID	rs771081314	rs150827255	rs150827255
cDNA substitution	c.C745T	c.C749G	c. C749G
Amino acid substitution	p.R249C	p.R249C	p.R249C
Diagnosis of patients with mutation	C3GN	C3GN	aHUS *
Genotype	HET	HET	HET
MCP risk polymorphism	NO	HET	HOM
CFH risk polymorphism	HET	NO	NO
Other potential pathogenic complement genetic variants	p.S115F:C1S:HET; p.D315N:C1S:HET	None	None
Anti FH antibodies	No	No	-
C3Nef	No	-	-
Autoantibodies	No	ANA #	-

HET – heterozygous, HOM – homozygous, ANA – anti-nuclear antibodies
, * - patient reported by *Urban et al.*,
- ANA were confirmed 6 years after the initial admission

Table S2. **Occurrence (in percent) of the most frequent amino acid types in selected positions**

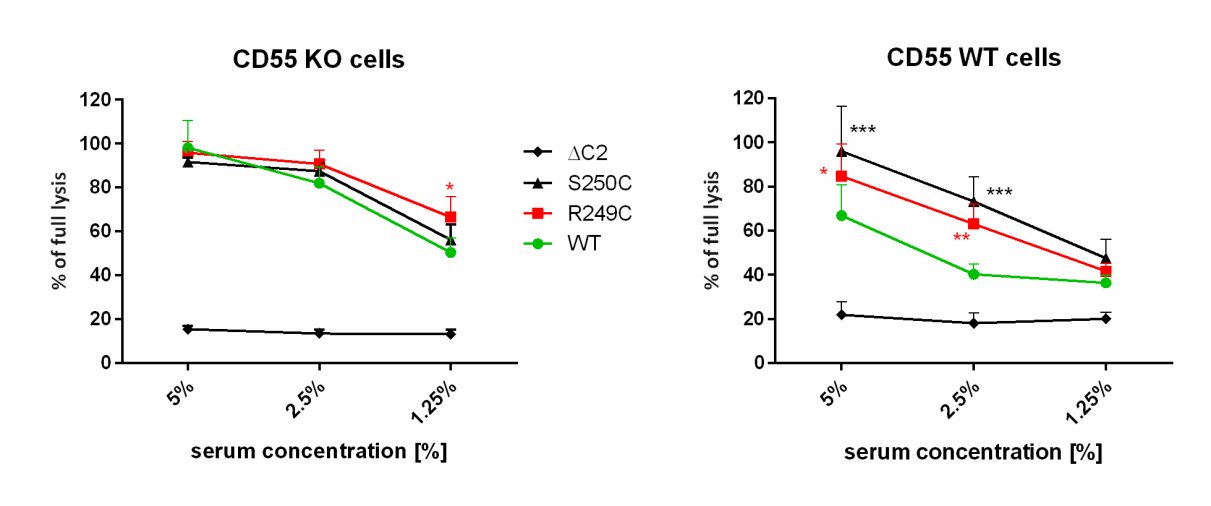
Amino acid position in C2 protein (Homo Sapiens)	Amino acid type occurrence	Remarks
250	S>99%	Data from reference 3
249	R 60%, K 3%	Observed mostly for C2 amino acid sequences
	P 30%	Observed mostly for Factor B amino acid sequence.

Fig. S1 Purification of R249C variant



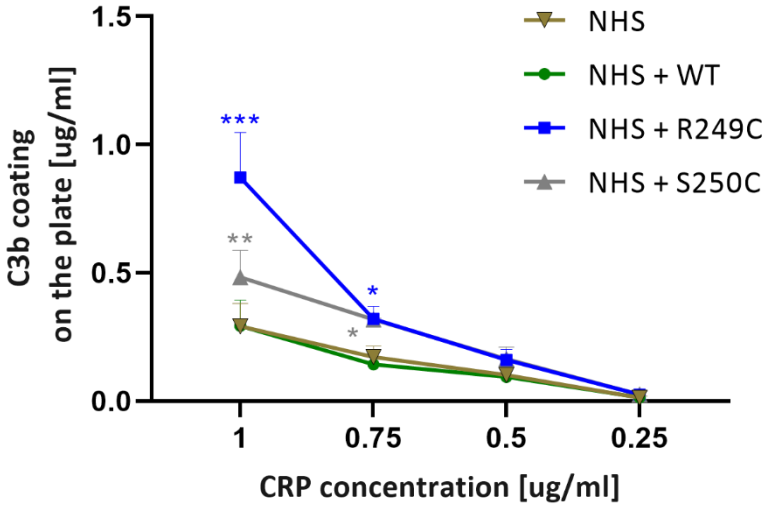
Purified R249C C2 variant was run on SDS-PAGE under reducing conditions and stained with Coomassie blue. Faint band below 70 kDa corresponds to C2a fragment, which occurred during spontaneous degradation, as concluded from Western Blotting (not shown).

Fig. S2 CDC assay on CD55 wild-type (WT) and CD55 KO Raji cells.



Raji CD55-knockout cells and control Raji cells were used for complement-dependent cytotoxicity (CDC) assay in ΔC2 serum supplemented with selected C2 mutants. All graphs show the results obtained from three independent experiments. Statistical significance of differences between the given C2 variant and the WT was analyzed by using the Dunnett multiple comparison test for non-repeated measures. *P < 0.05; **$P < 0.01$; ***$P < 0.001$.

Fig. S3 C3b deposition assay in the presence of CRP



Increasing concentrations of C-reactive protein (CRP) were coated onto ELISA microplate and overlaid with 0.25% normal human serum (NHS) +/- C2 variants. After 30 min incubation C3b deposition was detected by anti-C3b antibody. Purified C3b directly coated on the plate was used as a standard. Differences between the given C2 variant and the WT was analyzed by using the Dunnett multiple comparison test for non-repeated measures. *P < 0.05; **P < 0.01; ***P < 0.001.

Clinical synopsis of the C3G patient carrying the C2 p.S250C variant

He is a 51y-old male, with a diagnosis of Type 2 diabetes, who in 2010 was admitted to the clinic with a proteinuria in the nephrotic range that evolved to chronic renal insufficiency. He had no familial history of renal disease. Studies performed in 2016 showed a normal complement profile with a positive for anti-nuclear antibodies, but no anti DNA antibodies were detected.

A biopsy was performed with about 15 glomeruli useful for diagnosis, 8 of them were sclerosed in wafer-like shape. In the rest, an increase in global and diffuse mesangial matrix stand out, with mild mesangial hypercellularity and presence of segmental sclerosis with capsular adhesion affecting the vascular pole in 3 glomeruli. Occasional double contours were observed. No endocapillary or extracapillary proliferation was observed. Staining for Congo Red was negative. In the interstitium there was an extensive fibrosis affecting 40% of the cylinder with accompanying tubular atrophy. Three vascular sections with moderate intimal fibrosis were identified. Cryostat sections incubated with anti-IgG, anti-IgA, anti-IgM, anti-C1q, anti-C3, kappa and lambda sera labeled with fluorescence. Global and diffuse, linear deposits were observed in handles and mesangium with zones of subendothelial pattern for C3 (++) and kappa (++) and lower intensity for lambda (+) and IgM (+). Immunofluorescence was negative with the other antisera. Overall, it was a biopsy in which different lesions were observed. There was a clear predominance of nonspecific chronic changes, with 53% glomerular sclerosis and 40% of interstitial and tubular fibrosis and an important arteriosclerotic atrophy. Additionally, lesions of diffuse intercapillary glomerulosclerosis, of nephroangiosclerosis, focal and segmental glomerulosclerosis, as well as immune deposits compatible with C3 nephropathy were observed. The patient experienced a progressive loss of renal function that took him to hemodialysis on Nov, 2018. He was then referred to a main hospital to be included in the waiting list for kidney transplantation. In Feb 2020 he received a cadaver kidney transplant and up today presents normal kidney function.

Genetic studies of the complete complement gene set were performed in 2019 and the C2 p.S250C variant was the only relevant alteration found in the patient. He carries the *MCPggaac* polymorphism in heterozygosis and is negative for the *CFH-H3* aHUS risk polymorphism. At the time of these analyses, levels of C3, C4 and FH were within the normal range.

Clinical synopsis of the C3G patient carrying the C2 p.R249C variant

He is a 59y-old male with a previous history of nonspecific sediment alterations. He presented with a creatinine of 1.3mg/dl and FGxMDRD-4 57ml/min, microscopic hematuria and a proteinuria in the nephrotic range that flared up to chronic kidney disease. He was negative in the autoimmunity study and for the HBV, HCV and HIV serology. He has a brother under examination for presenting proteinuria.

A biopsy was performed that included seven glomeruli with sclerosis in three of them. The remaining showed mesangial hypercellularity with focal endocapillary proliferation. No intraglomerular inflammatory cells were observed. There were synechiae to the Bowman capsule and thickening of the glomerular basement membrane at some points. The interstitium presented a chronic inflammatory infiltrate that surrounded the sclerosed glomeruli and the tubules. Atrophy was also observed in part of the tubules and there were also accumulations of intraluminal polynuclear cells. The infiltrate also presented some plasma cells. Congo red staining was negative. The vessels showed moderate arteriosclerotic changes. In the immunofluorescence there was mesangial deposition of C3 (++) in the glomeruli without IgA, IgG, IgM or C1Q deposits. There was also absence of C4d deposits in the glomeruli, but few C5b9 deposits. Another biopsy on September 2020 showed mesangial and intramembranous dense deposits in the electron microscopy. Currently he is under treatment with MMF and corticoids, presents a monoclonal gammapathy and normal levels of C3.

Genetic studies of the complete complement gene set were performed in 2018 revealing the C2 p.R249C variant and two additional changes in the *C1S* gene (p.S115F, rs138764697; p.D315N; rs117907409), which significance is uncertain. He carries the *CFH-H3* polymorphism in heterozygosis and is negative for the *MCPggaac* aHUS risk polymorphism. At the time of the genetic analysis, levels of C3, C4 and FH were within the normal range.

Gain-of-function variants of complement C2 support cytocidal activity of anticancer monoclonal antibodies.

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The alternative complement pathway is induced constantly by a spontaneous breakdown of C3 protein into C3a anaphylatoxin and C3b, a fragment bondable non-specifically to cell surfaces. Deposition of C3b on the pathogen's surface lacking complement inhibitors fuels the cascade leading to osmotic lysis. Conversely, host cells are protected by the presence of endogenous inhibitors that prevent downstream processing.[1] Therefore, gain-of-function (GoF) mutations in the alternative pathway components that oppose physiological regulation by inhibitors, directly predispose carriers to autoimmune diseases. Our concept on how to turn potentially detrimental GoF variants into universal supporters of immunotherapeutics involves the modification of the classical complement pathway component, C2, to emulate the known GoF mutations in the analogous alternative pathway component, factor B. Since the molecular target for the classical complement pathway is defined by surface-bound immunoglobulins, GoF C2 variants enhance the cytocidal effect of anticancer antibodies, especially towards complement-resistant tumor cells.

Nearly half of the complement system proteins act as inhibitors that disable unwanted activation on self surfaces.[1] Most of these inhibitors control complement convertases – key enzymatic complexes that augment the complement activation by catalyzing the breakdown of C3 and C5 proteins. Mechanisms of convertase regulation involve either support of their proteolytic disruption or boosting their irreversible dissociation.[1] Factor B and C2 proteins are components of the alternative and classical pathway convertases, respectively. They share a high degree of amino acid similarity, almost identical structural organization [2] (Fig.1), and perform analogic roles in the complement cascade as their Bb and C2a subunits, respectively, act as enzymatic components providing a serine protease activity. Several missense mutations in factor B (e.g. p. D279G, p. Y363A, p. M458I) that result in the GoF phenotype were identified in patients with rare kidney diseases [3, 4] or designed *in silico*.[5] We translated these mutations to corresponding substitutions in C2 protein. Then, to obtain C2 variants providing several independent modes of the classical convertase enhancement, we combined these mutations into one construct.

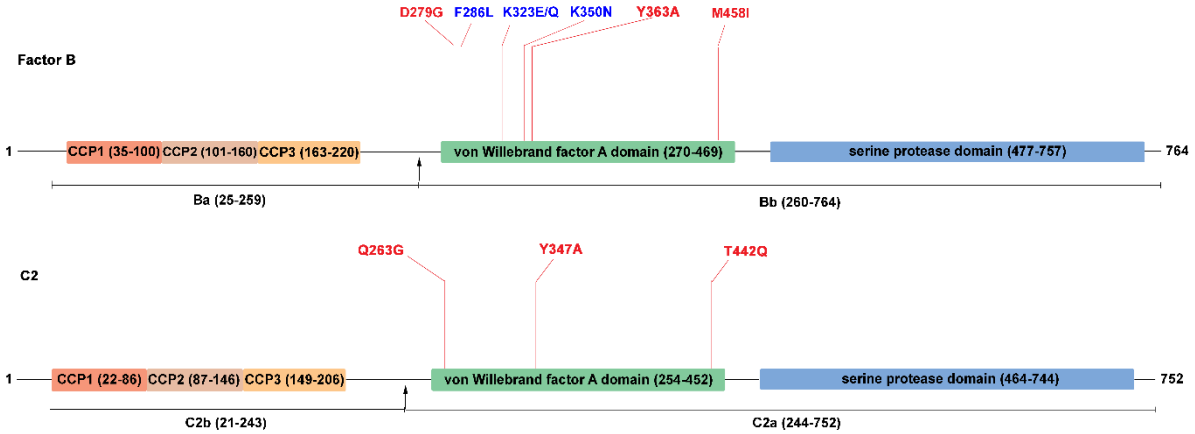


Fig. 1| Homology of Factor B and complement C2 proteins. The scheme presents the placement of domains within the primary structure of paralogs: factor B (upper graph) and C2 (bottom graph). Gain-of-function mutations in factor B confirmed with functional studies are indicated. Mutations chosen for translation into the corresponding substitutions in C2 are marked with red font. Black arrows mark cleavage sites in factor B and C2.

The introduction of complement-activating anticancer monoclonal antibodies (mAbs) as a means of targeted therapy opened a new era in oncology and hematology. The first-in-class drug, anti-CD20 rituximab approved in 1997, has markedly reduced the mortality of patients with non-Hodgkin lymphoma (NHL).[5] Despite an undoubted success, the number of patients poorly responding or refractory to rituximab, declining efficacy of subsequent rounds of therapy, and limited efficacy in other B cell malignancies imposed studies on new generations of anti-CD20 mAbs.[6] The second generation anti-CD20 mAb ofatumumab presents superior *in vitro* complement-dependent cytotoxicity (CDC).[7] However, data from randomized clinical studies demonstrate no superior efficacy versus rituximab [8] thus suggesting a dispensable role of complement activation in the therapeutic effect of anti-CD20 mAbs. On the other hand, a post-infusion drop in complement activity of patients' sera together with the observation that rituximab-nonresponsive patients achieved clinical response when the infusion was accompanied with fresh frozen plasma suggest complement exhaustion as an important factor limiting the efficacy of anti-CD20 mAbs (reviewed in [9]). Moreover, tumor cells overexpress [10] and hijack [11] complement inhibitors, which speed up unproductive complement consumption. Rituximab and ofatumumab are classified as type I anti-CD20 mAbs, i.e., strong activators of CDC and weak direct inducers of cell death. The opposite characteristics of type II specimens represented by glycoengineered, third-generation anti-CD20 mAb obinutuzumab [12] theoretically enable overcoming of resistance to type I antibodies. Improvements in progression-free survival (PFS) has been presented in clinical trials head-to-head comparing obinutuzumab and rituximab in patients with treatment-naïve chronic lymphocytic leukemia (CLL) and follicular lymphoma (FL).[13] However, recent data from a large phase 3 trial including patients with untreated diffuse large B cell lymphoma (DLBCL) demonstrated no advantage over rituximab treatment.[14] Therefore, there is still room for improvement of existing anti-CD20 therapies and unmet clinical need for the effective treatment of certain B cell malignancies. Herein we tested the suitability of C2 variants encompassing multiple GoF mutations as supporters of mAb-based immunotherapy.

First, we analyzed the effect of single and multiple substitutions in C2 on classical C5 convertase activity. CD20-positive human lymphoma cell line Ramos sensitized with ofatumumab was used as a model. Importantly, our experimental setup enabled convertase formation in a physiological milieu of human serum [15]. As expected, supplementation of human serum with C2 GoF mutants

resulted in increased convertase activity and some of the multiple GoF mutants outperformed single mutants (Fig.2). In the next step, we tested single GoF mutants and chosen multiple mutants in CDC assays on cells of different sensitivity to anti-CD20 mAbs that stem from both the composition of complement inhibitors and the expression of CD20 on the surface.[16] Based on our previous data, we selected three cell lines of high (Ramos), moderate (Raji), and low (Namalwa) sensitivity to anti-CD20-mediated CDC. A 10-fold increase of therapeutic antibodies rituximab or ofatumumab from 50 to 500 μg/ml did not significantly increase CDC in tested cell lines (Supplementary Fig.1), unlike the supplementation of serum with a physiological concentration of certain GoF mutants (Fig.3A-D). We repeated the same experiments with chosen C2 variant encompassing three single GoF mutations (Q263G,Y347A and T442Q, subsequently called "triple" mutant) on fresh cultures of CLL cells isolated from three treatment-naïve patients. CLL presents a major clinical challenge for anti-CD20 therapy as these cells express low levels of target antigen compared to non-Hodgkin's lymphomas. When tested in a serum concentration range from 10%-50%, triple GoF mutant significantly increased CDC exerted by ofatumumab in each CLL culture (Fig.3E-G). Obtained results indicate that C2 variants with multiple GOF mutations can markedly potentiate an *in vitro* activity of type I anti-CD20 mAbs.

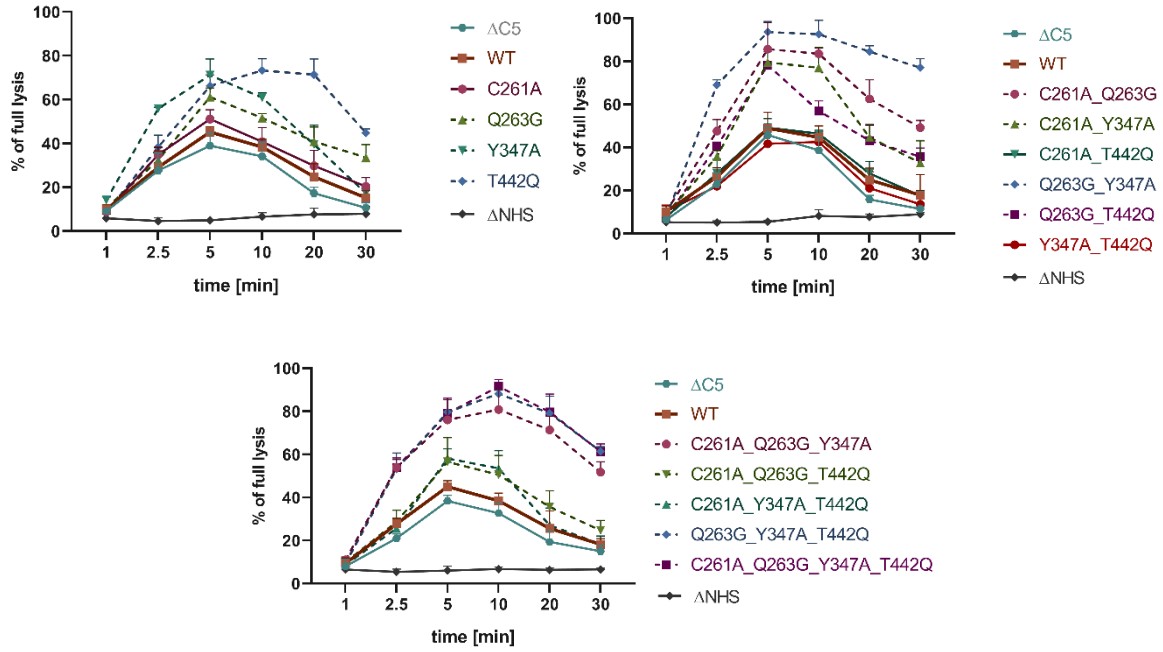


Fig. 2 The classical pathway convertase activity. Ramos cells were sensitized with ofatumumab and incubated for the indicated time with 20% of C5-depleted serum (ΔC5) supplemented with a particular C2 mutant. Heat-inactivated serum (ΔNHS) was applied as a negative control. Graphs present data from three independent experiments and error bars show standard deviation. Statistical significance for each variant vs WT protein are available in Supplementary Table 1.

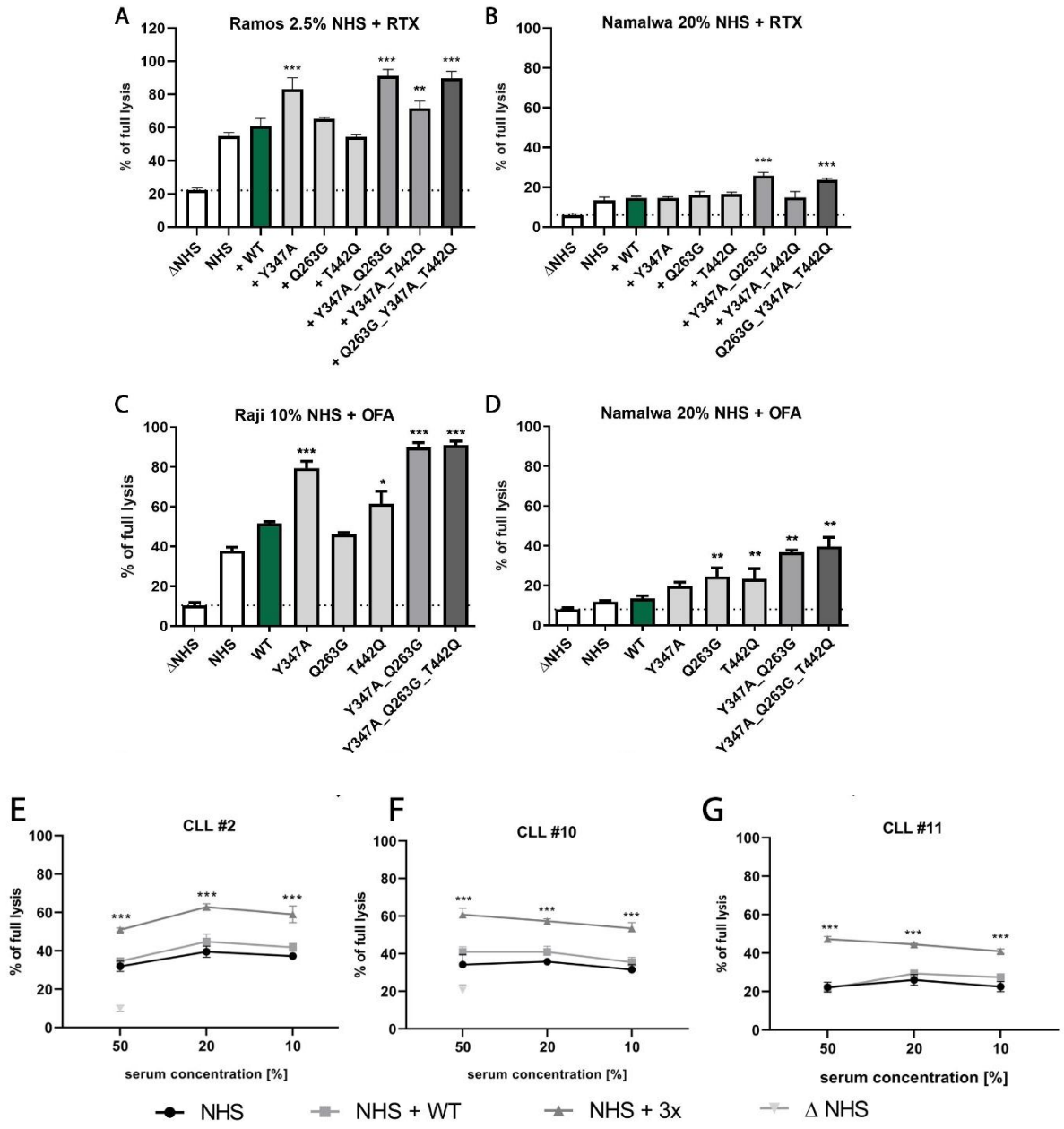
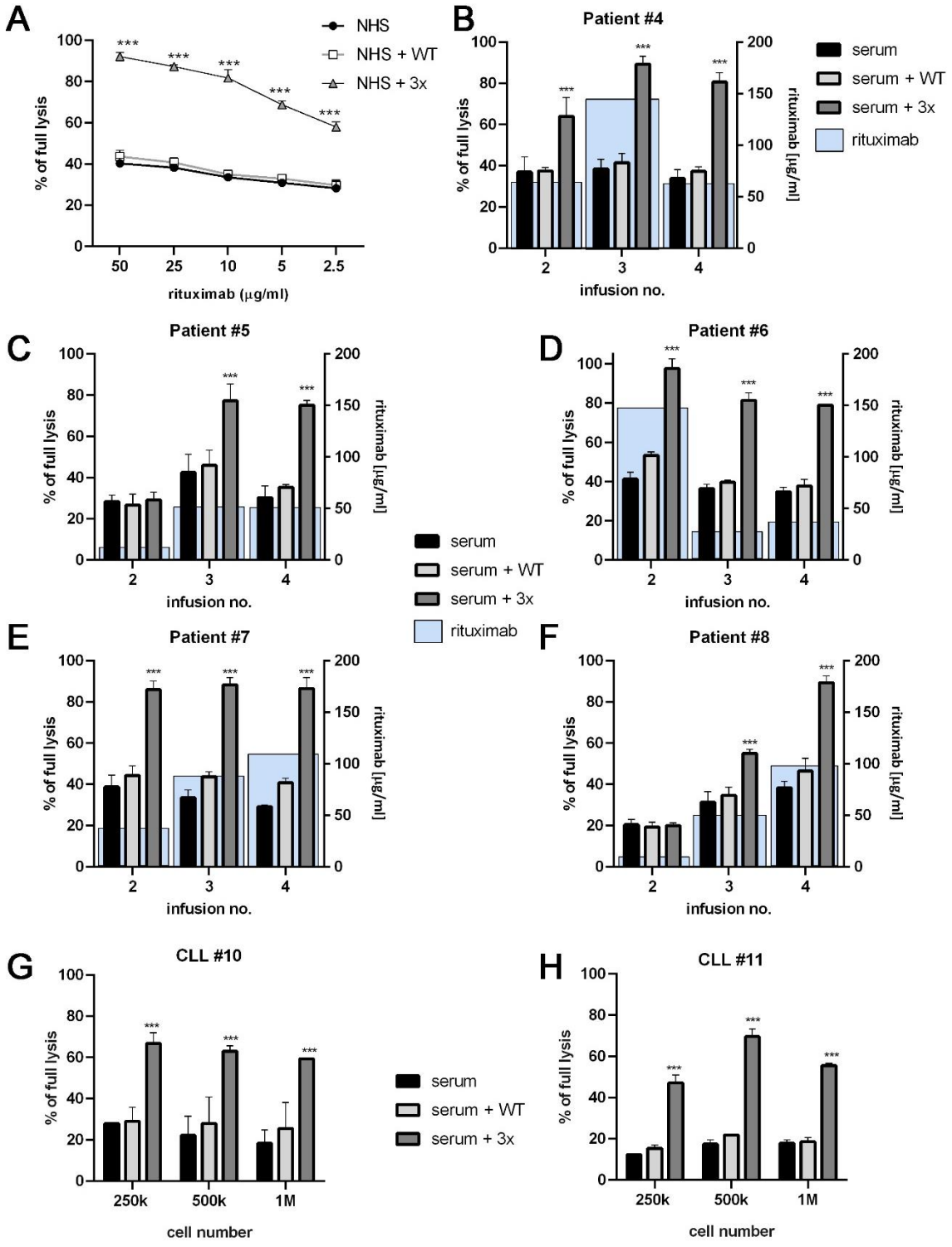


Fig. 3 Universal character of GOF C2 variants on CDC of anti-CD20 therapeutic antibodies. Calcein-AM-labelled cells were sensitized with type I anti-CD20 antibody (ofatumumab or rituximab), incubated with indicated concentration of normal human serum (NHS) and supplemented with a physiological concentration of particular C2 mutant A) CDC exerted in Ramos cell line after sensitized with rituximab, B) CDC exerted in rituximab-sensitized Namalwa cells, C) CDC exerted in ofatumumab-sensitized Raji cells, D) CDC exerted in ofatumumab-sensitized Namalwa cells. The green bar indicates supplementation with wild-type (WT) protein.E-G) Primary CLL cells employed for CDC assay and supplemented with physiological concentration of triple C2 variant (3x) at indicated concentration of NHS. Heat-inactivated serum (ΔNHS) was considered as a background, complement independent lysis and depicted by a dotted line. Statistical significance at p level < 0.05*, p < 0.01** and p < 0.001*** in comparison to cell lysis obtained by the addition of wild type C2 (WT, green bar) was calculated according to Dunnett's multiple comparison test (GraphPad Prism). Graphs present data from three independent experiments and error bars show standard deviation.

Afterward, we tested whether triple GoF C2 mutant can trigger efficient CDC at low rituximab concentrations. The effective therapeutic dose of type I anti-CD20 mAbs is a matter of ongoing debate. On one hand, increasing the standard rituximab dose (375 mg/m^2) was proposed in individuals with a high tumor burden that affects pharmacokinetic and can lead to suboptimal exposure.[17] On the other hand, *Kennedy et al.* reported that application of the standard rituximab dose can deplete complement in CLL patients for a week or longer.[18] *Beurskens et al*. extended these findings and showed that at physiologically relevant cell burdens, complement activity in 50% serum can be severely depleted by application of an excessive amount of type I mAbs.[19] In their two-step experiments, which mimicked repetitive dosing of mAb, large amounts of antibodies deposited C3 on the surface of CLL cells, however, a saturation of CDC was achieved at much lower antibody concentration than the saturation of C3 deposition. Application of the amount of antibody exceeding the CDC saturation level resulted in the exhaustion of complement activity and severely compromised CDC upon challenge with additional cells, even if adequate mAb concentrations were present. Another important observation made in rituximab-receiving CLL patients was that up to 80% of circulating tumor cells were eliminated after infusion of as little as 30 mg but the leukemic cells count rebounded shortly after completing the infusion with the remaining quantity of mAb.[20] Notably, these repopulating cells had markedly (90-95%) reduced levels of CD20 on their surface, which could stem from trogocytosis initiated by excessively administrated mAb.[20, 21] All the above-mentioned remarks speak for the necessity of maximizing cytocidal effect from the single anti-CD20 mAb. We tested whether supplementation of serum with triple GoF C2 mutant fulfils such a need and theoretically allows to reduce the effective antibody dose. Raji cells were treated with decreasing concentrations of rituximab, and the addition of triple mutant supported CDC double as effective as the addition of wild type C2 within the whole concentration range from 50 to 2.5 μg/ml. Even at the lowest rituximab concentration, the CDC activity of GoF mutant-containing serum was markedly higher than CDC at the highest rituximab concentration in non-supplemented or wild type C2-supplemented serum (Fig.4 A). To further exploit the potential of the triple C2 variant to maximize CDC at a limited concentration of anti-CD20 mAbs, we examined sera from five NHL patients treated with rituximab, which were collected before the second, third, and fourth infusion of the drug administered in four-week intervals. The only source of rituximab present in these samples was accumulation after the previous infusions. The results showed that upon supplementation of patients' sera with triple GoF C2 mutant, the leftovers of rituximab accumulated at the level above 30 μg/ml exerted substantial CDC in most of the cases (Fig.4 B-F), thus confirming that hyperactive classical convertases significantly decrease the threshold of mAb concentration necessary for the efficient killing of tumor cells by host immune effectors. Finally, we examined the effect of triple GoF mutant on increasing number of primary CLL cells derived from two patients. Previously, we observed a CDC-enhancing effect at 1 x 10^5 CLL cells per well (Fig.3 E-G) and now the same outcome was observed when cell number increased up to 1 × 10^6 per well (Fig.4 G,H). We conclude that C2 mutants can be effective at a high tumor burden. The resistance of CD20-positive cells to CDC is a derivative of a target antigen to complement inhibitors ratio, as shown in [16, 22]. *Takei et al.* have tracked the process of gaining resistance to rituximab by Ramos cells, which concurrently diminished CD20 expression and overexpressed complement inhibitors.[23] Using the siRNA technique aimed to knock-down CD20 expression, we succeed to isolate the clone of SU-DHL-4 cells bearing not only less target antigen but also more CD59 on the surface (Fig.4 I). While comparing the CDC exerted by rituximab on parental SU-DHL-4 cells and siRNA-edited cells, we observed significant differences in non-supplemented or wild type C2-supplemented serum but not in serum supplemented with triple GoF C2 variant (Fig. 4 I). In this experiment, we also included a quadruple GoF C2 mutant that encompassed the three previous mutations and the C261A mutation claimed to improve the intrinsic stability of classical convertase.[23] This new variant additionally flattened the differences between control and siRNA-modified cells. The final conclusion is that

multiple GoF C2 mutants not only ensure maximal CDC efficacy of rituximab but also counterbalance the selection of resistant cells.



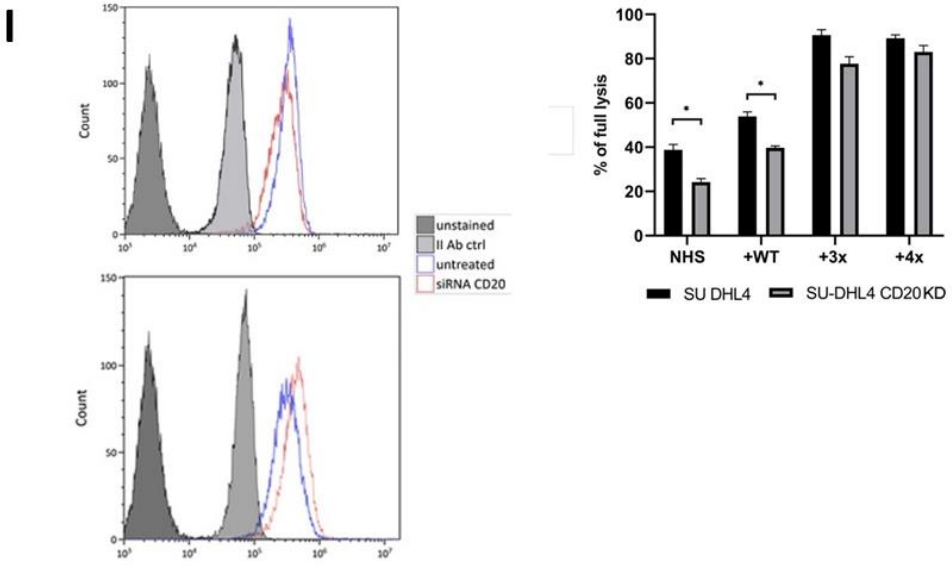


Fig.4 Demonstration on C2 triple GoF mutant capacity to support CDC in suboptimal conditions. The graphs show CDC mediated by rituximab on Raji cells upon A) a decreasing concentration of the drug after supplementation with either wild-type (WT) or triple (3x) C2 variant. Panels B-F) present the effect of supplementation of patients' sera collected before the 2nd, 3rd and 4th infusion of rituximab on CDC. Blue rectangles indicate the concentration of rituximab analysed by ELISA. G) and H) The graphs present CDC of primary CLL cells sensitized with rituximab upon increased cell number, and with addition of C2 variants. I) The left panels shows histograms comparing CD20 and CD59 levels exposed on the surface of SUDHL-4 line before and after CD20 siRNA-mediated knockdown. Dark grey peaks indicate unstained cells, while light grey peaks mark cells stained with secondary Ab only. Red and blue peaks present the level of CD20 (upper) or CD59 (lower) expression for either SUDHL parental or CD20 knockdown (KD) cells. Each experiment was run in triplicates and error bars show standard deviation. Statistical significance at p level < 0.05*, p < 0.01** and p < 0.001*** , in comparison to CDC with additional WT C2 is calculated according to Dunnett's multiple comparison test (GraphPad Prism). Graphs present data from three independent experiments and error bars show standard deviation

So far, our experiments were performed with type I anti-CD20 mAbs. We decided to examine if the concept of supporting CDC by GoF C2 variants can be extended to other anticancer antibodies. Recently *Kumar et al*. provided a model of binding of anti-CD20 therapeutics to its target.[24] The results revealed that type I mAb forms 1:2 or 2:1 (mAb:CD20) "seeding" complexes that enable subsequent concatenation of mAb or CD20 molecules, respectively. Conversely, type II antibodies upon binding to CD20 form 1:2 "terminal" complexes that preclude binding of additional mAb molecules, thus preventing the hexamerization required for C1q recruitment.[25] Therefore, obinutuzumab would require higher antigen densities for oligomerization and complement activation. This model explains the difference between the ability of type I and II anti-CD20 antibodies to activate CDC. However, our experiments show, that despite structural obstacles, the addition of triple GoF C2 mutant significantly boosted CDC exerted by obinutuzumab (Fig.5A). One may speculate that at a favourable local density of CD20, even minimal complement activation could be sufficient for effective CDC when the classical convertase is formed by hyperactive C2 protein. Inotuzumab ozogamicin is a humanized IgG4 anti-CD22 mAb bound covalently to calicheamicin dimethyl hydrazide, that mediates apoptosis in target cells. [26] Notably, the IgG4 isotype poorly induces Fc-dependent effector functions including CDC due to specific conformation of the Cγ2 domain.[27] However, triple and quadruple C2 mutants but not wild

type C2 added to 50% human serum exerted substantial lysis of Ramos cells (Fig.5B). Daratumumab, a human anti-CD38 IgG1 mAb approved for multiple myeloma treatment, does not show significant induction of CDC[28]. Our experiment showed that 80% of Daudi cells were killed when incubated with daratumumab in serum supplemented with triple or quadruple C2 mutant vs. 40% and 20% killing when wild-type C2 or no additional C2 was added, respectively (Fig.5C).

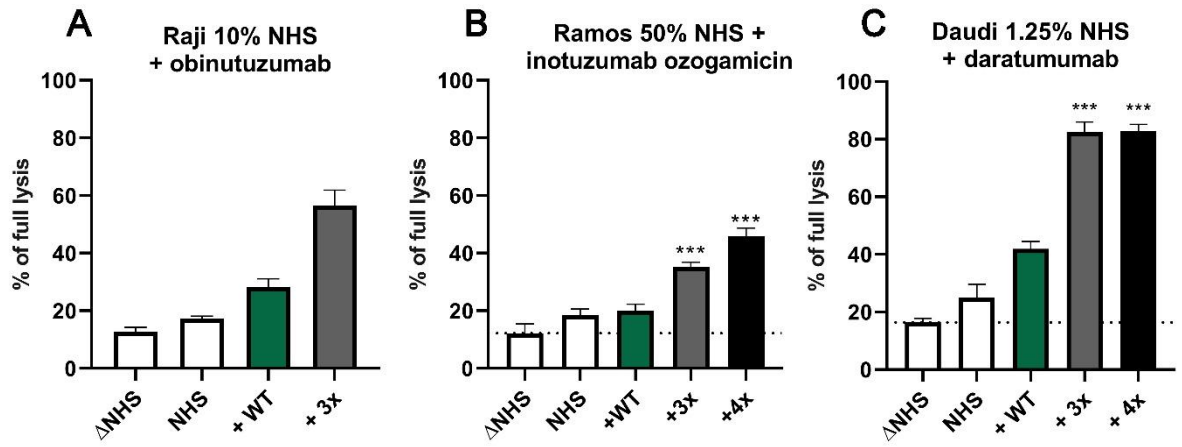


Fig. 5 Multiple GoF C2 variants induce CDC on cells sensitized with poor complement activators. Raji, Ramos or Daudi cells were incubated with normal human serum (NHS), supplemented with a physiological concentration of wild-type (WT) or triple (3x) C2 variant and indicated antibody to induce CDC. A) Raji cells were sensitized with type II anit-CD20 mAb obinutuzumab B) Ramos cells were incubated with anti-CD22 (inotuzumab ozogamicin) C) Daudi cells were sensitized with anti-CD38 antibody (daratumumab). Heat-inactivated serum (ΔNHS) was considered as a background, complement independent lysis and depicted by a dotted line. Statistical significance at p level < 0.05*, p < 0.01** and p < 0.001*** in comparison to cell lysis obtained by the addition of wild type C2 (WT, green bar) was calculated according to Dunnett's multiple comparison test (GraphPad Prism). Graphs present data from three independent experiments and error bars show standard deviation.

Our data confirmed the ability of GoF C2 variants to boost either the cytocidal activity of antibodies normally not engaged in complement activation or maximize the cytotoxic yield from a limited amount of type I anti-CD20 mAbs. The low physiological serum concentration of C2 protein (25 μg/ml) compared to other constituents of alternative and classical convertases makes it a bottleneck of the whole classical pathway and offers a possibility of effective supplementation with relatively low amounts of recombinant protein. The proposed strategy has several advantages over other attempts to overcome the resistance of tumor cells to CDC. Neutralization of complement inhibitors as a concept to increase the efficacy of therapeutic antibodies was exploited by testing bi-specific antibodies targeting CD20 and CD55 either *in vitro* or in a murine xenograft model of Burkitt lymphoma.[29] Also, the siRNA-mediated silencing of membrane complement inhibitors was proposed.[30] However, the affinity of the bi-specific antibody is limited to only one type of inhibitor and does not neutralize the activity of other inhibitors,, e.g. factor H, which is present in serum in micromolar concentration and, when hijacked by tumor cells, contributes to anti-CD20 mAb resistance.[11] Silencing of complement inhibitors by siRNA may be problematic due to the expression of target molecules on host cells, therefore requiring a specific delivery of siRNA only to tumor cells. A clear advantage of the herein

proposed strategy is the compatibility of GoF C2 with numerous therapeutic antibodies and the possibility to combine different mutations that oppose the activity of several complement inhibitors into a single construct.

References

1. Schmidt, C.Q., Lambris, J.D. & Ricklin, D. Protection of host cells by complement regulators. *Immunol Rev* **274**, 152-171 (2016).

2. Krishnan, V., Xu, Y., Macon, K., Volanakis, J.E. & Narayana, S.V. The structure of C2b, a fragment of complement component C2 produced during C3 convertase formation. *Acta Crystallogr D Biol Crystallogr* **65**, 266-274 (2009).

3. Marinozzi, M.C. *et al.* Complement factor B mutations in atypical hemolytic uremic syndrome-disease-relevant or benign? *J Am Soc Nephrol* **25**, 2053-2065 (2014).

4. Hourcade, D.E., Mitchell, L.M. & Oglesby, T.J. Mutations of the type A domain of complement factor B that promote high-affinity C3b-binding. *J Immunol* **162**, 2906-2911 (1999).

5. Salles, G. *et al.* Rituximab in B-Cell Hematologic Malignancies: A Review of 20 Years of Clinical Experience. *Adv Ther* **34**, 2232-2273 (2017).

6. Meyer, S. *et al.* New insights in Type I and II CD20 antibody mechanisms-of-action with a panel of novel CD20 antibodies. *Br J Haematol* **180**, 808-820 (2018).

7. Teeling, J.L. *et al.* Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood* **104**, 1793-1800 (2004).

8. Maloney, D.G. *et al.* A phase 3 randomized study (HOMER) of ofatumumab vs rituximab in iNHL relapsed after rituximab-containing therapy. *Blood Adv* **4**, 3886-3893 (2020).

9. Felberg, A. *et al.* Monitoring of the Complement System Status in Patients With B-Cell Malignancies Treated With Rituximab. *Front Immunol* **11**, 584509 (2020).

10. Gancz, D. & Fishelson, Z. Cancer resistance to complement-dependent cytotoxicity (CDC): Problem-oriented research and development. *Mol Immunol* **46**, 2794-2800 (2009).

11. Hörl, S. *et al.* Reduction of complement factor H binding to CLL cells improves the induction of rituximab-mediated complement-dependent cytotoxicity. *Leukemia* **27**, 2200-2208 (2013).

12. Tobinai, K., Klein, C., Oya, N. & Fingerle-Rowson, G. A Review of Obinutuzumab (GA101), a Novel Type II Anti-CD20 Monoclonal Antibody, for the Treatment of Patients with B-Cell Malignancies. *Adv Ther* **34**, 324-356 (2017).

13. Marcus, R. *et al.* Obinutuzumab for the First-Line Treatment of Follicular Lymphoma. *N Engl J Med* **377**, 1331-1344 (2017).

14. Sehn, L.H. *et al.* A randomized, open-label, Phase III study of obinutuzumab or rituximab plus CHOP in patients with previously untreated diffuse large B-Cell lymphoma: final analysis of GOYA. *J Hematol Oncol* **13**, 71 (2020).

15. Okroj, M., Holmquist, E., King, B.C. & Blom, A.M. Functional analyses of complement convertases using C3 and C5-depleted sera. *PLoS One* **7**, e47245 (2012).

16. Okroj, M., Eriksson, I., Osterborg, A. & Blom, A.M. Killing of CLL and NHL cells by rituximab and ofatumumab under limited availability of complement. *Med Oncol* **30**, 759 (2013).

17. Tout, M. *et al.* Rituximab exposure is influenced by baseline metabolic tumor volume and predicts outcome of DLBCL patients: a Lymphoma Study Association report. *Blood* **129**, 2616-2623 (2017).

18. Kennedy, A.D. *et al.* Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol* **172**, 3280-3288 (2004).

19. Beurskens, F.J. *et al.* Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J Immunol* **188**, 3532-3541 (2012).

20. Taylor, R.P. & Lindorfer, M.A. Fcgamma-receptor-mediated trogocytosis impacts mAb-based therapies: historical precedence and recent developments. *Blood* **125**, 762-766 (2015).

21. Williams, M.E. *et al.* Thrice-weekly low-dose rituximab decreases CD20 loss via shaving and promotes enhanced targeting in chronic lymphocytic leukemia. *J Immunol* **177**, 7435-7443 (2006).

22. Manches, O. *et al.* In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* **101**, 949-954 (2003).

23. Takei, K., Yamazaki, T., Sawada, U., Ishizuka, H. & Aizawa, S. Analysis of changes in CD20, CD55, and CD59 expression on established rituximab-resistant B-lymphoma cell lines. *Leuk Res* **30**, 625-631 (2006).

24. Kumar, A., Planchais, C., Fronzes, R., Mouquet, H. & Reyes, N. Binding mechanisms of therapeutic antibodies to human CD20. *Science* **369**, 793-799 (2020).

25. Diebolder, C.A. *et al.* Complement is activated by IgG hexamers assembled at the cell surface. *Science* **343**, 1260-1263 (2014).

26. DiJoseph, J.F. *et al.* Antitumor efficacy of a combination of CMC-544 (inotuzumab ozogamicin), a CD22-targeted cytotoxic immunoconjugate of calicheamicin, and rituximab against non-Hodgkin's B-cell lymphoma. *Clin Cancer Res* **12**, 242-249 (2006).

27. Davies, A.M. & Sutton, B.J. Human IgG4: a structural perspective. *Immunol Rev* **268**, 139-159 (2015).

28. Manna, A. *et al.* Targeting CD38 Enhances the Antileukemic Activity of Ibrutinib in Chronic Lymphocytic Leukemia. *Clin Cancer Res* **25**, 3974-3985 (2019).

29. Macor, P. *et al.* Bispecific antibodies targeting tumor-associated antigens and neutralizing complement regulators increase the efficacy of antibody-based immunotherapy in mice. *Leukemia* **29**, 406-414 (2015).

30. Mamidi, S. *et al.* Neutralization of membrane complement regulators improves complement-dependent effector functions of therapeutic anticancer antibodies targeting leukemic cells. *Oncoimmunology* **4**, e979688 (2015).

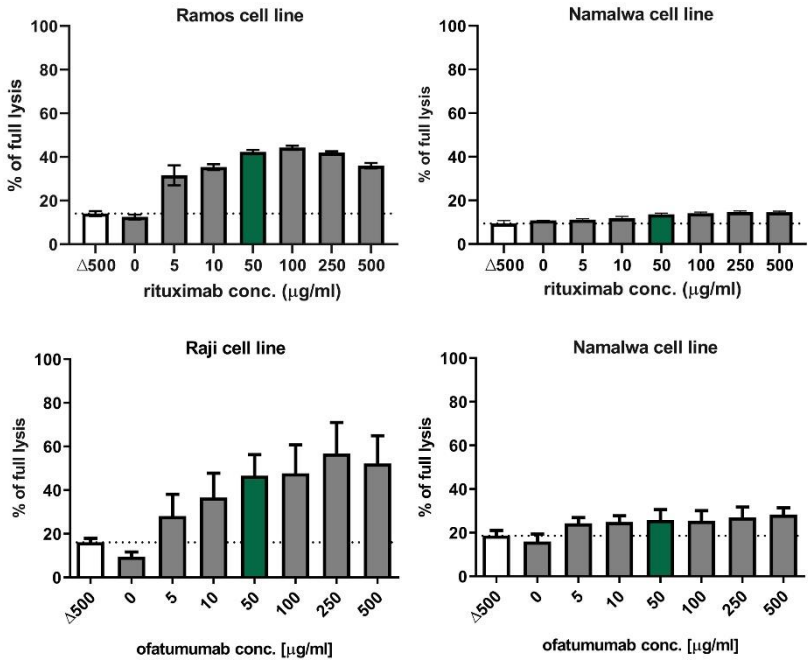
Supplementary data

significance vs. wild-type C2:	1 min	2.5 min	5 min	10 min	20 min	30 min
C261A	-	-	-	-	-	-
Q263G	-	-	**	**	**	***
Y347A	-	***	***	***	***	-
T442Q	-	-	***	***	***	***

	1 min	2.5 min	5 min	10 min	20 min	30 min
C261A_Y347A	-	-	***	***	***	**
C261A_Q263G	-	***	***	***	***	***
C261A_T442Q	-	-	-	-	-	-
Q263G_Y347A	-	***	***	***	***	***
Q263G_T442Q	-	**	***	*	***	***
Y347A_T442Q	-	-	-	-	-	-

	1 min	2.5 min	5 min	10 min	20 min	30 min
C261A_Q263G_Y347A	-	***	***	***	***	***
C261A_Q263G_T442Q	-	-	*	*	-	-
C261A_Y347A_T442Q	-	-	*	**	-	-
Q263G_Y347A_T442Q	-	***	***	***	***	***
C261A_Q263G_Y347A_T442Q	-	***	***	***	***	***

Supplementary Table 1. Statistical significance at p level < 0.05*, p < 0.01** and p < 0.001*** in comparison to cell lysis obtained by the addition of wild type C2 (WT) for the convertase activity assay (Fig.2) was calculated according to Dunnett's multiple comparison test (GraphPad Prism).



Supplementary Fig.1 Calcein-AM-labelled Ramos, Raji or Namalwa cells were sensitized with anti-CD20 antibody (ofa or rtx). The graphs present CDC of each cell line after titration of particular antibody. The green bar indicates saturating concentration of antibody (50 μg/ml), which was further applied in all experiments. Heat-inactivated serum supplemented with the highest concentration of the drug (Δ500) was considered as a background and is depicted by a dotted line. Graphs present data from three independent experiments and error bars show standard deviation.

Methods

Protein expression and purification

Wild-type C2 cDNA sequence (accession number NM_000063.5) additionally containing six histidine codons at 3' terminus, as well as sequences for single, double, triple and quadruple variants were codon-optimized, synthesized and cloned into pCEP4 vector in the framework of GeneArt Gene Synthesis service by Thermo Fisher. Proteins were expressed and purified as described [35]. Briefly, plasmid DNA was transfected into HEK293 Freestyle cells using Freestyle Max reagent (Thermo Fisher). Conditioned FreeStyle 293 expression medium (Thermo Fisher) was collected at 2nd, 4th and 7th days post-transfection and stored at − 80 °C until the protein purification. The resulting proteins were purified with HisTrap FF affinity column (GE Healthcare) and elution was carried out with an 0.7M imidazole gradient.

In vitro culture of human lymphoma cell lines and primary CLL cells

All cell lines (Raji, Ramos, Daudi, Namalwa) were cultured in RPMI 1640 medium with l-glutamine (ATCC) supplemented with 10% foetal bovine serum (ATCC) at 37 °C and humidified 5% CO_2 atmosphere. The culture of primary CLL cells was established as previously [25]. CLL cells were cultured in a 1:1 mixture of RPMI 1640: DMEM (HyCult) medium supplemented with 20% FBS. Cells were routinely checked for Mycoplasma contamination by DAPI staining [36] when cultured and never kept in continuous culture for more than 10 passages.

Clinical material

Serum samples were collected from patients admitted to Dept. of Hematology, Medical University of Gdańsk. The inclusion criterion was a diagnosis of B-cell malignancy with no prior treatment with anti-CD20 mAbs. Blood collection, sample handling and storage were performed as described in [18]. Briefly, blood was collected into Vacutainer tubes with clot activator (BD Biosciences) before and after each intra-venal infusion of standard rituximab dose (375 mg per 1 m^2 of body surface). Isolated blood was left at room temperature until clot formation, centrifuged at 700 × g for 12 min at 4 °C, then pooled, centrifuged again to get rid of residual cells, aliquoted, and finally stored at − 80 °C until needed. The same procedure was applied for blood collection from healthy volunteers used for the preparation of normal human serum (NHS) as described elsewhere [37].

CDC and classical convertase assays

Briefly, cells were harvested ($2*10^5$ cell per experimental point), loaded with Calcein-AM for 30 min at 37^{o}C (1 mg/ml diluted in complete medium). Afterwards, cells were washed two times with PBS and pelleted in the V-shaped plate. Complement-dependent cytotoxicity (CDC) assay was performed as described in [18]. For CP convertase assay cells were overlaid with 25 µl of PBS and incubated at 37 ^{o}C. Ofatumumab (100 µg/ml) and 20% of C5-depleted serum supplemented with 5 µg/ml of each C2 variant added at a specific time point. Then, cells were washed with EDTA buffer, pelleted and overlaid with EDTA buffer containing 1:20 dilution of guinea pig serum followed by 30 min incubation (37^{o}C, 600rpm). Fluorescence readout was performed at 490/520 nm. Cell lysis was calculated in reference to the readout obtained for cells treated with 30% DMSO.

Flow cytometry

Expression of CD20, CD46, CD55 and CD59 was analysed employing flow cytometry, as noted in [26]. Primary antibodies (clone UJ11 for CD35, clone MEM-258 for CD46, and clone HI-55a for CD55; Immunotools) and rituximab were used, followed by detection with secondary goat-anti mouse F(ab)2 labelled with AF488 antibody (Dako, 1:200 dilution).

Assessment of Rituximab Concentration

Rituximab concentration in serum samples collected just before and just after each infusion was measured using an enzyme-linked immunosorbent assay exactly as described previously [25].

CD20 siRNA-mediated knock-down

SU-DHL4 cells ($0.5×10^6$/ml per well) seeded onto 6-well plate the day prior transfection. Cells were transfected with CD20 siRNA duplexes (ON-TARGETplus Human MS4A1 siRNA, Dharmacon/Thermo Scientific) or siRNA negative control (Sigma) using FuGENE® 6 Transfection Reagent (Promega). After 48 hours cells were subjected to a second transfection using the same experimental conditions. Forty eight hours after re-transfection cells were harvested and analysed by flow cytometry for CD20, CD46, CD55 and CD59 expression, then subjected to CDC assay.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 software. The Kruskal-Wallis test was applied for column data, while the Dunnett multiple comparison test for non-repeated measures was applied for grouped analysis.

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ORIGINAL ARTICLE

Gain of function mutant of complement factor B K323E mimics pathogenic C3NeF autoantibodies in convertase assays

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ABSTRACT

Complement convertases are enzymatic complexes, which play a critical role in propagation and amplification of the complement cascade. Under physiological conditions, convertases decay shortly after being formed in either spontaneous or inhibitor-driven process. Prolongation of their half-life by C3NeF autoantibodies that prevent convertase dissociation results in pathogenic condition often manifested by renal diseases. However, the diagnosis of convertase abnormalities is difficult due to the labile nature of these enzymes and low credibility of existing methods. Only recently, two-step functional assays employing C5-depleted serum or C5 inhibitors were introduced. Their advantage is convertase formation in the physiological milieu of whole serum and the drawback is inter-assay variability due to variations in rabbit erythrocytes used for the haemolysis-based readout. Abovementioned problems demand the application of the internal standard in each experiment. Obtaining a defined preparation of autoantibodies is complicated due to ethical and practical considerations. We found that recombinant, his-tagged factor B (fB) variant K323E retains full hemolytic activity and possess the ability to form convertases with prolonged half-life either in fB-depleted serum or when mixed with normal human serum. Such dominant character of K323E mutation allows using recombinant protein as a reference in functional convertase assays, not limited to these using rabbit erythrocytes. Additionally, our results demonstrate that gain of function mutations in complement components mimic the phenotype of C3NeF. Hence, patients with such "genetic C3NeF" would not benefit from B-cell depletion (e.g. by rituximab) and therefore should be properly diagnosed in order to choose suitable therapeutic intervention.

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Introduction

Proper regulation of the complement system protects from misguided attack, eventually leading to damage of own cells and tissues. This aspect is underlined by the fact that half of the known complement proteins act as inhibitors. Majority of these regulatory proteins control the activity of complement convertases – enzymatic complexes which catalyze the breakdown of C3 (C3 convertases) and C5 molecules (C5 convertases) [1,2]. C3b fragment, a cleavage product of C3 molecule, can bind the parental convertase and switch its substrate specificity from C3 to C5, thus forming C5 convertase. Alternatively, C3b can initiate the alternative complement pathway and by doing so amplify the complement cascade [3]. There are two main mechanisms of regulation of convertase activity: proteolysis of active components C3b and C4b and acceleration of convertase decay (DAA, decay accelerating activity) [3]. The first mechanism involves serine protease factor I and appropriate cofactors supporting proteolytic activity, the latter engages proteins which speed up dissociation of the enzymatic complex. Protection of own cells stems from the presence of membrane-bound complement inhibitors on their surfaces but also from the ability to bind soluble complement inhibitors such as factor H (fH) [4]. Importantly, the alternative complement pathway initiation involves constant low-level activity and continuous probing of cell surfaces. Lack of inhibition on permissive (non-self) surfaces enables pathway propagation [3]. Therefore, loss of control of alternative pathway is much more dangerous comparing to the deregulation of classical and lectin pathways, which require specific stimuli (antibodies or specific sugar moieties) for activation. Imbalance of mechanisms controlling the alternative convertase very often leads to renal diseases like C3 glumerulopathies (C3G, including glomerulonephritis (C3GN) [5] and dense deposit disease (DDD) [6]) and atypical haemolytic uremic syndrome (aHUS) [7]. One of the possible etiological factors of these diseases is the presence of autoantibodies directed against assembled convertases (C3 nephritic factor, C3NeF) or complement inhibitors (e.g. autoantibodies against fH), which both prolong convertase half-life [8].

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Diagnosis of C3NeF is not a trivial task due to the labile character of convertases, the half-life of which under physiological conditions oscillates around 60 s. It is assumed that most of the C3NeF species bind neoepitopes on the assembled convertases [9,10], however, binding to single convertase components was also described [11]. Nonetheless, the binding of given antibody to the convertase complex does not automatically mean impairment of convertase function. The strength of the binding does not correlate with the conversion of C3 to C3b [10] and probably cannot be attributed to clinical consequences. The same is true for autoantibodies against fH, which is capable of either coordination of C3b proteolysis or decay acceleration. Yet, most of the pathogenic anti-fH autoantibodies associated with renal diseases bind the C-terminal site of fH [12]. Interestingly, the coexistence of C3NeF and anti-fH was also reported [13]. However, fH has several functional sites responsible for different effector activities (reviewed in Parente et al. [14]) and binding of given antibody to fH may alter different functions depending on specific epitope. Therefore, assays analyzing the function of convertases upon addition of a purified fraction of patients' antibodies have an advantage over the assays, which detect only the binding of given antibody to convertase complex. A panel of five assays analyzing C3NeF (including four functional and one binding assay) was recently evaluated by Paixao-Calvacante et al. [10]. Forty-eight out of 101 samples collected from the patients with histological and/or clinical evidence of renal pathologies (mostly DDD and C3GN) showed positive result in one or more assays. The best performance (36 positive samples) was achieved by ELISA-based test measuring the stabilization of alternative pathway convertase assembled from purified components (COS-P). Twelve samples negative in the convertase stabilization assay were found to be positive in the binding assay (COIg), while 27 samples were positive in both assays. A haemolytic assay based on modified Rother's protocol [15] identified 26 positive samples including five, which were found negative in COS-P assay and another five negative in the COIg assay. These data show inconsistency in C3NeF detection methods and suggest that artefacts may be responsible for both false positive and false negative results. Furthermore, standardization of C3NeF detection is challenging. The reference samples analyzed during the European quality assessment in 2015 were identified correctly only by 50% of the participating laboratories [16]. Recently, new methods for assessing convertase activity directly in serum were introduced [17,18]. The first one is designed to test purified components added to C5-depleted human serum [17] and the other enables analysis in patient serum or plasma supplemented with a C5 inhibitor: OmCI or eculizumab [18]. These methods have numerous advantages over those demanding purified complement components and convertase reconstruction on artificial surfaces [10,19]. Due to the employment of rabbit erythrocytes and physiological milieu of human serum, they limit the number of artefacts and help to select only the physiologically relevant effects. Moreover, these methods are relatively fast and much less expensive. However, one drawback stems from usage of rabbit erythrocytes, which cannot be standardized due to batch-to-batch variations and the age of cells at the time of the experiment. These parameters influence the vulnerability of red blood cells to osmotic lysis and may indirectly change the readout of the assay thus generating inter assay variability and making a comparison of data from different experiments problematic [18]. Obtaining high amounts of serum from patients, especially children (as a source of C3NeF standards) for diagnostic purposes is ethically controversial. On the other hand, the content of C3NeF in patient serum can be subjected to change over the time. Therefore, we looked for a compound which would mimic the effect of convertase-stabilizing antibody in functional assays. One promising approach was to test the known gain of function mutant of complement factor B (fB), namely, K323E [20]. Since glycosylation of fB may involve its secretion and catabolism [21], we expressed recombinant K323E variant in eukaryotic HEK293 Freestyle system. In order to facilitate the protein purification process, ideally to a single chromatography step, we added histidine tag and compared the effect of tagged recombinant fB to its wild-type recombinant and plasma-purified variants in a panel of functional assays.

Material and methods

Protein expression and purification

Wild-type fB (accession number NM_001710 for mRNA and NP_001701 for preproprotein) and K323E fB mutant cDNA sequences containing six histidines (6 x His-tag) at C terminus were codon optimized, synthesized and cloned into the pCEP4 vector in the framework of GeneArt Gene Synthesis service by Thermo Fisher, Waltham, MA. Obtained plasmids were amplified in *E. coli* DH5alpha strain, purified with Midi Prep Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and 30 µg of total purified vector DNA was transfected into HEK293 Freestyle cells using Freestyle Max reagent (Thermo Fisher). Conditioned FreeStyle 293 expression medium (Thermo Fisher) was collected at day 2, 4 and 7 post transfection and stored at −80 °C until the purification process. Prior loading onto HisTrap FF crude column (GE Healthcare, Little Chalfont, United Kingdom) medium was filtered through 0.45 nm membrane. Washing step was performed with 50 mM Tris-HCl pH 8.0 with the addition of 10 mM imidazole and elution was performed with 0.7 M imidazole in the same buffer. Protein-containing fractions were pooled, concentrated and buffer was exchanged to PBS with Vivaspin concentrating device (Millipore, Burlington, MA). The purity of obtained preparations was examined by SDS-PAGE followed by Coomassie staining. Human fB purified from human plasma (Complement Technology, Tyler, TX) was used as a standard in SDS-PAGE and further functional assays. OmCI protein was expressed and purified as described [18].

Haemolytic assay

Rabbit erythrocytes (Centre of Experimental Medicine, Silesian Medical University, Wrocław, Poland) were resuspended 1:1 in Alsever's solution (114 mM glucose, 28 mM Na-citrate dihydrate, 68 mM NaCl, 0.2 mM citric acid). Prior to experiment erythrocytes were washed with Mg^{2+}-EGTA buffer (2.075 mM veronal buffer pH 7.3, 10 mM EGTA, 7 mM $MgCl_2$, 0.083% gelatin, 116 mM glucose, 60 mM NaCl) and their concentration was adjusted to the point at which 10 µl of suspension lysed with 90 µl of water gave absorbance at 405 nm within a range of 1–1.5 AU. Erythrocytes were pelleted (1000 x G, 1 min) in V-shape wells in 96-well microplate (Nunc, Roskilde, Denmark) and overlaid with 5% of fB-depleted serum (Complement Technology) diluted in Mg^{2+}-EGTA buffer in the presence or absence of recombinant fB. After 30 min incubation at 37 °C with shaking the microplate was centrifuged, supernatant was transferred into a flat-bottom plate and absorbance at 405 nm was measured by Synergy H1 multidetection plate reader (BioTek, Winooski, VT).

Convertase assays

Functional assays measuring the activity of alternative convertase were performed according to the methods described in [17] (assay using C5-depleted serum) and in [18] (assay using C5 inhibitors). Briefly, rabbit erythrocytes were prepared in the same way as for haemolytic assay but instead of fB-depleted serum 5% C5-depleted serum (Complement Technology) or 5% normal human serum diluted in Mg^{2+}-EGTA buffer and supplemented with 20 µg/ml OmCI was used. Convertase formation was examined at six time points: 5, 10, 20, 30, 40 and 50 min. Lack of C5 or its inhibition by OmCI resulted in cascade progression up to C3bBbC3b level but not further. At the chosen time point erythrocytes were washed with ice-cold 40 mM EDTA-GVB buffer (40 mM EDTA, 5 mM veronal buffer, 0.1% gelatin, 145 mM NaCl) and then overlaid 1:1 with 40 mM EDTA-GVB buffer containing 1:40 dilution of guinea pig serum (Harlan Laboratories, Indianapolis, IN) and incubated for 20 min at 37 °C. Guinea pig serum was used as a source of terminal complement components, whereas the presence of EDTA prevented *de novo* convertase formation and allowed development of membrane attack complex formation only on the platform of preexisting convertases. The readout was performed in the same way as in haemolytic assay.

C3NeF-positive and control immunoglobulin preparations

The immunoglobulin fraction containing C3NeF was obtained from EDTA-plasma of the patient diagnosed with C3G and positive for C3NeF. Briefly, blood was collected and whole immunoglobulin fraction was purified by affinity chromatography using NAb™ protein A/G 5 ml spin columns (Thermo Fisher, Waltham, MA), according to manufacturer's protocol. Control immunoglobulin preparation was purified in the same way from normal human serum, pooled from 10 healthy individuals. This study was performed in accordance with the Declaration of Helsinki.

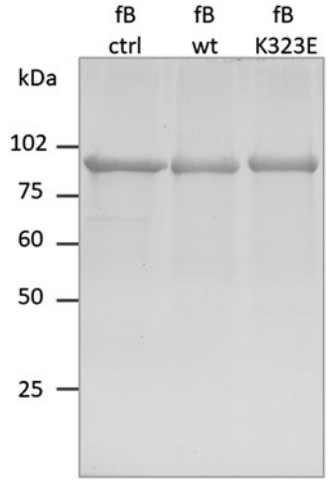

Figure 1. Expression and purity of recombinant wild-type factor B and K323E mutant. Purified, his-tagged recombinant wild-type (wt) and K323E mutant of factor B (fB) as well as plasma-purified fB (ctrl) were run on 10% SDS-PAGE and protein bands were stained with Coomassie dye.

Results

Protein expression and purification

Wild-type fB and its K323E mutant were purified to homogenicity as assessed by Coomassie staining (Figure 1). The final yield of purified protein was 11.3 mg and 4.7 mg from 1 litre of conditioned medium for wild type and K323E mutant, respectively.

Functional analysis of his-tagged recombinant fB proteins

In order to examine whether purified recombinant fB proteins with His-tag added at C terminus retain their alternative pathway (AP) haemolytic activity, we performed the haemolytic assay in fB-depleted serum. As expected, serum lacking factor B was devoid of AP haemolytic activity. Addition of either plasma-purified or recombinant, his-tagged proteins restored AP haemolytic activity in a dose-dependent manner (Figure 2). Dunnet's multiple comparison test showed no differences in haemolytic activity curves for plasma purified, recombinant wild-type and K323E proteins implying that expression and further purification process does not influence negatively the specific activity of fB.

The haemolytic assay shows only the overall effect of complement activation but provides no information about the efficiency of particular activation step. Therefore, we ran a preliminary experiment to find out whether K323E mutant is able to form convertases of extended stability. Factor B-depleted serum reconstituted with 20 µg/ml of K323E protein formed AP convertases with half-life twice longer than convertases formed in the same serum reconstituted with wild-type fB (Figure 3(A)). Next, we checked whether K323E mutant shows the same properties when mixed with human serum. Such dominant character of K323E variant would enable to use it as a positive control in assays, in

which AP convertases are built directly in patient serum. Normal human serum supplemented with K323E mutant formed AP convertases of significantly longer half-life in comparison to serum supplemented wild type fB (Figure 3(B)). Interestingly, the addition of 20 μg/ml of wild-type fB resulted in an increase of haemolysis at time points characterized by maximal convertase activity (10 and 20 min), but did not influence decay rate, which was the same as for normal human serum with no fB added.

His-tagged K323E factor B mutant mimics C3NeF in functional convertase assay

Knowing that K323E mutant supports the formation of resistant-to-decay AP convertase when mixed with whole serum, we compared its effect to the preparation of immunoglobulins isolated from the C3NeF positive patient. The similar preparation obtained from normal human serum was used as a negative control. The assay was performed in C5-depleted serum. Such experimental conditions are ideal for testing the influence of purified components on the activity of complement convertases. At the time points corresponding to the decay phase (≥30 min) there were no significant differences in the activity of convertases formed in C5-depleted serum and the same serum supplemented with 300 μg/ml of control immunoglobulins (Figure 4). Conversely, the addition of 300 μg/ml of C3NeF preparation resulted in significant increase of convertase activity at 30, 40 and 50 min. The similar but even more pronounced effect was observed when as little as 20 μg/ml of the K323E mutant was added to C5-depleted serum (Figure 4).

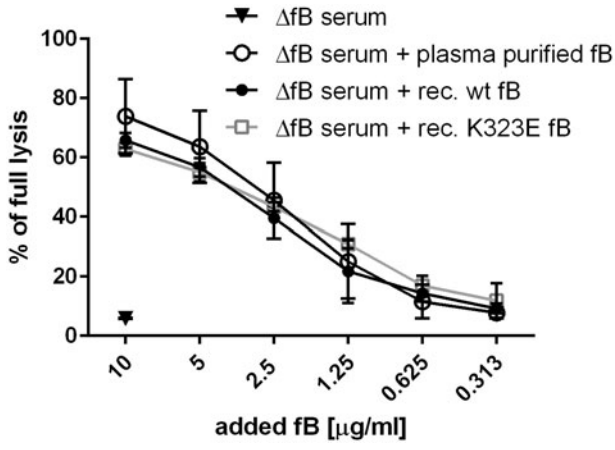

Figure 2. Haemolytic potential of recombinant, his-tagged complement factor B. Rabbit erythrocytes were mixed with 5% factor B (fB)-depleted serum (Δ fB, negative control) or alternatively with Δ fB serum supplemented with various concentrations of plasma-purified fB, wild type recombinant fB (rec. wt fB) or recombinant K323E mutant (rec. K323E fB). After 30 min erythrocytes were centrifuged and the amount of released haemoglobin was measured at 405 nm. The graph shows the mean results obtained from three independent experiments and error bars show standard deviation.

Discussion and conclusion

Renal diseases such as aHUS or C3G are classified as very rare [22] but life-threatening conditions, which lead to loss of renal function. Uncontrolled activation of the complement alternative pathway has been described for all subtypes of C3G. In most of the cases, this deregulation is

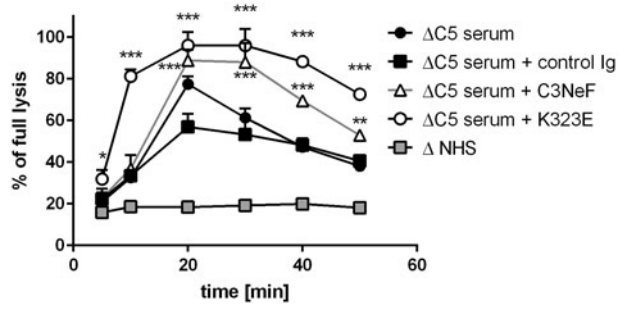

Figure 4. K323E mutant of complement factor B mimics C3NeF in convertase functional assay. C5-depleted serum was supplemented with 300 μg/ml of a preparation of control immunoglobulin (control Ig) obtained from healthy individuals or C3NeF-positive preparation. Alternatively, C5-depleted serum was mixed with 20 μg/ml of K323E mutant of factor B. The activity of alternative convertase was measured as described in [17]. The graph shows the results obtained from three independent experiments. Symbols ** and *** depicts statistically significant differences in relation to C5-depleted serum, according to Dunnett's multiple comparison test. Heat-inactivated normal human serum (Δ NHS) was used as a negative control.

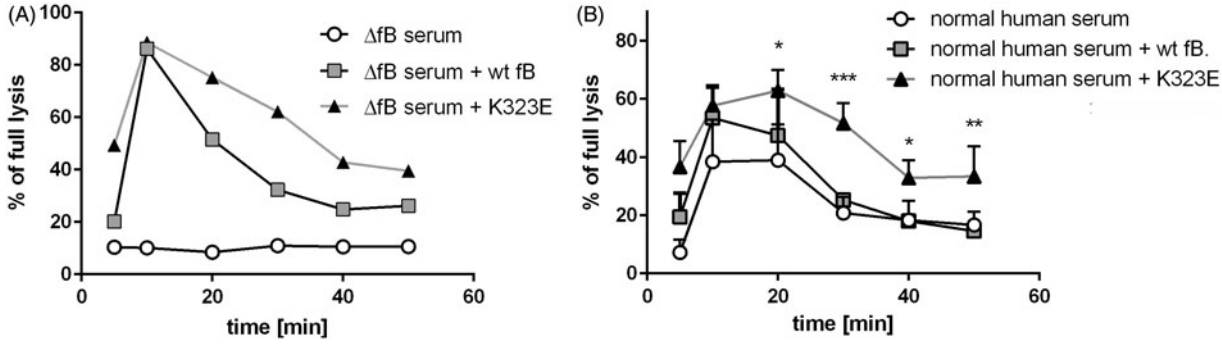

Figure 3. Recombinant his-tagged factor B forms functional convertases. Ability to convertase formation by recombinant, his-tagged factor B (fB) and its K323E variant was examined in two-step functional convertase assay with OmCI as a C5 blocker. In the preliminary experiment (A, single repetition) the standard procedure (18) was modified in a way that 5% fB-depleted serum supplemented with 20 μg/ml of tested, recombinant fB proteins replaced normal human serum. Thereafter, we checked whether K323E mutant forms convertase of prolonged half-life when mixed with normal human serum (B). Twenty micrograms per milliliter of recombinant wild type or K323E mutant of complement factor B were added to 5% normal human serum and subsequently, the activity of alternative complement convertase was assessed as described in [18]. The graph shows the results obtained from three independent experiments. Symbols *, ** and *** depict statistically significant differences between K323E and wild type factor B at $p < .05$; ,01 and $p < .001$ respectively, according to Dunnett's multiple comparison test.

accompanied by the presence of C3NeF with detection levels of approximately 80% among DDD patients [23] and 60% in C3GN [10,24]. Given that binding of the antibody to convertase complex does not automatically implicate the effect on complement regulation, that autoantibodies binding C3/C5 alternative convertases were also found in healthy individuals [25] and that anti-idiotypic response to C3NeF was reported [26], functional convertase assays seem to be the most reliable diagnostic methods.

Nonetheless, functional analyses of complement convertases are vulnerable to a number of pitfalls, which generate either false positive and false negative results. This statement is confirmed by the fact, that substantial number of the participating laboratories are not capable to correctly report the presence of C3NeF in reference material [16]. There are several approaches to measure convertase activity. Stepwise reconstruction of the enzymatic complex from purified components has been performed on artificial surfaces like biosensor chip [19] or ELISA microplate [10] or, alternatively on the surface of sheep erythrocytes previously sensitized with antibodies, and opsonized with C3b deposited due to activation of classical pathway [27]. A potential source of artefacts in these methods stems from non-physiological conditions of convertase formation and lack of other serum components capable to interfere with the complement cascade. The assay detecting C3NeF, which utilizes full serum as an analyte was originally designed by Rother [28] and further modified [29]. This concept assumes that complement activation by human serum on the surface of sheep erythrocytes allows separation of activation phase (up to the step of convertase formation) from terminal phase at the time point of 20 min. Thereafter addition of rat serum in the presence of EDTA creates conditions permissive only for MAC formation from preexisting convertases. Sheep erythrocytes are not an optimal target for spontaneous activation of the alternative complement pathway due to elevated presence of sialic acid residues [30] and therefore such model theoretically enables discrimination between convertases of normal and prolonged half-life. However, as elaborated by Blom et al. [18], convertase formation and dissociation, even if slowed down, are dynamic processes and setting the exact time point of transition from activation to terminal phase is purely arbitrary. In practice, upon stimulation some sera reach their maximal convertase activity (so-called T_{max} time) earlier or later than anticipated from Gaussian distribution, which disables fair conclusions from the results of Rother's assay. This may explain why Paixao-Calvacante and coworkers classified less than half of the samples as C3NeF positive by Rother's assay in comparison to the results of the COS-P assay [10]. Being aware of the abovementioned problems we introduced a new concept for measuring complement convertase activity, which is based on C5-depleted serum [17] or C5 blockers [18]. Disabling complement propagation beyond the level of C5 cleavage gives a possibility to measure convertase activity at optimal time point, whereas using serum concentration much lower than physiological (e.g. 5%) pushes T_{max} values from seconds to minutes range. The assay with C5 blockers is capable to screen for convertase abnormalities but cannot point out the cause of abnormality when whole serum is used as a test sample. We chose this method to demonstrate a dominant character of K323E mutation, which was originally identified by Goicoechea de Jorge and coworkers in patients suffering from aHUS [20]. According to their study K323E mutant forms C3bBb convertase resistant to decay by fH, the main soluble inhibitor of the alternative complement pathway as well as DAF/CD55 – a membrane-bound complement inhibitor with DAA function. Consequently, convertase assembled from K323E mutant should be characterized by increased half-life either in soluble or membrane-bound form, and such phenotype would result in the same outcome as the presence of C3NeF autoantibodies. Having checked that the effect of the K323E mutant is visible when mixed even with prevailing concentrations of wild type fB in normal human serum, we applied the assay based on C5-depleted serum, which was originally designed to test the influence of purified components on convertase stability. The activity curve of convertase formed upon addition of immunoglobulins from C3NeF-positive individual showed significantly prolonged decay phase comparing to conditions, where immunoglobulins from healthy individuals were added and this effect was mimicked by addition of K323E mutant (Figure 3).

Performance of the haemolysis-based functional assays depends on many variables. It is worth notifying that different batches of depleted sera (e.g. C5–depleted serum) may generate different T_{max} values depending on a degree of complement consumption during immunodepletion. We reported previously [17,18] that T_{max} point is reached faster in sera of higher effective complement content. This putative effect is visible when comparing activity profiles of convertases formed in normal human serum (Figure 2) and C5-depleted serum (Figure 3). Therefore, an internal standard addible to each reference serum used for functional assay would be an advantage. Another reason for applying an internal standard is variability of rabbit erythrocytes, which are difficult to normalize due to rabbit-to-rabbit differences, delivery/handling issues and time within the term of validity when erythrocytes are used for experiments. We noticed that experiments performed on different batches of rabbit erythrocytes (or even on the same batch but after several days) generate different T_{max} values in spite of the application of the same protocol and this is the main source of inter-assay deviations. The putative readout of the internal standard would mirror these changes and enable to distinguish between prolonged convertase decay due to late T_{max} from prolongation due to, e.g. C3NeF presence. In our opinion such serum and erythrocyte-based inconsistencies are the main obstacles in the reliable C3NeF analysis by haemolysis-based methods. However, the utility of K323E fB protein is not limited to haemolysis-based assays since this variant may be applicable as a standard in any other functional assay including e.g. COS-P or FPC tests [10].

Recently, international standards for various complement assays were introduced in order to achieve consensus between diagnostic laboratories [31]. In terms of C3NeF assays, there is still no acknowledged internal standard and usage of chosen, highly C3NeF-positive preparation of

immunoglobulins or whole serum is a common practice [10]. This approach is however practically and ethically problematic since it demands additional blood collection from patients only for standardization purposes. Additionally, the level of C3NeF may change during consecutive blood collections and ongoing therapies [24]. Instead, we propose to use recombinant K323E mutant expressed in HEK293 Freestyle cells. These cells are adjusted to high-density, suspension culture in a defined serum-free medium thus allowing to obtain a high yield of recombinant fB. The protocol for cell maintenance and transfection is well described and has been successfully used by several groups [32,33]. According to our results, this system provides a substantial amount of K323E mutant that retains its full haemolytic activity. Noteworthy, amount of K323E mutant obtained from 1 litre of conditioned medium from transfected cells is sufficient to perform approximately 5000 analyses. Furthermore, the C-terminal his-tag has no influence on protein's function but reduces the purification process to a single affinity chromatography step, which is an undoubted advantage.

Our findings are important in the context of proposed therapeutic options to treat complement-mediated renal diseases, which among others include B-cell depleting agents (e.g. rituximab) and C5 blockers (eculizumab) [34–36]. Elimination of B cells as a source of autoantibodies should theoretically reduce the disease activity in C3NeF-positive individuals. However, data regarding potential benefits of such strategy is limited to a single case report by Giaime et al. [37] who achieved complete remission of DDD. Other reports show that application of rituximab in analogous cases was insufficient (reviewed in [35]), even despite complete depletion of B cells and reducing C3NeF to an undetectable level [38]. Importantly, there is much bigger evidence that eculizumab may ameliorate disease in certain C3G patients (reviewed in [39]), including rituximab non-responders [35]. Mimicking C3NeF by a gain of function mutations in early complement components is an issue significant for clinical interventions. Although we documented such phenomenon for mutation in FB, we stress that mimicking C3NeF phenotype in functional assays can be a case for other autoantibodies (e.g. anti-FH) as well as genetic changes in FH or C3 proteins. Of note, approximately 20% of the patients with C3G and 70% of the patients with aHUS carry mutations in complement components, which may influence half-life of AP convertases [40]. Such C3Nef-like activity caused by mutation but not autoantibody (or due to the coexistence of both conditions) cannot be controlled by B-cell depletion. Thus the possibility of "genetic C3NeF" should be investigated in rituximab non-responders. Nonetheless, eculizumab theoretically disables further propagation of complement cascade in such patients and therefore may be considered as a preferred therapeutic. Only recently Marinozzi et al. further subclassified nephritic factors into three groups: antibodies that stabilized C3 but not C5 alternative convertase, antibodies stabilizing both alternative convertases and antibodies stabilizing C5 convertase only [41]. Stabilization of C5 convertases (so-called C5NeF activity) was associated with elevated level of soluble terminal complement components (sC5b-9, attributable to downstream processing of C5 molecule) in plasma and significant overrepresentation in C3GN over DDD patients group [41]. This is another example of abnormalities in the complement system, which are indistinguishable in some functional assays but result in different functional or pathological consequences or may need different therapeutic approach. Therefore, we underline the importance of proper diagnosis, not limited to general screening of complement aberrations but discriminating between particular types of autoantibodies and genetic changes as possible causative factors.

Disclosure statement

The authors report no conflicts of interest.

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References

[1] Ricklin D, Hajishengallis G, Yang K, et al. Complement: a key system for immune surveillance and homeostasis. Nat Immunol. 2010;11:785–797.
[2] Fleming SD. Natural antibodies, autoantibodies and complement activation in tissue injury. Autoimmunity. 2006;39:379–386.
[3] Schmidt CQ, Lambris JD, Ricklin D. Protection of host cells by complement regulators. Immunol Rev. 2016;274:152–171.
[4] Kajander T, Lehtinen MJ, Hyvarinen S, et al. Dual interaction of factor H with C3d and glycosaminoglycans in host-nonhost discrimination by complement. Proc Natl Acad Sci USA. 2011;108:2897–2902.
[5] Sethi S, Nester CM, Smith RJ. Membranoproliferative glomerulonephritis and C3 glomerulopathy: resolving the confusion. Kidney Int. 2012;81:434–441.
[6] Zhang Y, Meyer NC, Wang K, et al. Causes of alternative pathway dysregulation in dense deposit disease. Clin J Am Soc Nephrol. 2012;7:265–274.
[7] Servais A, Fremeaux-Bacchi V, Lequintrec M, et al. Primary glomerulonephritis with isolated C3 deposits: a new entity which shares common genetic risk factors with haemolytic uraemic syndrome. J Med Genet. 2007;44:193–199.
[8] Seelen MA, Daha MR. The role of complement in autoimmune renal disease. Autoimmunity. 2006;39:411–415.
[9] Daha MR, Austen KF, Fearon DT. Heterogeneity, polypeptide chain composition and antigenic reactivity of C3 nephritic factor. J Immunol. 1978;120:1389–1394.
[10] Paixao-Cavalcante D, Lopez-Trascasa M, Skattum L, et al. Sensitive and specific assays for C3 nephritic factors clarify mechanisms underlying complement dysregulation. Kidney Int. 2012;82:1084–1092.
[11] Daha MR, Van Es LA. Stabilization of homologous and heterologous cell-bound amplification convertases, C3bBb, by C3 nephritic factor. Immunology. 1981;43:33–38.
[12] Jozsi M, Strobel S, Dahse HM, et al. Anti factor H autoantibodies block C-terminal recognition function of factor H in hemolytic uremic syndrome. Blood. 2007;110:1516–1518.

[13] Goodship TH, Pappworth IY, Toth T, et al. Factor H autoantibodies in membranoproliferative glomerulonephritis. Mol Immunol. 2012;52:200–206.
[14] Parente R, Clark SJ, Inforzato A, et al. Complement factor H in host defense and immune evasion. Cell Mol Life Sci. 2017;74:1605–1624.
[15] Lopez-Trascasa M, Marin MA, Fontan G. C3 nephritic factor determination. A comparison between two methods. J Immunol Methods. 1987;98:77–82.
[16] Prohaszka Z, Nilsson B, Frazer-Abel A, et al. Complement analysis 2016: clinical indications, laboratory diagnostics and quality control. Immunobiology. 2016;221:1247–1258.
[17] Okroj M, Holmquist E, King BC, et al. Functional analyses of complement convertases using C3 and C5-depleted sera. PLoS One. 2012;7:e47245.
[18] Blom AM, Volokhina EB, Fransson V, et al. A novel method for direct measurement of complement convertases activity in human serum. Clin Exp Immunol. 2014;178:142–153.
[19] Harris CL, Pettigrew DM, Lea SM, et al. Decay-accelerating factor must bind both components of the complement alternative pathway C3 convertase to mediate efficient decay. J Immunol. 2007;178:352–359.
[20] Goicoechea de Jorge E, Harris CL, Esparza-Gordillo J, et al. Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. Proc Natl Acad Sci USA. 2007;104:240–245.
[21] Matthews WJ Jr, Goldberger G, Marino JT Jr, et al. Complement proteins C2, C4 and factor B. Effect of glycosylation on their secretion and catabolism. Biochem J. 1982; 204:839–846.
[22] Goodship TH, Cook HT, Fakhouri F, et al. Atypical hemolytic uremic syndrome and C3 glomerulopathy: conclusions from a "Kidney Disease: Improving Global Outcomes" (KDIGO) controversies conference. Kidney Int. 2017;91:539–551.
[23] Leroy V, Fremeaux-Bacchi V, Peuchmaur M, et al. Membranoproliferative glomerulonephritis with C3NeF and genetic complement dysregulation. Pediatr Nephrol. 2011;26: 419–424.
[24] Schwertz R, Rother U, Anders D, et al. Complement analysis in children with idiopathic membranoproliferative glomerulonephritis: a long-term follow-up. Pediatr Allergy Immunol. 2001;12:166–172.
[25] Spitzer RE, Stitzel AE, Tsokos GC. Evidence that production of autoantibody to the alternative pathway C3 convertase is a normal physiologic event. J Pediatr. 1990;116:S103–S108.
[26] Spitzer RE, Stitzel AE, Tsokos GC. Study of the idiotypic response to autoantibody to the alternative pathway C3/C5 convertase in normal individuals, patients with membranoproliferative glomerulonephritis, and experimental animals. Clin Immunol Immunopathol. 1992;62:291–294.
[27] Okroj M, Mark L, Stokowska A, et al. Characterization of the complement inhibitory function of rhesus rhadinovirus complement control protein (RCP). J Biol Chem. 2009;284:505–514.
[28] Rother U. A new screening test for C3 nephritis factor based on a stable cell bound convertase on sheep erythrocytes. J Immunol Methods. 1982;51:101–107.
[29] West CD. A hemolytic method for the measurement of nephritic factor. J Immunol Methods. 2008;335:1–7.
[30] Van Dijk H, Rademaker PM, Willers JM. Determination of alternative pathway of complement activity in mouse serum using rabbit erythrocytes. J Immunol Methods. 1980;36:29–39.
[31] Bergseth G, Ludviksen JK, Kirschfink M, et al. An international serum standard for application in assays to detect human complement activation products. Mol Immunol. 2013;56:232–239.
[32] Liu C, Dalby B, Chen W, et al. Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. Mol Biotechnol. 2008;39:141–153.
[33] Ido H, Harada K, Futaki S, et al. Molecular dissection of the alpha-dystroglycan- and integrin-binding sites within the globular domain of human laminin-10. J Biol Chem. 2004; 279:10946–10954.
[34] Smith RJ, Alexander J, Barlow PN, et al. and G. Dense Deposit Disease Focus. New approaches to the treatment of dense deposit disease. J Am Soc Nephrol. 2007;18:2447–2456.
[35] Rudnicki M. Rituximab for treatment of membranoproliferative Glomerulonephritis and C3 Glomerulopathies. Biomed Res Int. 2017;2017:2180508.
[36] Rousset-Rouviere C, Cailliez M, Garaix F, et al. Rituximab fails where eculizumab restores renal function in C3nef-related DDD. Pediatr Nephrol. 2014;29:1107–1111.
[37] Giaime P, Daniel L, Burtey S. Remission of C3 glomerulopathy with rituximab as only immunosuppressive therapy. Clin Nephrol. 2015;83:57–60.
[38] McCaughan JA, O'Rourke DM, Courtney AE. Recurrent dense deposit disease after renal transplantation: an emerging role for complementary therapies. Am J Transplant. 2012;12:1046–1051.
[39] Nester CM, Smith RJ. Treatment options for C3 glomerulopathy. Curr Opin Nephrol Hypertens. 2013;22:231–237.
[40] Noris M, Remuzzi G. Glomerular diseases dependent on complement activation, including atypical hemolytic uremic syndrome, membranoproliferative Glomerulonephritis, and C3 Glomerulopathy: core curriculum 2015. Am J Kidney Dis. 2015;66:359–375.
[41] Marinozzi MC, Chauvet S, Le Quintrec M, et al. C5 nephritic factors drive the biological phenotype of C3 glomerulopathies. Kidney Int. 2017;92:1232–1241.