Międzyuczelniany Wydział Biotechnologii
Uniwersytetu Gdańskiego i Gdańskiego Uniwersytetu Medycznego

mgr Anna Felberg-Miętka

Znaczenie układu dopełniacza w powstawaniu nowotworów oraz w terapii przeciwnowotworowej

Role of the complement system in cancer development and anticancer therapy

Praca przedstawiona
Radzie Dyscypliny Nauki Biologiczne Uniwersytetu Gdańskiego
celem uzyskania stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych
w dyscyplinie nauki biologiczne

Promotor: dr hab. Marcin Okrój, prof. GUMed
Instytut Biotechnologii Medycznej i Okologii Doświadczalnej
Zakład Biologii Komórki i Immunologii MWB UG-GUMed

Gdańsk 2022

Spis treści

Spis rysunków

Streszczenie

Układ dopełniacza stanowi jeden z podstawowych składników odporności nieswoistej organizmu. W jego skład wchodzi kilkadziesiąt białek obecnych w surowicy krwi i w płynach ustrojowych, wraz z receptorami i regulatorami zlokalizowanymi na powierzchni komórek. Tradycyjnie dopełniacz był postrzegany jako jedna z pierwszych linii obrony przed patogenami. Wzrastająca liczba doniesień naukowych sugeruje, iż zaburzenia w funkcjonowaniu układu dopełniacza prowadzą do wielu patologii, w tym powstawania i stworzenia warunków permisywnych dla wzrostu nowotworów. W świetle dostępnych badań nie jest jednak jasne czy układ dopełniacza sprzyja, czy też przeciwdziała powstawaniu nowotworów. Z jednej strony istnieje wiele immunoterapeutyków wykorzystujących dopełniacz jako mechanizm działania. Z drugiej strony, w świetle ostatnich badań, pojawiła się hipoteza, iż w pewnych warunkach aktywność układu dopełniacza może stanowić element wspomagający progresję nowotworu.

Celem realizowanej pracy doktorskiej jest poszerzenie wiedzy na temat znaczenia układu dopełniacza w nowotworzeniu oraz roli jaką spełnia w terapiach opartych o przeciwciała antynowotworowe. Pracę podzielono na trzy części:

I Analiza markerów aktywacji układu dopełniacza powstających w czasie leczenia chorych na białaczki B-limfocytarne przy pomocy przeciwciał anty-CD20. Korelacja otrzymanych wyników z danymi klinicznymi.

Badaliśmy aktywność cytotoksyczną surowic pobranych przed kolejnymi wlewami przeciwciał terapeutycznych alemtuzumabu i ofatumumabu od grupy dwunastu pacjentów biorących udział w badaniu klinicznym. Do badań wykorzystaliśmy nową, opisaną przez nasz zespół metodę pomiaru aktywności cytotoksycznej zależnej od dopełniacza (ang. complement-dependent cytotoxicity, CDC). Zbadaliśmy także konsumpcję dopełniacza, poziomy markerów aktywacji dopełniacza i akumulacje leku w grupie pacjentów poddanej leczeniu przeciwciałem anty-CD20, rytuksymab. We wspomnianych badaniach wykorzystano surowicę i osocze pacjentów z przewlekłą białaczką limfocytarną oraz chłoniakami B-komórkowymi, uzyskane dzięki współpracy z Katedrą i Kliniką Hematologii i Transplantologii Gdańskiego Uniwersytetu Medycznego oraz Kliniką Hematologii Instytutu Karolinska w Sztokholmie.

II Zbadanie możliwości wykorzystania czynników patogennych powodujących schorzenia autoimmunologiczne zależne od układu dopełniacza jako uniwersalnych wspomagaczy immunoterapii.

W tej części mojej pracy doktorskiej zaproponowałam nowatorskie rozwiązanie polegające na wykorzystaniu zmutowanych ludzkich białek układu dopełniacza w celu wzmocnienia aktywności cytotoksycznej immunoterapeutyków. Wyniki zostały zaprezentowane na międzynarodowej konferencji European Meetings on Complement in Human Diseases (EMCHD, Madryt 2019, na której prezentacja uzyskała wyróżnienie) i stanowią przedmiot ochrony patentowej na terenie Polski oraz analogicznej aplikacji patentowej złożonej do Europejskiego Biura Patentowego oraz jego amerykańskiego odpowiednika.

III Badanie roli czynnika I (inhibitora układu dopełniacza) w rozwoju ludzkiego niedrobnokomórkowego raka płuca.

Zbadaliśmy związek między występowaniem czynnika I (FI) w tkance nowotworowej pacjentów z niedrobnokomórkowym rakiem płuca z danymi klinicznymi. Materiał kliniczny wraz z dostępną bazą danych został udostępniony przez prof. Rubena Pio oraz prof. Luisa Montuengę z Uniwersytetu Nawarryw Pamplonie. Podczas stażu EMBO Short-Term Fellowships w instytucie Sanquin w Amsterdamie wykorzystałam metodę CRISPR/Cas9 w celu trwałego usunięcia czynnika I z komórek linii komórkowej H2087 ludzkiego niedrobnokomórkowego raka płuc, w której białko to naturalnie występuje. Uzyskane klony komórek H2087 posłużyły do dalszych badań nad rolą czynnika I w rozwoju raka płuc, wykorzystujących m.in. analizę transkryptomu oraz testy funkcjonalne.

Abstract

The complement system acts in the framework of innate immunity, playing a key role in defense against pathogens but also in the maintenance of the body's homeostasis. Several activation pathways and various pattern-recognition molecules, as well as multiple effector mechanisms including opsonization, anaphylaxis, and direct cell lysis, ensure a wide spectrum of direct and indirect cytocidal activities. The complement system is initiated by conformational and/or proteolytic changes upon the recognition of invaders. The subsequent cascade of enzymatic reactions is tightly regulated to assure that complement is activated only at specific locations currently invaded by pathogens, thus avoiding a misguided attack on host cells and tissues. Noteworthy, complement can be also targeted onto tumor cells since the transformation from normal to malignant phenotype is reflected in cell membrane composition due to accompanying metabolic changes or appearance of mutated proteins. Exposure of such novel tumor epitopes distinguishes cancer cells from their normal counterparts and makes them visible to the immune system. There is an increasing number of scientific reports suggesting that disturbances in the functioning of the complement system lead to many pathologies, including the formation of tumors. However, is still not clear how exactly complement interferes with tumorigenesis and whether the complement is a friend or foe of tumor cells. On the one hand, therapeutic modulation of complement activity emerges as an attractive target in clinical approaches and there are several anti-cancer drugs approved, which utilize the complement system as their effector mechanism. On the other hand, research shows that cancer cells can also benefit from complement activation under certain conditions.

In my Ph.D. project I propose three objectives aimed at expanding knowledge of the role of the complement system in cancer development together with basic and practical issues regarding complement role in therapeutic approaches based on anti-cancer antibodies:

I Analysis of the complement activation markers during treatment of B-cell leukemia patients with anti-CD20 antibodies. Correlation of the obtained results with clinical data.

We have analyzed the cytotoxic activity of sera collected before subsequent infusions of therapeutic antibodies alemtuzumab and ofatumumab from twelve patients participating in the clinical trial. We used a new method, described by our team, to measure complement-dependent cytotoxicity (CDC). In the next study, we investigated the complement consumption and drug accumulation in patients during anti-CD20 antibody therapy with rituximab. Serum and plasma samples from patients with chronic lymphocytic leukemia and B-cell lymphomas were obtained thanks to the collaboration with the Department of Hematology and Transplantology, Medical University of Gdańsk and Department of Hematology, Karolinska University Hospital in Stockholm.

II Exploring the applicability of pathogenic factors causing complement-dependent autoimmune diseases as universal supporters of anti-CD20 immunotherapeutics.

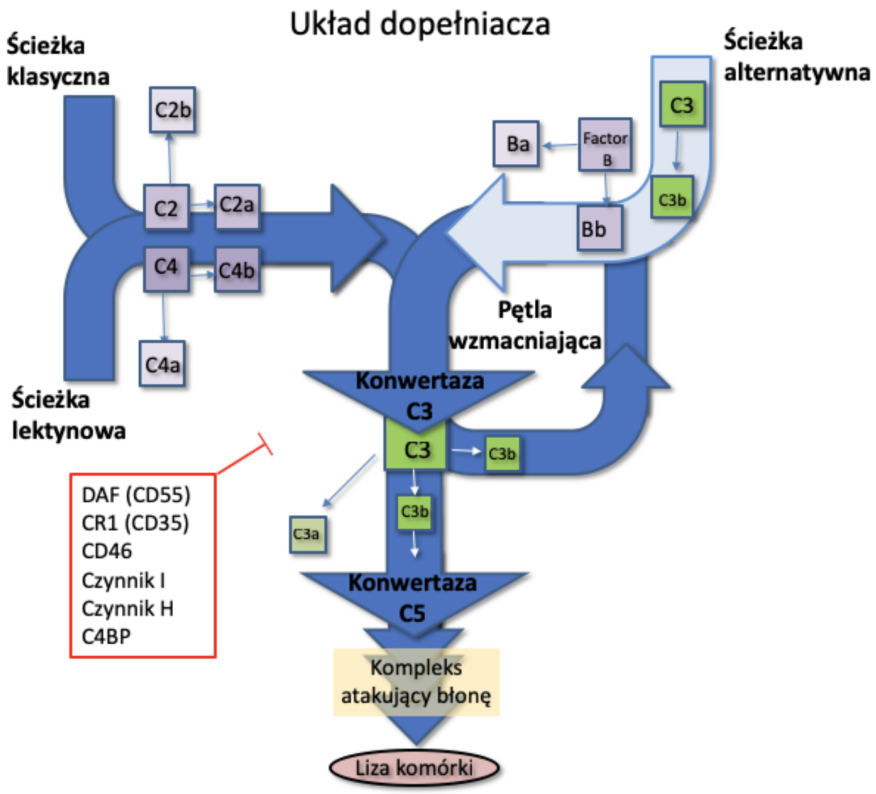
In this part, I propose an innovative idea based on the use of the gain-of-function variants of human complement proteins to enhance the cytocidal effect of immunotherapeutics. Results were presented at the European Meetings on Complement in Human Diseases (EMCHD, Madrid 2019, where the presentation was awarded) and they are either protected by a patent in Poland or become the subject of analogous patent applications submitted in the EU and US.

III Understanding the role of factor I (complement inhibitor) in the development of solid tumors using a model of human non-small cell lung cancer.

Recent research shows that cancer cells can benefit from complement activation under certain conditions. We examined the link between the expression of FI in cancer cells of patients with non-small cell lung cancer (NSCLC) and clinical outcome. The clinical material with the available database was received from prof. Ruben Pio and prof. Luis Montuenga from the University of Navarre in Pamplona. During the EMBO Short-Term Fellowships at the Sanquin Institute in Amsterdam, I used the CRISPR / Cas9 method to permanently remove factor I from cells of cell lines in which this protein is naturally present. The obtained cell lines were used for further research including functional assays and transcriptome analyses aimed to elucidate the role of factor I (FI) in the development of lung cancer.

1 Wstęp

Układ dopełniacza stanowi jeden z podstawowych składników odporności nieswoistej organizmu. Grupa kilkudziesięciu białek surowicy wchodzących w jego skład tworzy kaskadę enzymatyczną (ryc. 1), która chroni organizm gospodarza przed patogenami poprzez opsonizację i lizę komórek docelowych, a także poprzez wywołanie reakcji anafilaktycznej i nakierowanie oraz modulację odpowiedzi swoistej wobec patogenu. Wzrastająca liczba doniesień naukowych sugeruje, iż zaburzenia w funkcjonowaniu układu dopełniacza prowadzą do wielu patologii, w tym do stworzenia warunków permisywnych dla wzrostu nowotworów.



Rycina 1: Uproszczony schemat układu dopełniacza

Aktywacja oraz działanie układu dopełniacza odbywa się na drodze kaskady enzymatycznej. Poza składnikami samej kaskady, układ dopełniacza obejmuje także białka regulatorowe rozpuszczalne obecne w osoczu oraz związane z błona komórek. Podstawowe inhibitory układu dopełniacza zaznaczono na schemacie w czerwonej ramce. Wyróżnia się trzy drogi aktywacji układu dopełniacza: klasyczną, lektynową oraz alternatywną. Niezależnie od sposobu aktywacji, wszystkie trzy ścieżki prowadzą do wytworzenia konwertazy C3 – kompleksu enzymatycznego, który rozkłada składnik C3 do biologicznie aktywnych fragmentów C3a i C3b. Aktywacja składnika C3 inicjuje wszystkie funkcje efektorowe dopełniacza. Ostatecznym efektem aktywacji układu dopełniacza jest wbudowanie w błonę komórki docelowej kompleksu atakującego błonę, skutkujące utworzeniem porów i lizą osmotyczną komórki docelowej.

Działanie układu dopełniacza było dotychczas postrzegane jako mechanizm przeciwnowotworowy, co znajduje potwierdzenie w fakcie istnienia szeregu immunoterapeutyków wykorzystujących dopełniacz jako efektor. W świetle ostatnich badań pojawiła się hipoteza, iż wpewnych warunkach aktywność układu dopełniacza może stanowić element wspomagający progresję nowotworu.

Badania wykonane w pracy doktorskiej podzielono na trzy części, które dotyczyły podstawowej wiedzy na temat znaczenia układu dopełniacza w nowotworzeniu oraz roli jaką spełnia w terapiach opartych o przeciwciała antynowotworowe.

Badania miały następujące cele i zostały opisane w wymienionych publikacjach:

I Analiza markerów aktywacji układu dopełniacza powstających w czasie leczenia chorych na białaczki B-limfocytarne przy pomocy przeciwciał anty-CD20. Korelacja otrzymanych wyników z danymi klinicznymi.

G. Stasiłojć*, A. Felberg*, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin, A. Österborg, M. Okrój
"Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies"
Journal of Immunological Methods, Vol. 476, 112675, 2020
*autorzy maja równy wkład w przygotowanie publikacji
[Publikacja nr 1]

A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój
"Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab"
Frontiers in Immunology, Vol. 11, 584509, 2020
[Publikacja nr 2]

G. Stasiłojć, A. Felberg, M. Okrój
"Parameters critical for the effector mechanism of anti-CD20 antibodies revisited"
British Journal of Haematology, Vol. 180, Issue 6, 777-779, 2018
[Publikacja nr 3]

II Zbadanie możliwości wykorzystania czynników patogennych powodujących schorzenia autoimmunologiczne zależne od układu dopełniacza jako uniwersalnych wspomagaczy immunoterapii.

A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój
"Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics"
Cancer Immunology, Immunotherapy, Vol. 68, 587–598, 2019
[Publikacja nr 4]

III Poznanie roli czynnika I (inhibitora układu dopełniacza) w rozwoju guzów litych z wykorzystaniem modelu ludzkiego niedrobnokomórkowego raka płuca.

A. Felberg, M. Bieńkowski, T. Stokowy, F. Mohlin, S. Nilsson,
I. Jongerius, R. Spaapen, L. M. Montuenga, A. M. Blom, R. Pio, M. Okrój
"Elevated expression of complement factor I in lung cancer cells is associated with shorter progression-free survival and disease-specific survival"
[Manuskrypt nr 5, złożony do czasopisma]

Układ dopełniacza jest kaskadą enzymatyczną, której głównym miejscem działania jest surowica krwi oraz płyny przestrzeni pozanaczyniowej. Białka wchodzące w jego skład występują w formie proenzymow, ktore zostają sekwencyjnie aktywowane po związaniu molekuł rozpoznawczych (ang. pattern-recognition molecules, PRM) z powierzchnią komórki docelowej. Tradycyjnie dopełniacz był postrzegany jako wspomagająca, pierwsza linia obrony przed patogenami. Ostatnie dwie dekady pokazały, że oprócz zaangażowania w eliminację drobnoustrojów, pełni też wiele innych funkcji. Przykładowo, dzięki różnym białkom rozpoznawczym jest w stanie rozróżnić zdrową tkankę gospodarza od szczątków komórkowych, komórek apoptotycznych czy komórek nowotworowych. Po rozpoznaniu celu molekularnego kaskada dopełniacza jest aktywowana a następnie amplifikowana w przypadku braku działania specyficznych inhibitorów [1]. Istnienie kilkunastu PRM oraz trzech niezależnych dróg aktywacji dopełniacza zapewnia szeroki zakres ochrony organizmu.

Wyróżnia się trzy drogi aktywacji układu dopełniacza: klasyczną, lektynową oraz alternatywną (ryc. 1). Dwie pierwsze wymagają obecności odpowiednich stymulantów. W przypadku ścieżki klasycznej są to przeciwciała wiążące się z błoną komórki docelowej, a w przypadku ścieżki lektynowej lektyna wiążąca mannozę (ang. mannose binding lectin, MBL) oraz fikoliny. Aktywacja drogi alternatywnej następuje spontanicznie, a jej aktywność utrzymywana jest w sposób ciągły na niskim poziomie i kontrolowana przez działanie licznych inhibitorów zlokalizowanych na powierzchni własnych komórek oraz występujących w formie rozpuszczalnej w surowicy. Niezależnie od sposobu aktywacji, wszystkie trzy ścieżki prowadzą do wytworzenia enzymu konwertazy C3, która rozkłada składnik C3 do biologicznie aktywnych fragmentów C3a i C3b. Aktywacja składnika C3 warunkuje większość funkcji efektorowych dopełniacza. Ostatecznym ogniwem kaskady dopełniacza jest ścieżka końcowa (ang. terminal pathway) i wbudowanie w błonę komórki docelowej elementów kompleksu białkowego MAC (ang. membrane attack complex) skutkujące utworzeniem porów i lizą osmotyczną komórki.

Zmiany metaboliczne zachodzące w komórce podczas transformacji nowotworowej prowadzą do różnic w składzie fosfolipidowym błony oraz ekspozycji nowopowstałych epitopow charakterystycznych dla nowotworów, co czyni je bezpośrednio lub pośrednio rozpoznawalnymi dla układu dopełniacza. Niestety, miano naturalnie występujących przeciwciał przeciwnowotworowych (inicjujacych klasyczna droge aktywacji dopełniacza) jest zazwyczaj niskie, przez co wydajna aktywacja układu dopełniacza na komorkach nowotworowych jest utrudniona. Układ dopełniacza zapewnia również szereg pośrednich mechanizmów eliminacji patogenów np. takich jak opsonizacja (opłaszczanie), anafilaksja, pobudzanie fagocytozy, naprowadzanie i wzmacnianie odpornosci swoistej. Przy eliminacji komórek nowotworowych istotne są zarówno mechanizmy bezpośrednie działania układu dopełniacza jak i pośrednie, po-

nieważ opłaszczenie komórki nowotworowej przez składniki dopełniacza ułatwia jej pochłonięcie przez fagocyty. Aby unikać ataku ze strony układu dopełniacza, komórki nowotworowe zaadoptowały kilka strategii obrony m.in. nadekspresję inhibitorów dopełniacza zwiazanych z błoną komórkową.

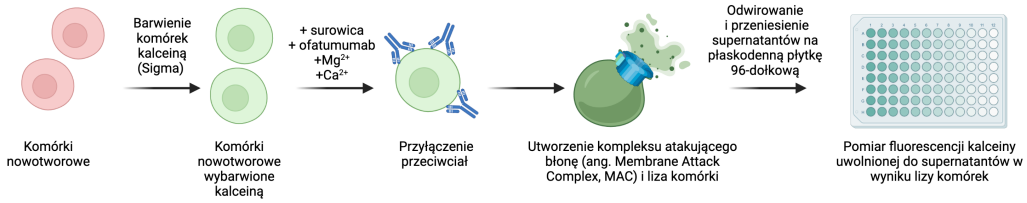
Zainteresowanie układem dopełniacza w kontekście nowotworów powróciło wraz z wprowadzeniem do użytku klinicznego przeciwciał antynowotworowych takich jak anty-CD20. Wprowadzenie do standardowej terapii przeciwnowotworowej przeciwciał rozpoznających determinanty komórek rakowych przyczyniło się do znacznego wydłużenia czasu przeżycia pacjentów oraz obniżenia tempa progresji nowotworów, a wprowadzenie rytuksymabu zostało uznane za największa innowacje w leczeniu chłoniakow w ciagu ostatniego półwiecza [2]. Pomimo tego, że przeciwciała przeciwnowotworowe zrewolucjonizowały leczenie, nie u wszystkich pacjentów występuje odpowiedź kliniczna, a u części pojawia się oporność na terapię.

Znanym problemem podczas terapii przeciwciałami anty-CD20 jest wyczerpanie puli białek dopełniacza z powodu stosowania wysokich dawek przeciwciał terapeutycznych [3], ale istnieje ograniczona liczba danych eksperymentalnych, szczegółowo opisujących aktywność układu dopełniacza między kolejnymi dawkami leku. Co więcej, nie było do tej pory danych w odniesieniu do terapii dwoma przeciwciałami jednocześnie, co teoretycznie mogłoby skutkować z jednej strony wzmocnionym efektem cytotoksycznym z uwagi na dwa niezależne cele molekularne, a z drugiej strony wzmożoną konsumpcją białek układu dopełniacza. Podczas terapii ważne jest określenie, jak długo stan wyczerpania białek układu dopełniacza utrzymuje się po wlewie przeciwciał oraz czy pula białek zostaje odnowiona przed kolejnym podaniem leku.

Powszechnie stosowaną metodą pomiaru aktywności cytotoksycznej zależnej od dopełniacza (ang. complement-dependent cytotoxicity, CDC) surowicy pacjentów jest test hemolityczny (CH50) na erytrocytach owczych opłaszczonych specyficznymi przeciwciałami. Jego niewątpliwą zaletą jest prostota, natomiast brak ludzkich inhibitorów dopełniacza na powierzchni erytrocytów owczych może nie odzwierciedlać kluczowych interakcji między surowicą pacjenta a inhibitorami dopełniacza na powierzchni komórek docelowych, prowadząc tym samym do niedoszacowania niektórych istotnych efektów. Biorąc pod uwagę fakt, że jest to istotny problem, w swojej pracy doktorskiej proponuję alternatywną metodę pomiaru aktywności cytotoksycznej (CDC) surowicy pacjentów. W opracowanej metodzie (ryc. 3) zamiast erytrocytów wykorzystujemy ludzkie komórki nowotworowe znakowane kalceiną, rozpoznawane przez identyczne przeciwciała jak te stosowane podczas terapii. **Test cytotoksyczny z uwolnieniem kalceiny jest metodą pozwalającą na ilościowe oszacowanie zjawiska śmierci komórek. Metoda została dokładnie opisana przez nasz zespół jako test przydatny do pomiaru lizy zależnej od układu dopełniacza oraz aktywności cytotoksycznej surowicy [4]**, oraz była wielokrotnie wykorzystana w mojej pracy doktorskiej. Po krótce, komórki CD20-pozytywne inkubujemy 30 minut w pożywce z dodatkiem kalceiny. Żywe komórki aktywnie pobierają kalceinę i metabolizują ją do fluorescencyjnej pochodnej akumulowanej w cytoplazmie. Po skończonej inkubacji komórki płuczemy w celu usunięcia wolnej kalceiny z roztworu. Do studzienek V-kształtnej płytki 96-dołkowej dodajemy komórki w ilości 10^5/dołek i zawieszamy w roztworze zawierającym pożądane stężenie surowicy i przeciwciała terapeutycznego. Następnie, płytki inkubujemy przez 30 minut w 37°C

z wytrząsaniem. Po inkubacji odwirowujemy, a supernatant przenosimy na płaskodenną płytkę i mierzymy fluorescencję przy 485/515 nm. Jako 100% lizy przyjmujemy odczyt fluorescencji uzyskany dla komórek lizowanych 2% NP40 lub 30% DMSO.

W pracy [4] pokazaliśmy aktywność cytotoksyczną surowic pobranych od dwunastu pacjentów biorących udział w badaniu klinicznym, w którym sprawdzano zastosowanie jako terapii pierwszego rzutu alemtuzumabu (przeciwciało anty-CD52) razem z wysoką (2000 mg) dawką ofatumumabu (przeciwciało anty-CD20). Zbadaliśmy funkcjonalność układu dopełniacza przed kolejnymi wlewami przeciwciał terapeutycznych alemtuzumabu i ofatumumabu, stosowanych w terapii łączonej. Wyniki wykazały, że podawanie samego alemtuzumabu lub w połączeniu z ofatumumabem (nawet w wysokiej dawce 2000 mg) nie zmniejszało trwale dostępnej puli białek dopełniacza u większości pacjentów. Porównaliśmy i skorelowaliśmy wyniki uzyskane w teście hemolitycznym i w teście cytotoksycznym wykorzystującym zjawisko uwolnienia kalceiny. Zbiorcze wyniki testu CDC z uwolnieniem kalceiny, przeprowadzonego na komórkach CD20-pozytywnych oraz wyniki testu hemolitycznego korelowały ze sobą, ale różniły się w przypadku analizy danych dla poszczególnych pacjentów.



Rycina 2: Schemat testu CDC z wykorzystaniem komórek nowotworowych znakowanych kalceiną

Komórki nowotworowe (np. Raji) w stężeniu do $1 \times 10^6/ml$ w pożywce hodowlanej wybarwiano kalceiną (Sigma), po półgodzinnej inkubacji resztki kalceiny wypłukano buforem PBS z dodatkiem 1 mM $CaCl_2$ i $MgCl_2$. Następnie komórki inkubowano z roztworem surowicy z dodatkiem przeciwciała terapeutycznego ofatumumabu w buforze PBS z dodatkiem 1 mM $CaCl_2$ i $MgCl_2$. Po 30 minutach inkubacji zebrano supernatanty i zmierzono fluorescencje kalceiny uwolnionej do supernatantów w wyniku lizy komórek.

Rytuksymab (przeciwciało anty-CD20) jest przeciwciałem chimerycznym ludzko-mysim zawierającym ludzkie sekwencje stałe IgG1 oraz złożone z łańcuchów lekkich i ciężkich mysie sekwencje zmienne. Nie ma wątpliwości, że rytuksymab aktywuje układ dopełniacza *in vitro*, niemniej jednak również inne mechanizmy efektorowe np. ADCC, bezpośrednia śmierć apoptotyczna czy wzmożona fagocytoza mogą mieć znaczenie w jego działaniu terapeutycznym *in vivo*. W środowisku onkologów toczy się debata, który z mechanizmów jest kluczowy dla działania rytuksymabu. Wyniki doświadczeń w ramach różnych badań wydają się być sprzeczne. Różnice mogą wynikać z różnych stadiów zaawansowania nowotworów, z różnego stosunku komórek docelowych do komórek efektorowych u poszczególnych pacjentów i w poszczególnych modelach *in vivo*, z tła genetycznego zwierząt użytych do doświadczeń, w końcu z różnic w metodyce i zastosowania nieadekwatnych metod. Dlatego też konieczne jest zidentyfikowanie znaczenia powyższych zmiennych tak szczegółowo, jak to możliwe a następnie zaordynowanie terapii najbardziej wskazanej dla danego przypadku.

W ramach pracy doktorskiej podsumowałam obecny stan wiedzy na temat mechanizmów działania przeciwciał anty-CD20 [5]. CD20 jest cząsteczką obecną na powierzchni większości stadiów rozwojowych limfocytów B. Jej funkcja nie jest w pełni poznana, ale odkryto, że ma związek z dojrzewaniem komórek B oraz sygnalizacją wapniową [6, 7]. Liczba potencjalnych epitopów jest ograniczona, ponieważ cząsteczka CD20 ma jedynie dwa zewnątrzkomórkowe fragmenty: reszty 72–80 (mała pętla) i reszty 140–186 (większa pętla). Pomimo tego, że miejsca wiązania większości znanych przeciwciał anty-CD20 pokrywają się, ich mechanizmy efektorowe mogą być różne. W niektórych przypadkach przeciwciała mogą bezpośrednio indukować działanie cytotoksyczne, aktywując szlaki sygnalizacyjne prowadzące do programowanej śmierci komórki (ang. programmed cell death, PCD). Alternatywnie, mogą wykorzystywać komponenty układu immunologicznego gospodarza, takie jak układ dopełniacza czy komórki z receptorem Fc, zdolne do indukowania odpowiedzi cytotoksycznej – w drugim przypadku mówimy o cytotoksyczności komórkowej zależnej od przeciwciał (ang. antibody-dependent cellular cytotoxicity, ADCC).

Przeciwciała anty-CD20 dzielą się na trzy typy: przeciwciała typu I skutecznie aktywują układ dopełniacza, a słabo indukują PCD podczas gdy przeciwciała typu II skutecznie aktywują PCD, natomiast słabo aktywują układ dopełniacza. Nieliczne przeciwciała typu III łączą w sobie cechy typu I i typu II [8, 3, 9]. Co ważne, zarówno przeciwciała typu I jak i II są zdolne do wywoływania ADCC. ADCC zachodzi efektywnie przy niskim poziomie ekspresji CD20, który nie jest wystarczający do optymalnej aktywacji dopełniacza [10]. Większość przeciwciał anty-CD20 zalicza się do typu I, jednak różnią się między sobą siłą wywoływania efektu cytotoksycznego zależnego od dopełniacza. Na podstawie porównania dwóch popularnych przeciwciał monoklonalnych anty-CD20 typu I rytuksymabu i ofatumumabu wstępnie określono, że na skuteczną aktywację CDC wpływa kilka czynników, takich jak szybkość dysocjacji (ang. off-rate), gęstość cząsteczek będących celem molekularnym czy wzajemne interakcje między fragmentami Fc przeciwciał i odległość docelowego epitopu od błony komórkowej [11]. Ofatumumab, który jest lepszym induktorem CDC, rozpoznaje epitop położony bliżej błony komórkowej i dysocjuje wolniej niż rytuksymab. Wymienione wyżej przeciwciała anty-CD20 (rytuksymab i ofatumumab), jak również inne, w tym alemtuzumab (przeciwciało anty-CD52), stosowane są w leczeniu białaczek i chłoniaków B-komórkowych jako terapeutyki pierwszego rzutu.

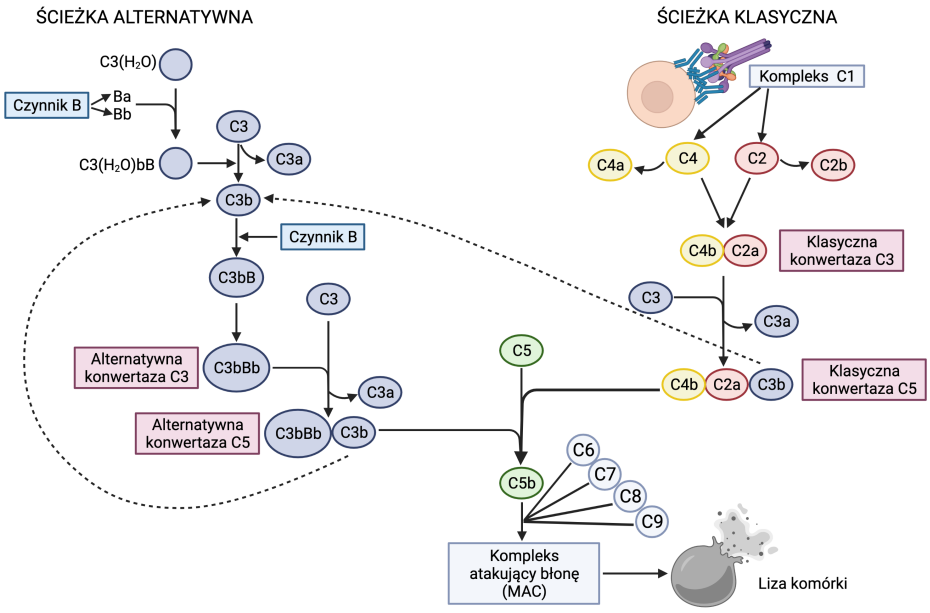
Niewiele przeciwciał typu III zostało opisanych, a badania nad ich zastosowaniem nadal trwają, ale wydaje się, że połączenie cech przeciwciał typu I i II mogłoby być efektywniejsze w zwalczaniu komórek nowotworowych [8, 12, 9]. Co więcej, jest wysoce prawdopodobne, że mechanizm działania mógłby się zmieniać w zależności od czynników komórkowych (np. gęstość celu molekularnego na powierzchni komórki) czy typu nowotworu (nowotwory krążące a niekrążące).

Obecne wytyczne dla immunoterapii są podyktowane głównie przesłankami natury genetycznej i histologicznej, a nie wynikami badań ujmujących czynniki mogące wpływać na efektywność aktywacji dopełniacza lub ADCC. **Uzupełnienie wiedzy na temat mechanizmów efektorowych immunoterapeutyków jest ważne nie tylko z punktu widzenia naukowego, ale może mieć również znaczenie praktyczne w leczeniu chorych na białaczki i chłoniaki.**

W pracy doktorskiej pokazano dane dotyczące markerów aktywacji układu dopełniacza, zużycia komponentów dopełniacza oraz kumulacji rytuksymabu w osoczu pacjentów z przewlekłą białaczką limfocytarną (ang. chronic lymphocytic leukemia, CLL) oraz chłoniakami nieziarniczymi (ang. non-Hodgkin's lymphomas, NHL) [13]. Otrzymane wyniki badań przeprowadzonych na materiale pozyskanym w sumie od 28 pacjentów wykazały wzrost stężenia markerów, takich jak C4d i TCC, a także konsumpcję dopełniacza po pierwszej infuzji oraz mniejszą intensywność ww. zjawisk po kolejnych infuzjach. W przypadku pacjentów z CLL wiązało się to ze znaczącym spadkiem ilości krążących komórek białaczkowych. Analiza stężenia rytuksymabu w surowicach pobranych przed kolejnym podaniem leku wykazała iż znaczna liczba pacjentów akumuluje lek przy interwale 4-tygodniowym. Efektem tego zjawiska jest wysoka aktywność cytotoksyczna surowic pacjentów (badana poprzez test funkcjonalny CDC z uwolnieniem kalceiny) która w dużym stopniu pokrywa się z wysokim stężeniem rytuksymabu w ww. surowicach oraz fakt, iż nowe podanie leku nie poprawia już parametrów CDC w modelowym doświadczeniu *in vitro*. Otrzymany wynik wskazuje na konieczność indywidualnego, bieżącego monitorowania stężenia rytuksymabu i/lub aktywności CDC w surowicach pacjentów poddanych leczeniu.

Kolejną ważną przyczyną oporności na terapeutyki działające za pośrednictwem układu dopełniacza jest nadekspresja inhibitorów tego układu na powierzchni komórek nowotworowych. Powstało kilka koncepcji przeciwdziałania temu mechanizmowi oporności, zaproponowano m.in. zastosowanie przeciwciał bispecyficznych wiążących jednocześnie determinantę antygenową będącą celem terapeutycznym oraz inhibitory dopełniacza zlokalizowane w bliskim sąsiedztwie na powierzchni komórki nowotworowej [14] czy wyciszenie ekspresji inhibitorów dopełniacza poprzez transfekcję konstruktami siRNA [15].

W moim projekcie doktorskim proponuję nowatorskie rozwiązanie polegające na wykorzystaniu zmutowanych ludzkich białek układu dopełniacza, wchodzących w skład konwertaz [16]. Konwertazy są nietrwałymi kompleksami enzymatycznymi kluczowymi w całej kaskadzie. Odpowiadają za produkcję opsonin, anafilatoksyn i inicjację ścieżki terminalnej dzięki proteolizie białek dopełniacza C3 i C5 do ich aktywnych fragmentów C3b i C3a oraz C5b i C5a. Kompleksy te składają się z podjednostek enzymatycznych czynnika B dla szlaku alternatywnego (C3bBb, C3bBbC3b) lub białka C2 dla konwertaz klasycznych/lektynowych (C4bC2a, C4bC2aC3b) (ryc. 2). Konwertazy C3 katalizują rozpad fragmentów C3 do C3a i C3b. Fragment C3b może przyłączyć się do konwertazy „rodzicielskiej" i w następstwie tego zmienić jej specyficzność substratową z białka C3 na C5, tworząc w ten sposób konwertazę C5. W innym scenariuszu, C3b może utworzyć nowe kompleksy alternatywnej konwertazy C3 (ścieżki alternatywnej), które w krótkim czasie mogą spowodować powstanie dużej ilości C3b. Zjawisko to, nazywane pętlą amplifikacji, jest mechanizmem wzmacniana wszystkich trzech ścieżek aktywacji dopełniacza na zasadzie dodatniego sprzężenia zwrotnego (ryc. 2) [1].



Rycina 3: Schemat aktywacji układu dopełniacza, ścieżka alternatywna i klasyczna.

Związanie przeciwciał do błony komórkowej inicjuje ścieżkę klasyczną układu dopełniacza. Kompleks C1 (kompleks podjednostek C1q – C1r – C1s) po związaniu fragmentow Fc przeciwciał ulega autokatalizie. C1s wykazuje aktywnosc enzymatyczna w stosunku do C4 oraz C2, rozkładajac je do fragmentow C4b, C2a, ktore tworza klasyczną konwertaze C3 (kompleks C4b2a).Konwertaza C3 rozszczepia cząsteczkę C3 na fragmenty C3a i C3b. Wiązanie C3b z macierzystym kompleksem konwertazy C3 (C4b2a) prowadzi do wytworzenia nowego enzymu – konwertazy klasycznej C5 (C4b2a3b). Alternatywnie bezpośrednie wiązanie C3b do powierzchni komórki uruchamia tzw. pętlę amplifikacji (zaznaczona na schemacie strzałką przerywaną) prowadzącą do powstania alternatywnej konwertazy C3 i C5. Rozkład C5 przez klasyczną lub alternatywną konwertazę C5 inicjuje wspólny szlak terminalny prowadząc do osmotycznej lizy komórki docelowej. (W oparciu o: Chen et al., Nat Rev Nephrol 2017 i Felberg et al., Cancer Immunol Immunother. 2019).

Czynnik B (ang. factor B, FB) jest elementem enzymatycznym konwertazy C3 oraz C5 ścieżki alternatywnej układu dopełniacza. Fakt, iż większość znanych inhibitorów układu dopełniacza działa na poziomie konwertaz, sugeruje, że kompleksy te są kluczowymi elementami kaskady a ich kontrola odgrywa znaczącą rolę w utrzymaniu homeostazy układu.

Mutacje w genach kodujących białka ścieżki alternatywnej są dobrze poznanym czynnikiem etiologicznym wielu schorzeń autoimmunologicznych lub o charakterze zapalnym. Mutacje w czynniku B stanowią temat licznych publikacji, których większość dotyczy mutacji typu GOF (od ang. gain-of-function)[17, 18]. Mutacje GOF mogą prowadzić do oporności na działanie inhibitorów takich jak takich jak CD55, CD46, CD35, czynnik H lub do wyższego powinowactwa elementów składowych konwertazy do siebie. Skutkiem jest zwiększona aktywność konwertaz, co z jednej strony może się przekładać na wydajniejszą lizę komórki docelowej a z drugiej na zwiększone ryzyko rozwinięcia stanów patologicznych.

Zidentyfikowane mutacje GOF w czynniku B zostały opisane jako czynnik sprawczy chorób takich jak glomerulopatie C3 (C3G) oraz nietypowy zespół hemolityczno-mocznicowy (aHUS) i były w literaturze światowej jednoznacznie kojarzone ze zjawiskami patologicznymi.

Moja hipoteza badawcza zakłada, że suplementacja surowicy przy pomocy wariantu GOF czyn-

nika B może wzmocnić efekt cytotoksyczny terapeutycznych przeciwciał anty-CD20 dzięki mechanizmowi pętli amplifikacji, wzmacniającej aktywność ścieżki klasycznej. Badania *in vitro* prowadzone na liniach komórkowych chłoniaka oraz z wykorzystaniem surowic pobranych od pacjentów leczonych przeciwciałem anty-CD20 potwierdziły hipotezę [16] i pozwoliły naszemu zespołowi rozwijać nową strategię maksymalizującą skuteczność immunoterapeutyków działających za pośrednictwem dopełniacza. Odkrycia te zostały objęte ochroną patentową w Polsce (nr zgłoszenia 425133) a analogiczne wnioski o ochronę patentową w innych krajach są w trakcie procedowania (zgłoszenia: EP19726774.3; USAppl.np.17045058; PCT/PL2019/000024).

Zastosowanie praktyczne prowadzonych badań może polegać na obniżeniu dawek przeciwciał anty-CD20 przy zachowaniu oczekiwanej odpowiedzi klinicznej. Obecnie przeciwciała anty-CD20 są stosowane w dużych nadmiarach, aby przezwyciężyć mechanizmy obronne komórek nowotworowych. Taka strategia powoduje jednak szybkie zużycie składników dopełniacza oraz przyspieszenie usuwania antygenu CD20 z powierzchni komórek nowotworowych [19, 20], co przekłada się na obniżenie efektywności kolejnych dawek leku. Obniżenie dawki przy jednoczesnym zniwelowaniu działania inhibitorów dopełniacza na komórkach nowotworowych spowodowałoby mniejsze zużycie komponentów układu odpornościowego i zachowanie jego zasobów zarówno na dalsze etapy leczenia jak i do ochrony organizmu przez drobnoustrojami, co jest szczególnie ważne w przypadku limfopenii spowodowanej użyciem przeciwciał anty-CD20.

Przez długi czas sądzono, że aktywacja układu dopełniacza przez komórki nowotworowe jest korzystna jedynie z punktu widzenia gospodarza. Taka hipoteza wydawała się być potwierdzona przez kilka faktów. Po pierwsze, komórki nowotworowe wypracowały liczne strategie przeciwdziałania aktywacji dopełniacza [21, 22, 23], po drugie guzy nowotworowe z wyciszoną ekspresją inhibitorów dopełniacza rosły wolniej w modelach zwierzęcych ksenotransplantów [24]. Z najnowszych badań wynika jednak, że komórki nowotworowe mogą również korzystać z aktywacji dopełniacza dzięki mobilizacji komórek supresorowych układu immunologicznego ze szpiku kostnego [25], tudzież indukcji angiogenezy [26]. Fakt, że wiele linii komórek nowotworowych produkuje nie tylko inhibitory, ale również białka aktywujące dopełniacz, zdaje się uprawdopodabniać jego korzystny wpływ na rozwój nowotworu w pewnych warunkach. Ostatnie badania pokazują, że komponenty układu dopełniacza są istotnym modulatorem mikrośrodowiska guza ważnym w kontekście m.in. progresji, nawrotów, przerzutów i aktywności komórek odpornościowych naciekających nowotwór [27, 28, 29, 30]. Co ciekawe, ten sam typ komórek nowotworowych może wytwarzać zarówno aktywatory [30], jak i rozpuszczalne inhibitory dopełniacza [31, 32] sugerując tym samym, że dynamiczna regulacja, a nie konstytutywne hamowanie lub aktywacja układu dopełniacza jest cechą charakterystyczną dla guzów.

Rozpuszczalne inhibitory dopełniacza rzadko wytwarzane są w miejscach innych niż wątroba. Nasze wcześniejsze badania wykazały natomiast, że komórki raka płuc wydzielają szereg rozpuszczalnych inhibitorów dopełniacza takich jak czynnik I (ang. factor I, FI), czynnik H (FH od ang. factor H) i białko wiążące C4b (C4BP od ang. C4b-Binding Protein), zapewniających komórkom ochronę przed układem dopełniacza [32]. Zbadaliśmy związek między ekspresją FI w komórkach nowotworowych pacjentów z niedrobnokomórkowym rakiem płuca a danymi klinicznymi. Materiał kliniczny wraz

z dostępną bazą danych został udostępniony przez prof. Rubena Pio oraz prof. Luisa Montuengę z Uniwersytetu Nawarry w Pamplonie. W pracy [Manuskrypt nr 5] pokazałam, że intensywność barwienia immunohistochemicznego na obecność FI w komórkach nowotworowych koreluje z przeżyciem wolnym od progresji (ang. progression-free survival, PFS) oraz czasem przeżycia zależnym od choroby nowotworowej (ang. cancer-specific survival, CSS). C4d jest końcowym produktem degradacji aktywnego składnika dopełniacza C4b, w której bierze udział czynnik I. Aby odpowiedzieć na pytanie, czy ekspresja FI przez komórki nowotworowe miała na celu ochronę przed wrodzoną odpornością gospodarza, przeanalizowaliśmy odkładanie C4d w tkance nowotworowej. Wyniki nie wykazały tutaj istotnego związku i pokazały niską obecność C4d jedynie w nielicznych bioptatach tkanki nowotworowej. Chcąc dokładniej zbadać rolę czynnika I w rozwoju raka płuc, wykorzystałam do dalszych badań linię komórkową niedrobnokomórkowego raka płuc produkującą FI. Podczas stażu w instytucie Sanquin w Amsterdamie pod opieką dr Robberta Spaapena i dr Ilse Jongerius wykorzystałam metodę CRISPR/Cas9 w celu trwałego usunięcia czynnika I (ang. FI knock-out, FI-KO) z komórek linii komórkowej H2087, w których białko to naturalnie występuje i jest wydzielane na zewnątrz. Wykorzystując uzyskane linie komórkowe FI-KO przeprowadziliśmy sekwencjonowanie RNA wyizolowanego z komórek pozbawionych i niepozbawionych czynnika I. Porównanie transkryptomu ww. komórek ujawniło znaczące różnicę ekspresji ponad 60 genów a dalsza analiza bioinformatyczna wykazała przełożenie tych różnic na aktywność kilku szlaków sygnalizacyjnych. W celu dalszego porównania otrzymanych komórek ze zmodyfikowanym genomem oraz komórek typu dzikiego przeprowadzałam testy funkcjonalne *in vitro* badające zdolności migracyjne jak i wielkości kolonii tworzonych przez obydwa typy komórek. Wobec uzyskania statystycznie istotnych różnic oraz wykazanych wcześniej różnic w transkryptomie komórek pozbawionych FI wobec komórek niemodyfikowanych postulujemy, że niekanoniczna aktywność FI wpływa na fizjologię komórek raka płuc i może tłumaczyć złe rokowanie u chorych, które wykazaliśmy analizując wyniki barwienia immunohistochemicznego.

2 Publikacja nr 1

G. Stasiłojć*, A. Felberg*, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin, A. Österborg, M. Okrój

"Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies"

Journal of Immunological Methods, Vol. 476, 112675, 2020

*autorzy maja ten sam wkład w przygotowanie publikacji

Wkład autorów:

G. Stasiłojć , A. Felberg, A. Urban, D. Kowalska przeprowadzili eksperymenty prezentowane w pracy. S. Ma, J. Lundin , A. Österborg zdiagnozowali pacjentów i zebrali materiał kliniczny. A. Blom, M. Okrój napisali wstępną wersję manuskryptu, w edycję której zaangażowani byli wszyscy autorzy.

Journal of Immunological Methods 476 (2020) 112675

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies

Grzegorz Stasiłojć[a,1], Anna Felberg[a,1], Aleksandra Urban[a], Daria Kowalska[a], Shuo Ma[b], Anna M. Blom[c], Jeanette Lundin[d], Anders Österborg[d], Marcin Okrój[a,*]

[a] Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Dębinki 1 street, 80-211 Gdańsk, Poland

[b] Northwestern University Feinberg School of Medicine and the Robert H. Lurie Comprehensive Cancer Center, Northwestern University, 675 N St. Clair, Chicago, IL 60611, USA

[c] Department of Translational Medicine, Lund University, Inga Marie Nilssons street 53, 214 28 Malmö, Sweden

[d] Department of Hematology, Karolinska University Hospital Solna, 171 76 Stockholm, Sweden

ARTICLE INFO

Keywords:
Ofatumumab
Alemtuzumab
CLL
Complement system

ABSTRACT

Monoclonal antibodies ofatumumab (anti-CD20) and alemtuzumab (anti-CD52) which are approved for usage in patients with chronic lymphocytic leukemia (CLL), efficiently activate the classical complement pathway. However complement is an exhaustible component and high doses of its activators may deplete complement-dependent cytotoxicity (CDC) potential, thus reducing the effect of repeated mAb dosing. Widely used method to measure CDC activity of patients' serum is hemolytic assay (CH50) on sheep erythrocytes. Despite its simplicity, such CH50 assay may not reflect pivotal interactions between patient serum and human complement inhibitors on the surface of target cells. We propose calcein release assay performed on tumor cells similar to those targeted by therapeutic antibodies as an alternative method. We analyzed serum samples collected from 12 patients participating in the clinical study, receiving s.c. 30 mg alemtuzumab three times per week combined with i.v. ofatumumab at an initial dose of 300 mg in week 3 further escalated to 2000 mg every other week. All serum samples were measured by hemolytic assay on sheep erythrocytes as well as using calcein release assay on CD20-positive Raji cells. Our data show that results obtained from both assays are related to each other at the level of the whole group ($n = 96$ samples, Spearman $r = 0.504$, $p < .001$) but may substantially differ when analyzing individual patients. Furthermore, by using CDC assay on Raji cells, we found that in the presented clinical study CDC serum potential was not significantly affected when measured before consecutive administrations in most of the patients.

1. Introduction

Introduction of immunotherapeutics bondable to a specific molecule expressed on tumor cells is considered as a milestone in the field of hematology and oncology. A good example of such molecule being a promising target for immunotherapy is CD20, a surface protein present on mature B lymphocytes and B cell malignancies. Rituximab, the anti-CD20 mAb was approved in 1997 and since that time many other anti-CD20 agents with enhanced effector functions like ibritumomab tiuxetan, ofatumumab, obinutuzumab or tositumomab were developed (Okroj et al., 2013b). Depending on effector functions, anti-CD20 antibodies are divided into class I and class II compounds. Type I antibodies represented by rituximab and ofatumumab are efficient activators of the classical complement pathway and complement-dependent cytotoxicity (CDC) whereas type II antibodies are capable of activating intracellular signalling pathways leading to programmed cell death, a feature hardly attainable for type I mAbs (Winiarska et al., 2010). Also, the other surface molecules on malignant B cells such as CD52 were exploited as a target for immunotherapy. Anti-CD52 mAb alemtuzumab was shown to lyse freshly isolated CLL cells more efficiently than

Abbreviations: ADCC, antibody-dependent cellular cytototoxicity; AUC, area under curve; CDC, complement-dependent cytotoxicity; CH50, hemolytic complement (classical) pathway assay; CLL, chronic lymphocytic leukemia; DGVB++, dextrose-gelatin veronal buffer; DMSO, dimethyl sulphoxide; FU 2 M, follow-up two months; mAb, monoclonal antibody; NHS, normal human serum; Δ NHS, heat-inactivated normal human serum

* Corresponding author.
E-mail address: marcin.okroj@gumed.edu.pl (M. Okrój).

[1] Equally contributed.

https://doi.org/10.1016/j.jim.2019.112675
Received 17 July 2019; Received in revised form 9 September 2019; Accepted 23 September 2019
Available online 17 October 2019

rituximab (Golay et al., 2004) and after positive evaluation in clinical trials eventually approved as a single agent for CLL therapy. The introduction of antitumor mAbs markedly improved prognosis for patients suffering from B cell malignancies but still, a substantial number of patients will relapse and become refractory. This fact not only stimulated research on more efficient complement activators but also initiated a discussion about the limitations of complement activation by type I mAbs. One of the well-defined obstacles in complement-mediated cells killing is overexpression of complement inhibitors by tumor cells (Jurianz et al., 1999). Application of anti-CD20 mAbs at high doses aims to override endogenous complement inhibition but it may not be an optimal strategy as excessive mAbs promote either internalization or trogocytic removal/shedding of CD20 and act as a selective pressure factor for establishing a population with low expression of CD20 cells (Taylor and Lindorfer, 2014). Another problem postulated in literature is the exhaustion of the complement system, which renders further doses/cycles of type I anti-CD20 mAbs ineffective. Substantial consumption of complement during anti-CD20 immunotherapy was first demonstrated ex vivo and followed by in vivo observations that supplementation of patients with fresh frozen plasma improved clinical outcome, even when previous administrations of rituximab alone or in combination with chemotherapy were ineffective (Klepfish et al., 2009). Taken together, an important question is how long a state of the complement system depletion persists after infusion of complement-activating mAbs and whether it overlaps with another dosing. Nowadays, the most frequently used method to estimate the activity of the classical complement pathway is CH50 assay performed using sensitized sheep erythrocytes. Sheep erythrocytes lack human complement inhibitors, which are defined as one of the main obstacles in successful therapy with type-I mAbs. We propose a novel approach to measure complement activity in the serum of patients using cytotoxic assay on tumor cells similar to those targeted by therapeutic antibodies. We have studied complement activity of serum collected from twelve patients, who participated in a clinical study on combined alemtuzumab (an anti-CD52 mAb) and high (2000 mg) dose of ofatumumab as 1st line therapy. Results obtained from CH50 and CDC assays were compared and correlated.

2. Materials and methods

2.1. Patients' characteristics

The cohort consisted of twelve CLL patients recruited in the framework of a clinical study (spin-off project). These patients coded with B1-B12 received three weekly (30 mg) subcutaneous injections of alemtuzumab from week 1 to week 18. Starting from week 3, they also received 300 mg ofatumumab intravenously further escalated to 2000 mg from week 5 and then every other week. We had a complete set of serum samples before infusions collected in week 1 (baseline), week 3, 5, 7, 9, 13, 17 and two months follow-up (FU 2 M) from twelve patients available for our study. Patients demographics are depicted in Supplementary Table 1. All patient samples were obtained in accordance with the Declaration of Helsinki and with approval from The Local Ethics Committee in Stockholm (approval number: 2011/485-31/2). Written informed consent was obtained from all individual participants included in the study.

2.2. Sample handling

Upon collection serum samples were kept at −80 °C until the time of experiment. Repetitive freezing and thawing were avoided and the same rule applied for normal human serum (NHS) and heat-inactivated normal human serum (Δ NHS) used as positive and negative controls, respectively. Working dilutions of sera were prepared only before experiments in chilled tubes or microplates put onto ice.

2.3. Hemolytic complement (classical) pathway assay

Sheep erythrocytes (Biomaxima) were washed 3× with DGVB++ (dextrose-gelatin veronal buffer: 2.5 mM veronal buffer pH 7.3, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$) and incubated with amboceptor (Behring) at 1:1000 dilution at 30 °C with shaking. After 30 min cells were washed with DGVB++ until the red color disappeared from the supernatant. Erythrocytes were suspended in DGVB++ at the amount set up in a way that 10 µl of suspension added to 90 µl of water resulted in absorbance of the supernatant at 405 nm within a range 1.5 to 2.0 units. Thereafter 10 µl of adjusted suspension was transferred to V-bottom microplate wells and overlaid with 90 µl of DGVB++ buffer containing patients' serum in dilutions 1:100, 1:200, 1:400 and 1:800. The same dilutions of NHS and Δ NHS were used as a references. After 30 min incubation with shaking at 37 °C erythrocytes were centrifuged and absorbance of the supernatant at 405 nm was measured.

2.4. Cells lines

Raji cells were obtained from the American Type Culture Collection. Cells were aliquoted and cryopreserved after the first few passages. Cells used for experiments were grown from such stock aliquots in RPMI 1640 medium with L-glutamine (Corning) supplemented with 10% fetal bovine serum (PANBiotech) at 37 °C and humidified 5% CO_2 atmosphere. Cells were routinely checked for *Mycoplasma* contamination by DAPI staining when cultured and never kept in continuous culture for > 10 passages.

2.5. CDC assay

Raji cells were diluted to 1 million per ml in culture media and stained with calcein-AM (Sigma) for 30 min at standard culture conditions. Leftovers of calcein were washed out with PBS buffer supplemented with 1 mM $CaCl_2$ and $MgCl_2$ and (if not stated otherwise) 1×10^5 cells were pelleted onto V-shape microplate wells and overlaid with 40 µl of the indicated concentration of serum and 50 µg/ml ofatumumab diluted in the same PBS buffer. Fluorescence of calcein released into the supernatant was measured at 490 / 520 excitation/emission wavelength in Synergy H1 microplate reader (Biotek).

2.6. Statistical analysis

GraphPad Prism 7 software was used for calculations of Sidak's multiple comparison test, r Spearman coefficient of correlation and area under the hemolytic curves (AUC).

3. Results

3.1. Technical performance of CDC assays

In order to provide a proof of principle for our CDC assays later on used for evaluation of complement consumption in clinical samples, we tested the experimental conditions. In the first step of the experiment Raji cells, (non-labelled with calcein-AM) were subjected to 50% NHS supplemented with 50 µg/ml ofatumumab and incubated for 30 min at 37 °C. Cells were tested at three different densities: 1×10^5, 2.5×10^5 and 5×10^5 cells per well (Fig. 1A). In parallel, NHS and Δ NHS without cells were incubated at 37 °C too. Incubated sera and supernatants collected from non-labelled Raji cells served as a source of serum used in the second step of the experiment. One hundred thousand of calcein-labelled Raji cells were subjected either to non-diluted supernatant (equivalent of 50% serum) or 5× diluted supernatant (equivalent of 10% serum). NHS and Δ NHS incubated at 37 °C without cells served as positive and negative controls, respectively. Differences in calcein release after the second step of the experiment were

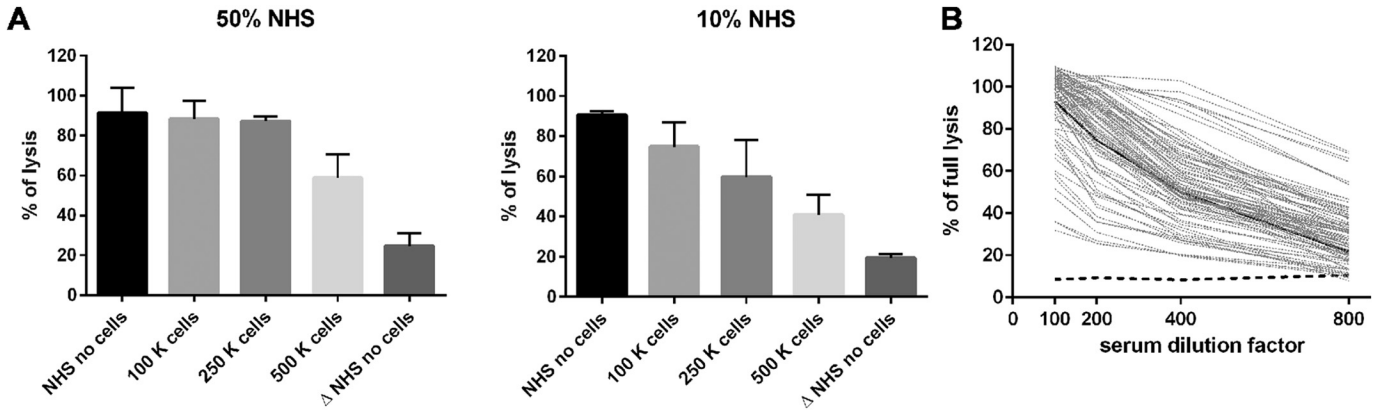


Fig. 1. A) Step 1 of cytotoxic assay was performed on different number of Raji cells (non-labelled) incubated with 50% of normal human serum (NHS) and 50 μg/ml of ofatumumab. Supernatants collected after 30 min incubation at 37 °C were applied in Step 2) either non-diluted (50% NHS) or 5× diluted (10% serum). Additionally, NHS or heat-inactivated NHS (Δ NHS) incubated without any cells in step 1 of the experiment were used as controls. Supernatant collected from calcein-labelled cells lysed with 30% DMSO diluted in PBS served as the indicator of 100% (full) lysis. Readout obtained for heat-inactivated serum (Δ NHS) served as negative control, i.e. background lysis independent on complement activation. Data are presented as means of three independent experiments and error bars represent standard deviation. B) Hemolytic curves (means of two independent experiments) obtained for all samples available from 12 patients participating in NCT0131711 study. Each sample was assayed at 1:100, 1:200, 1:400 and 1:800 dilution. The thick solid line shows a hemolytic curve obtained for normal human serum, while the dashed line presents a curve obtained for heat-inactivated normal human serum.

attributable to complement consumption during the first step. The more cells were incubated with serum in the first step, fewer cells were lysed in the second step, especially when 5×-diluted supernatant was used (Fig. 1A). Therefore, we decided to apply 10% of serum samples collected from CLL patients in the further assessment of complement consumption/activity assays in clinical samples.

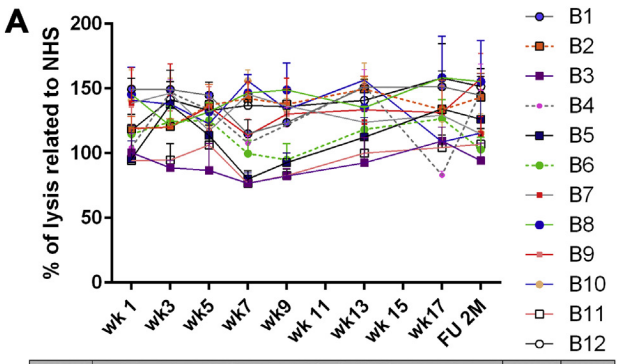
The result of CH50 assay is delivered as serum dilution, at which 50% lysis of sheep erythrocytes (standardized for either quantity and sensitization) takes place. In Fig. 1B we plotted with faint grey lines the lytic curves obtained for every available sample from 12 patients (B1-B12) diluted 1:100 (1%), 1:200 (0.5%), 1:400 (0.25%) and 1:800 (0.0125%). Full lysis was calculated as a value obtained after incubation with water instead of serum. The lytic curve obtained for NHS is indicated with a solid thick line and the lytic curve obtained for Δ NHS is indicated with a stripped thick line. While the calculation of serum dilution attributable to 50% full lysis was possible for most of the samples, some did not reach the value of 50% full lysis at the highest concentration and some did not lyse < 50% erythrocytes even at the lowest concentration. Extrapolation could be affected by the linear vs. non-linear character of the individual curves (Fig. 1B). Therefore, instead of calculation of CH50 value, we calculated the area under each curve (AUC) representing individual patient sample as a readout surrogate for CH50 and free of extrapolation-related problems, which seems to be more suitable for the samples from a given cohort.

3.2. Complement activity in pre-infusion serum samples from alemtuzumab / ofatumumab treated patients - comparison of CDC assay on Raji cells and hemolytic-based assay

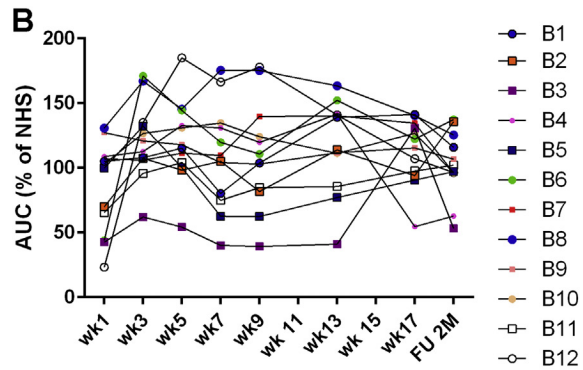
We first aimed to determine by CDC assay on Raji cells how co-administration of efficient complement activators (alemtuzumab and ofatumumab) affects complement activity of patients' serum measured before next dosing. In case of persistent complement depletion one would expect diminishment of CDC activity in consecutive serum samples, especially samples from week 3 (after administration of alemtuzumab and before ofatumumab), week 5 (after adding an initial dose of ofatumumab) and week 7 (sample reflecting ofatumumab dose escalation from 300 mg in week 3 to 2000 mg in week 5). Our results (Fig. 2A) show that none of the patients showed a significant decrease of complement activity after two weeks of alemtuzumab administration (week 3), in spite of the fact that all but two patients (B9 with 55 G/l and B12 with 30× G/l) became lymphocytopenic already at this stage. None of the patients had decreased complement activity following the introduction of ofatumumab (week 5). One patient (B1) showed a significant decrease in complement activity in her serum sample collected in week 7 when compared to the baseline level. Moreover, serum complement activity in samples collected from patients B1, B4, B5 and B6 in week 7 was weaker when compared to prior samples from week 5 (Fig. 2). Importantly, all these patients showed a rebound in complement activity in week 9 (B1, B4, B5) or after week 9 (B6). All remaining patients did not show any clear tendency towards diminished complement activity correlated with the introduction or dose escalation of ofatumumab. In parallel, we determined CDC potential of the same samples by hemolytic assay and expresses obtained results as AUC for each patient's serum at a given time point in % relation to AUC readout obtained for NHS (Fig. 2B). These hemolytic curves had much higher amplitude and the range of extreme values was twice higher than the range obtained in test on Raji cells. Next, we calculated the correlation between CDC assay and hemolytic assay either for the whole patients' cohort (Fig. 2C) or samples from each individual patient (Fig. 2D). The overall correlation coefficient calculated for the whole cohort remained significant with Spearman r-value of 0.504 and p level < 0.001 (Fig. 2C) thus showing that at group level the results of both assays were related to each other. Notably, such correlation does not hold at the level of individual patients, since p level < 0.05 was achieved only for patients B1 and B11. However, patients B1, B5 and B6 who showed a reduction of complement activity in week 7 in CDC assay on Raji cells also showed a drop in hemolytic potential at this time point (Fig. 2A,B).

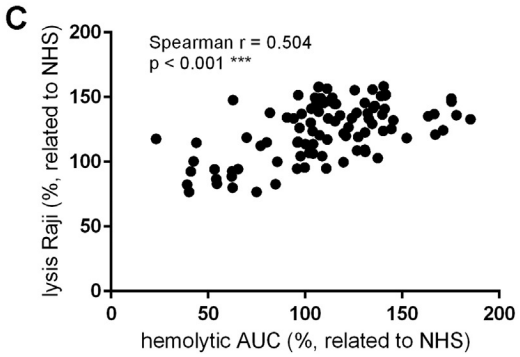
4. Discussion and conclusions

We postulate value in checking the activity of the classical complement pathway by cytotoxic assay performed on tumor cells similar to those targeted by therapeutic antibodies rather than by well-established methods using hemolytic reactions (e.g. CH50 assay). Sheep erythrocytes lack human complement inhibitors, which are defined as one of the main obstacles in successful therapy with type-I mAbs. Instead, they are equipped with putative homologues of human counterparts but of unknown efficacy towards the human serum. Of note, sensitized sheep erythrocytes are highly vulnerable to CDC at very low serum concentration (0,25% - 1%) (Blom et al., 2014) in contrast to the majority of leukemia cells (Beurskens et al., 2012; Okroj et al., 2013a). Operating on low serum dilutions and highly sensitive cells predisposes for inter-experiment deviations of results and in fact, we can observe



patient	significance vs. week 1 (baseline)							w.3 vs. w.5	w.5 vs. w.7
	w. 3	w.5	w.7	w.9	w.13	w.17	FU 2M		
B1	n.s.	n.s.	* ↓	n.s.	n.s.	n.s.	n.s.	n.s.	** ↓
B2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B4	** ↑	* ↑	n.s.	n.s.	*** ↑	n.s.	** ↑	n.s.	* ↓
B5	** ↑	* ↑	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	* ↓
B6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	* ↓
B7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s
B8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s
B9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	* ↑
B11	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B12	n.s.	n.s.	n.s.	n.s.	n.s.	* ↑	n.s.	n.s.	n.s.





D

Patient #	Spearman r value	p value
B1	0,785	0,027 *
B2	0,666	0,083
B3	0,595	0,132
B4	0,476	0,241
B5	0,69	0,069
B6	0,529	0,196
B7	-0,142	0,75
B8	-0,285	0,5
B9	0,095	0,84
B10	0	>0,99
B11	0,881	0,007 **
B12	-0,309	0,461

Fig. 2. A) Results of the cytotoxic assay performed on Raji cell in 10% serum of patients B1-B12 collected at baseline (week 1) and before consecutive ofatumumab infusions at week 3, 5, 7, 9, 13, 17 and at 2-months follow-up (FU 2 M). Data are collected from three independent experiments. Error bars represent standard deviation. Statistical differences depicted in the table below the graph are calculated according to Sidak's multiple comparison test. B) Results obtained in hemolytic assay on sheep erythrocytes and presented as % of AUC calculated for NHS sample. AUC embraces average value calculated from two curves (four experimental points each) obtained in two independent experiments. Correlation between the results of the CDC assay on Raji cells and the values of AUC determined from hemolytic curves, C) for the whole patients' cohort D) or serum samples from each individual patient.

much bigger fluctuations in individual patients' readouts of the hemolytic assay (Fig. 2B) when compared to CDC assay on Raji cells (Fig. 2A). Another reason is that hemolytic assay may not accurately reflect the impact of individual serum component on lysis of nucleated cells. This was recently demonstrated in a study, which employed gain of function variants of complement components. Addition of such hyperactive proteins to sensitized sheep erythrocytes had a limited effect on hemolysis but resulted in a spectacular increase of Raji cells sensitivity to CDC (Felberg et al., 2019). Our results showed that readouts of CDC assay on CD20-positive cells and hemolytic assay are related to each other at the level of the whole group (Fig. 2C) but may differ substantially when analyzing individual patients (Fig. 2D).

The problem of complement exhaustion due to excessive administration of type I anti-CD20 mAbs is known (Beurskens et al., 2012) but there is a limited number of experimental data detailing the complement activity between dosing intervals. Importantly, there is not such data in relation to treatment with two mAbs simultaneously, a situation which may theoretically result in extraordinary pressure on the complement system and its recovery before consecutive administration. We examined complement recovery before consecutive administrations of complement activators alemtuzumab and ofatumumab used in combination. Examination of pre-infusion samples from consecutive administrations of the drugs showed that administration of alemtuzumab alone or in combination with ofatumumab, even in a high dose of 2000 mg did not persistently reduce available complement pool in most of the patients. Moreover, in this particular cohort, the drop of serum CDC observed in four patients in coincidence to ofatumumab dose escalation was not dramatically deep and usually CDC level measured before next infusion was comparable to the level attainable for NHS. Serum samples were the only material available for us from this clinical study and we could not analyze either CD20 and CD52 expression or deposition of complement activation markers like C3 and/or C4d on CLL cells. This fact precludes firm conclusions on whether described therapeutic schedule was optimal for the restoration of complement before consecutive infusions or whether the minimal reduction of CDC potential observed only in some patients was due to low expression of the molecular target resulting in limited complement activation. Nonetheless, we argue that measurement of serum CDC before administrations of type I immunotherapeutics is important not only to determine that effector for given antibodies is present but also to ensure that patient (already depleted from lymphcytes by anti-CD20 or anti-CD52 antibodies) is not additionally devoid of important defense line of innate immunity, which may increase the risk of infections and undesired side effects. CDC test performed on nucleated cells being

a potential target of applied immunotherapeutic seems to be a more reliable method than CH50 or related hemolytic-based assays. We also underline a need for proper handling of clinical material devoted to functional complement assays. Storing in temperature higher than −80 °C and/or repetitive thawing and freezing introduce a risk for complement activation in the tube resulting in artificial complement exhaustion, which produces false negative results in CDC assay.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.112675.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgements

This work was supported by National Science Centre (Poland) grant no. 2014/14/E/NZ6/00182 and by grant from Cancerfonden (Sweden).

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Supplementary Table 1

Patient characteristics

#	WBC count at baseline (G/l)	1st line treatment	Rai stage	age	sex	high-risk cytogenetics (11q-, 17p- or p53 abberation)
B1	147,2	alem + ofa	3	53	F	none
B2	153,9	alem + ofa	4	71	F	none
B3	131,3	alem + ofa	3	63	F	none
B4	148	alem + ofa	4	63	M	Del 17p
B5	251,7	alem + ofa	4	62	M	Del 17p
B6	43,5	alem + ofa	1B	51	F	*TP53* mut
B7	113,6	alem + ofa	4	50	M	none
B8	133,9	alem + ofa	4	71	M	none
B9	181,3	alem + ofa	4	59	M	none
B10	15,8	alem + ofa	1B	67	F	none
B11	312,5	alem + ofa	4	59	F	*TP53* mut
B12	709	alem + ofa	4	54	M	none

Abbreviaitions: alem = alemtuzumab; ofa = ofatumumab

3 Publikacja nr 2

A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój

"Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab"

Frontiers in Immunology, Vol. 11, 584509, 2020

Wkład autorów:

A. Felberg, A. Urban, K. Jaskuła, A. Jurkiewicz i G. Stasiłojć przeprowadzili eksperymenty i/lub zoptymalizowali testy zastosowane w badaniach. M. Taszner, A. Majeranowski i J. Zaucha zdiagnozowali pacjentów i zebrali materiał kliniczny. A. Blom, M. Okrój i J. Zaucha napisali pierwotną wersję manuskryptu.

frontiers in Immunology

ORIGINAL RESEARCH
published: 19 November 2020
doi: 10.3389/fimmu.2020.584509

Monitoring of the Complement System Status in Patients With B-Cell Malignancies Treated With Rituximab

*Anna Felberg[1], Michał Taszner[2], Aleksandra Urban[1], Alan Majeranowski[2], Kinga Jaskuła[1], Aleksandra Jurkiewicz[1], Grzegorz Stasiłojć[1], Anna M. Blom[3], Jan M. Zaucha[2] and Marcin Okrój[1]**

[1] Department of Cell Biology and Immunology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland, [2] Department of Hematology and Transplantology, Medical University of Gdańsk, Gdańsk, Poland, [3] Department of Translational Medicine, Lund University, Malmö, Sweden

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Edited by:
Zvi Fishelson, Tel Aviv University, Israel

Reviewed by:
Mark S. Cragg, University of Southampton, United Kingdom
Christian Drouet, INSERM U1016 Institut Cochin, France

***Correspondence:**
Marcin Okrój
marcin.okroj@gumed.edu.pl

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: *17 July 2020*
Accepted: *19 October 2020*
Published: *19 November 2020*

Citation:
Felberg A, Taszner M, Urban A, Majeranowski A, Jaskuła K, Jurkiewicz A, Stasiłojć G, Blom AM, Zaucha JM and Okrój M (2020) Monitoring of the Complement System Status in Patients With B-Cell Malignancies Treated With Rituximab. Front. Immunol. 11:584509. doi: 10.3389/fimmu.2020.584509

Rituximab is a pioneering anti-CD20 monoclonal antibody that became the first-line drug used in immunotherapy of B-cell malignancies over the last twenty years. Rituximab activates the complement system *in vitro*, but there is an ongoing debate on the exact role of this effector mechanism in therapeutic effect. Results of both *in vitro* and *in vivo* studies are model-dependent and preclude clear clinical conclusions. Additional confounding factors like complement inhibition by tumor cells, loss of target antigen and complement depletion due to excessively applied immunotherapeutics, intrapersonal variability in the concentration of main complement components and differences in tumor burden all suggest that a personalized approach is the best strategy for optimization of rituximab dosage and therapeutic schedule. Herein we critically review the existing knowledge in support of such concept and present original data on markers of complement activation, complement consumption, and rituximab accumulation in plasma of patients with chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphomas (NHL). The increase of markers such as C4d and terminal complement complex (TCC) suggest the strongest complement activation after the first administration of rituximab, but not indicative of clinical outcome in patients receiving rituximab in combination with chemotherapy. Both ELISA and complement-dependent cytotoxicity (CDC) functional assay showed that a substantial number of patients accumulate rituximab to the extent that consecutive infusions do not improve the cytotoxic capacity of their sera. Our data suggest that individual assessment of CDC activity and rituximab concentration in plasma may support clinicians' decisions on further drug infusions, or instead prescribing a therapy with anti-CD20 antibodies like obinutuzumab that more efficiently activate effector mechanisms other than complement.

Keywords: obinutuzumab (GA101), non-Hodgkin's lymphoma, complement system, chronic lymphocytic leukemia, rituximab

INTRODUCTION

CD20, a surface molecule present on most developmental stages of B lymphocytes, fulfills many conditions attributable to being a promising target for immunotherapy (1–5). The first anti-CD20 immunotherapeutic rituximab was clinically approved in 1997 (6). It became the first-line drug (usually in combination with chemotherapy), which significantly improved the survival of patients suffering from B cell leukemias and lymphomas (7, 8). Rituximab contains a human IgG1 Fc portion capable of activating immune effector mechanisms in man and rodents, including activation of the complement system and complement-dependent cytotoxicity (CDC) next to antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis mediated by either Fc—or complement receptors (2, 9). On the other hand, immune escape and modulation of immune response by tumor cells and supracellular factors like the number of tumor cells and bioavailability of the drug influence the effectiveness of cancer eradication. Accordingly, indications that many patients are refractory to rituximab (10) reasoned the studies on the pivotal effector and resistance mechanisms, which often brought contradictory results. Our goal was to form coherent conclusions in the light of published data, with an emphasis on the role of the complement system. We also supplement these conclusions with original data showing the status of the complement system and the retention of the drug in patients with B cell malignancies receiving rituximab. In our opinion, monitoring of such parameters contributes to a personalized therapeutic approach highly appreciated in patients undergoing treatment with anti-CD20 antibodies.

An Interplay Between Effector Mechanisms of Rituximab

Based on predominant effector mechanisms, anti-CD20 mAbs are classified into type I and type II antibodies (1, 3). Type I specimens are potent complement activators in contrast to type II, which directly exert cell death upon binding to the target cell. There are reports on limited rituximab-induced cell death in certain tumor B cell lines (11), nonetheless, rituximab is more efficient in the complement-mediated killing and categorized as a representative of type I. Notably, both type I and type II anti-CD20 mAbs can support ADCC induced by the binding of the Fc portion of antibody to Fc receptors localized on effector cells (predominantly NK cells). ADCC and CDC mechanisms may compete with each other as complement activation on the platform of cell-bound rituximab imposes the occupation of its Fc portion and results in a steric hindrance for the interaction with FcγRIII. This phenomenon was proven for the first time *in vitro* by Wang et al., who noticed that normal human serum or C5-depleted serum but not heat-inactivated serum, C1- and C3-depleted serum blocks NK cell activation (12). Further experiments in a syngeneic murine lymphoma model showed that complement depletion by application of cobra venom factor (CVF) before mAb administration resulted in longer survival than the application of mAb alone, thus suggesting that the ADCC mechanism is pivotal and complement activation is detrimental for the therapeutic effect of type I mAbs (13). However, one limitation of this and many other syngeneic mouse models is the usage of anti-CD20 other than rituximab whereas even subtle differences in target epitope or Ig structure outside of CDR regions may be critical for type I/II characteristics (14). A few studies analyzed effector mechanisms of type I anti-CD20 antibodies in transgenic mice expressing human CD20 (15–17). Beers et al. reported a dispensable role of the complement system in the elimination of CD20-positive cells by rituximab converted to mouse IgG2a isotype (equally efficient in CDC as the original rituximab) (16). Results of Tipton and colleagues suggest that antibody-mediated phagocytosis is the crucial effector mechanism (17) whereas Gong et al. showed that effective depletion of B cells may need different effectors depending on their location. Complement was found crucial for the elimination of B cells from the marginal zone in the spleen but not important in other sites (15). The other limitation in the context of the translational potential of *in vivo* studies in mouse models is the fact that mouse complement is very weak compared to other mammals (18, 19), and therefore experiments performed in the mouse model introduce the risk of under-appreciation of CDC as an effector mechanism. Nonetheless, there is a number of the mouse *in vivo* studies that either support (20–22) or question (16, 17, 23, 24) the critical role of complement in the therapeutic effect of rituximab. There is a lack of conclusive *in vivo* studies performed in animal models with complement activity comparable to humans (e.g., rat, guinea pig, and dog). A single study in nude rats with intracerebral lymphoma xenograft successively treated with rituximab suggests complement involvement (25). However, a separate and more detailed investigation must ensure the extrapolation of this conclusion.

Observations from clinics and *ex vivo* experiments in man also bring ambiguous conclusions. ADCC reactions may play a role in the therapeutic effect of rituximab as a low number of NK cells correlated with poor clinical outcome (26). A higher response rate to rituximab and higher progression-free survival of patients with follicular lymphoma was shown in individuals with a polymorphism in FcγRIIIa (CD16), which renders a high affinity to IgG1 (27, 28) but these findings were not confirmed in a larger clinical study (29). Additionally, clinical response and duration of response to rituximab were correlated with polymorphism of the C1qA gene that associates with low levels of C1q—the first component of the classical complement pathway (30). Contrarily, addition of fresh frozen plasma to CLL patients markedly improved their clinical outcome, even when previous administrations of rituximab were ineffective (31, 32). These data suggest that the CDC/ADCC interplay depends either on model or supracellular factors like the number of tumor cells and the expression of the target antigen. Since the threshold necessary for effective ADCC is lower than that for CDC (33), these two competitive effector mechanisms may act cooperatively, i.e. in case of a heterogeneous population of tumor cells, ADCC eliminates these of low CD20

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukemia; CR3, complement receptor 3; CVF, cobra venom factor; MAC, membrane attack complex; NHL, non-Hodgkin's lymphoma; NHP, normal human plasma; NHS, normal human serum; TCC, terminal complement complex; Δ NHS, heat-inactivated normal human serum.

expression whereas complement eradicates cells with high CD20 content. The number of tumor cells is another parameter important in the context of rituximab's effector mechanisms. Boross et al. showed that injection of rituximab to FcRγ-deficient mice was ineffective at a high load of tumor cells and that, in contrast to a challenge with a low number of tumor cells, effective elimination demands the cooperation of complement and ADCC and the presence of functional complement receptor 3 (CR3) on blood phagocytes (34). The role of receptors for complement-derived opsonins is also underlined by Lee at al., who developed rituximab RA801 mutant non-bondable to human or mouse Fc receptors but retaining complement activation potential (35). While PBMC and PMN were not able to eliminate RA801-opsonized CD20-positive cells *ex vivo* without the addition of serum depleted of the C9 component, there was no difference in human CD20-positive Ramos cells' eradication in *in vivo* nude mouse model between original rituximab and RA801 mutant (35). Yet, eradication of mouse EL4 lymphoma cells expressing human CD20 by rituximab, but not RA801, was impaired in mice additionally lacking all Fc receptors. This can be explained by the higher CDC efficacy of RA801 (4.5-fold lower CH_{50} value) compared to rituximab. Nonetheless, such results underline two important issues: i) extrapolation of conclusions obtained from the studies on one mAb to the other, even closely related mAb, is not reliable, and ii) the relative importance of rituximab's effector mechanisms heavily depends on the target cells. Therefore the seemingly contradictory results showing successful depletion of B cells by rituximab-like antibodies in mice with functional macrophages and Fcγreceptor-dependent pathways but lacking functional complement or ADCC mechanism (16, 23, 36) should not be surprising.

Rituximab (Type I) or Type II Anti-CD20 Immunotherapeutics?

Since both type I and type II anti-CD20 antibodies are nowadays available in clinics, a relevant dilemma is which of these two types is superior for particular patients. Complicated interplay between effector mechanisms and heterogeneity of targets in B cell malignancies in conjunction with supracellular factors make a unanimous answer problematic. Due to the same reason, the role of the complement system in the therapeutic effect cannot be generally ruled out or confirmed. However, assuming that under certain circumstances patients may benefit from complement activation by rituximab, parallel monitoring of the complement system parameters enables selecting subjects with functional impairment, saturation, or unresponsiveness of this effector mechanism, who may benefit more from type II antibodies, e.g., obinutuzumab that more efficiently activates effectors other than complement (37). Another parameter deserving control in case of usage of type I anti-CD20 antibodies is their retention in blood. When excessively administered, they may lead to loss of target antigen *via* internalization (3) and trogocytic removal (38, 39). Conversely, administering type II antibodies results in higher stability of surface CD20 antigen (40). In experimental models, the saturation of the CDC takes place much faster than the saturation of C3b deposition on target cells, thus overdosing provokes exhaustion of the complement system (41, 42). Such exhaustion affects mostly the initial components of the classical pathway, namely, C1 and C2, which are present in serum at much lower molar concentrations than C3 and act as a bottleneck of the whole pathway. Since malignant B cells are typically equipped with a set of complement inhibitors that affect C3/C5 convertases (43, 44), their activity will also lead to the consumption of downstream components C1 and C2. Therefore, too high concentration of rituximab and potent intrinsic complement inhibition by tumor cells may not only dampen CDC at consecutive infusions of the drug but also lead to the selection of tumor cells with low expression of CD20 antigen. Transient loss of CD20 on tumor B cells following rituximab infusion was observed in CLL patients and considered as one of the causes of the limited efficacy of antitumor mAbs (42, 45).

Previously we proposed a calcein release assay on Raji cells as a method for monitoring CDC potential of serum collected from patients treated with type I anti-CD20 antibodies (46). There are several advantages of this method over the routinely used CH_{50} assay performed on sensitized sheep erythrocytes: i) usage of human tumor cells bearing both molecular target (CD20) for dedicated immunotherapeutics and human complement inhibitors (CD46, CD55, CD59) (43) fully compatible with human complement, ii) adequate sensitivity of target cells to complement-mediated lysis and iii) lower inter-assay variability compared to CH_{50} assay (46). Using this approach, we measured the CDC potential of serum samples collected before and after each infusion of rituximab in 17 patients with various B cell malignancies. In another version of these experiments, we supplemented the analyzed sera with saturating concentration of rituximab to evaluate whether putative post-infusion complement depression overlapped with consecutive infusions. In parallel, we measured rituximab concentrations in each sample. The combined results of these experiments reveal the net functional effect of rituximab retention and individual competence of the complement system, which altogether may support the clinician's decision on modification of the therapeutic schedule or switch into type II anti-CD20 antibodies.

METHODS

Patients and Treatment

All samples collected from patients and healthy volunteers were obtained after written informed consent, in accordance with the Declaration of Helsinki and with approval from The Local Bioethical Committee at Medical University of Gdańsk (approval number: NKBBN/500/2016). The cohort consisted of 17 patients admitted to Dept. of Hematology and Transplantology of Medical University of Gdańsk, 7 of which were diagnosed with CLL and 10 with different forms of NHL. All patients had no prior therapies. They were administered with 375 mg/m2 rituximab over the period from 2 to 5 h in four-week intervals for 4 to 8 cycles. All but two patients received concomitant chemotherapy. Detailed patients' characteristics are given in **Table 1**. Response to treatment was assessed according to iwCLL guidelines (47). Blood drawn immediately before and after rituximab infusions was used for

TABLE 1 | Patients' characteristics.

Patient #	Diagnosis	Combined chemotherapy	Clinical response (the way of assessment)	Lymphocyte count before infusions 1-4 (CLL patients only) [10^9/ml]			
				1st	2nd	3rd	4th
1	DLBCL	CHOP	mCR (PET)				
6	HGL	EPOCH	PROG (PET)				
8	PMBCL	EPOCH	mCR (PET)				
9	DLBCL	CHOP	PROG (CT)				
10	BL	codox/ivac	mCR (PET)				
11	FL	COP	PR (CT)				
12	MZL	none	mCR (PET)				
17	CLL	none	PR (clinical)	91.44	42.79	15.03	9.44
18	CLL	FC	CR (clinical)	46.16	3.42	1.73	0.75
19	HGL	CHOP	PROG (CT)				
20	MZL	COP/bendamustine	PR (CT)				
21	CLL	FC	CR (clinical)	64.76	0.83	0.23	0.34
23	CLL	FC	CR (clinical)	128.0	4.37	2.58	0.85
26	CLL	FC	CR (clinical)	81.63	0.48	0.56	0.32
27	CLL	FC	PR (clinical)	90.96	2.29	2.63	0.93
31	FL	COP	CR (CT)				
33	CLL	FC	CR (MRD -)	6.67	2.82	0.17	1.05

Diagnosis: DLBCL, diffused large B cell lymphoma; HGL, high grade lymphoma; PMBCL, primary mediastinal B cell lymphoma; FL, follicular lymphoma; BL, Burkitt lymphoma; MZL, marginal zone B cell lymphoma; CLL, chronic lymphocytic leukemia.

Chemotherapy: CHOP, cyclophosphamide + hydroxydaunorubicin + oncovin + prednisone; EPOCH, etoposide + prednisone + oncovin + cyclophosphamide + hydroxydaunorubicin; COP, cyclophosphamide + oncovin + prednisone; FC, fludarbine + cyclophosphamide.

Clinical response: CR, complete response; mCR, metabolic clinical response; PR, partial response, PROG, progression; PET, positron emission tomography; CT, computed tomography; MRD, minimal residual disease.

serum preparation, as described in (48) and for preparation of EDTA-plasma.

Sample Handling

After collection and preparation, which was accomplished in approximately 30 min after blood collection, serum and plasma samples were aliquoted and kept at −80°C until the time of the experiment. Repetitive freezing and thawing were avoided, and the same rule was applied to normal human serum (NHS) and normal human plasma (NHP), which were prepared from the blood of healthy volunteers and pooled. NHS was then used as a positive control in the CDC assay. NHP was used as a milieu for the preparation of the calibration curve in ELISA-based measurements of C4d and TCC. Heat-inactivated normal human serum (Δ NHS) was prepared from NHS heated to 56°C for 30 min and then cleared by centrifugation at 3000 x G for 5 min. Δ NHS was used as a negative control in CDC assays as heat-inactivation depletes complement activity. Working dilutions of serum and plasma were prepared only before experiments in chilled tubes or microplates kept on ice.

Cell Lines

Raji, Ramos, Namalwa, SU-DHL-4 cells were obtained from the American Type Culture Collection. Cells were aliquoted and cryopreserved after the first few passages. Cells used for experiments were grown from such stock aliquots in RPMI 1640 medium with l-glutamine (ATCC) supplemented with 10% fetal bovine serum (ATCC) at 37 °C and humidified 5% CO_2 atmosphere. Cells were routinely checked for Mycoplasma contamination by DAPI staining (49) when cultured and never kept in continuous culture for more than 10 passages. The primary culture of CLL cells was established from heparinized patients' blood. Lymphocyte fraction was isolated using Lymphoprep (Stemcell Technologies) according to the manufacturer's instruction and assessed as a homogenous population by flow cytometry (>98% of gated objects) showing CD20 expression. Then CLL cells were cultured in a 1:1 mixture of RPMI 1640: DMEM (HyCult) medium supplemented with 10% FBS.

Assessment of Rituximab Concentrations

Rituximab concentration in samples collected just before and just after each infusion was measured using an enzyme-linked immunosorbent assay. 96-well ELISA MaxiSorp plates (ThermoFisher Scientific) were coated with 1 μg/ml of anti-rituximab (anti-idiotype) antibody RB01 (R&D Systems) and blocked with washing buffer (50 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween, pH 7.5) supplemented with 3% fish skin gelatin (Sigma-Aldrich). Patients' serum was diluted to the final concentration of 0,125% in PBS with 0.02% Tween-20 and 0.02M EDTA. Rituximab (Roche) serially diluted in NHS was used for the preparation of the calibration curve. The horseradish peroxidase-conjugated goat anti-mouse antibody (Dako, P0447) was used for detection. The assay was developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich), and absorbance readout at 450 nm was measured using a Synergy H1 microplate reader (Biotek).

CDC Assay

Complement-dependent cytotoxicity (CDC) functional assay was performed as described in (46). Briefly, cells previously loaded with calcein-AM (Sigma) were pelleted onto V-shape microplate wells and overlaid with 50 μl of the indicated serum with or without addition of rituximab. After 30 min of

incubation fluorescence of calcein released into the supernatant was measured at 490 nm/520 nm excitation/emission wavelength in Synergy H1 microplate reader (Biotek).

Measurement of Complement Activation Markers

Measurements of the early activation marker of the classical complement pathway, C4d, and the marker of terminal complement pathway activation TCC were performed as described in (50), with slight modifications regarding the TCC sandwich ELISA assay. Instead of zymosan-activated serum, serial dilutions of purified sC5b-9 complex (Complement Technology) in 5% NHP solution in PBS with 0.02% Tween-20 and 0.02M EDTA were used for calibration curve. Detection was achieved using polyclonal rabbit anti-human sC5b-9 neo antibody (Complement Technology) followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (Dako).

Statistics

The grouped analyses of differences in CDC potential and concentration of complement activation markers between pre- and post-infusion serum samples collected at each infusion were performed by multiple Sidak's comparison tests. Calculations were supported by GraphPad 6 software (Prism).

RESULTS

We analyzed the CDC activity of patients' sera collected immediately before and after each infusion of rituximab in two different experimental settings: i) without the addition of a new dose of rituximab and ii) with saturating concentration of rituximab added to patients' serum. The first measurement aimed to assess the cytotoxic activity of serum during the treatment, which reflected the retention of rituximab and the competence of the complement system. The second measurement was performed upon conditions, which imposed complement activity but not rituximab concentration, as a CDC-limiting factor. Thus, the latter assessed the immediate post-infusion complement depletion and whether such putative depletion overlapped with the next infusion. The results obtained for CLL and NHL patients are shown in **Figures 1** and **2**, respectively.

Only one CLL patient (#18) showed spectacular, significantly lower CDC of rituximab-supplemented post-infusion serum sample compared to the analogical pre-infusion sample and such CDC depletion was only observed at the first infusion (**Figure 1** and **Supplementary Statistics File**). Out of seven CLL patients included in the study, four accumulated more than 100 mg/ml of rituximab before infusion 3 (patient #27), infusion 4 (patients #18 and #33), and infusion 6 (patient #26). Similarly, six out of ten patients with NHL accumulated rituximab at the level of 100 mg/ml before infusion 2 (patient #12), infusion 3 (#1, #8, and #11) infusion 5 (#9) and infusion 8 (#20), respectively (**Figure 2**). In both groups of patients, there was a significant correlation between rituximab concentration and CDC exerted on Raji cells, with the saturation level of CDC achieved at rituximab concentration around 50 mg/ml (inlets in **Figures 1** and **2**). We found one NHL patient (#19) who presented depressed CDC throughout all infusions, even when serum samples were supplemented with extra rituximab (**Figure 2**). Interestingly, this patient did not respond to the therapy. One NHL patient (#31) exhibited a low level of CDC in both pre- and post-infusion serum samples, but all his samples regained functionality when supplemented with extra rituximab. Nonetheless, patient #31 achieved a complete response to the treatment.

Previously we characterized Raji as a cell line moderately sensitive to CDC exerted by anti-CD20 mAbs. Incubation of Raji cells in 50% NHS supplemented with CDC-saturating concentration of rituximab (50 μg/ml) yielded in c.a. 50% of lysis (43). In the current experiments performed in 10% of patients' sera (**Figures 1** and **2**), we observed the highest impact of rituximab on the CDC in a concentration range from 10 to 100 μg/ml. Therefore we attempted to assess the effect of the same concentration range either at the different load of tumor cells or on other CD20-positive tumor cells of different sensitivity to CDC (**Figure 3**). Experiments performed in 50% NHS should demonstrate the highest CDC effect theoretically attainable in blood. Raji cells showed CDC increase from 35% to 53% at 100.000 cells and from 25 to 35% at 1M cells when rituximab concentration increased from 10 to 100 μg/ml. Ramos cells showed increased CDC from 44 to 61% of full lysis but there was no effect of increased cell number. Similarly, a 10-fold increase of cell number did not significantly affect the lysis of SU-DHL-4 cells, where the CDC oscillated from 65% at 10 μg/ml of rituximab to 77% at 100 μg/ml of rituximab. Rituximab was ineffective in the killing of Namalwa cells and fresh culture of CLL cells, irrespectively on concentration (**Figure 3**).

CLL patients possess tumor cells circulating in their bloodstream, which are much better accessible for effector mechanisms than tumor cells residing in bone marrow, lymph nodes or other extravascular locations. We analysed appearance of complement activation markers in plasma samples from the CLL patients during the first four infusions (when available). Significant increase of either C4d and TCC were observed (if any) mainly after the first infusions (**Figure 4**), corresponding with the high number of circulating tumor cells further eliminated during the treatment (see **Table 1**). However, patients #21 and #27 did not show signs of strong systemic complement activation, despite the ability of their sera to exert CDC *in vitro* (**Figure 1**). Importantly, levels of both C4d and TCC markers do not correlate with CDC exerted on different target cells (**Supplementary Figure 1**) and should be considered as qualitative rather than quantitative measures of CDC *in vivo*.

DISCUSSION

There is no unanimous opinion on the role of complement in the therapeutic effect of type I anti-CD20 antibodies. Results of *in vivo* animal studies seem to be model-dependent (reviewed in (4)), and the predictive value of *ex vivo* CDC assays in uncertain. Bordon et al. reported the vulnerability of isolated CD20-positive tumor cells to the CDC as a predictor of clinical response to rituximab (51), but

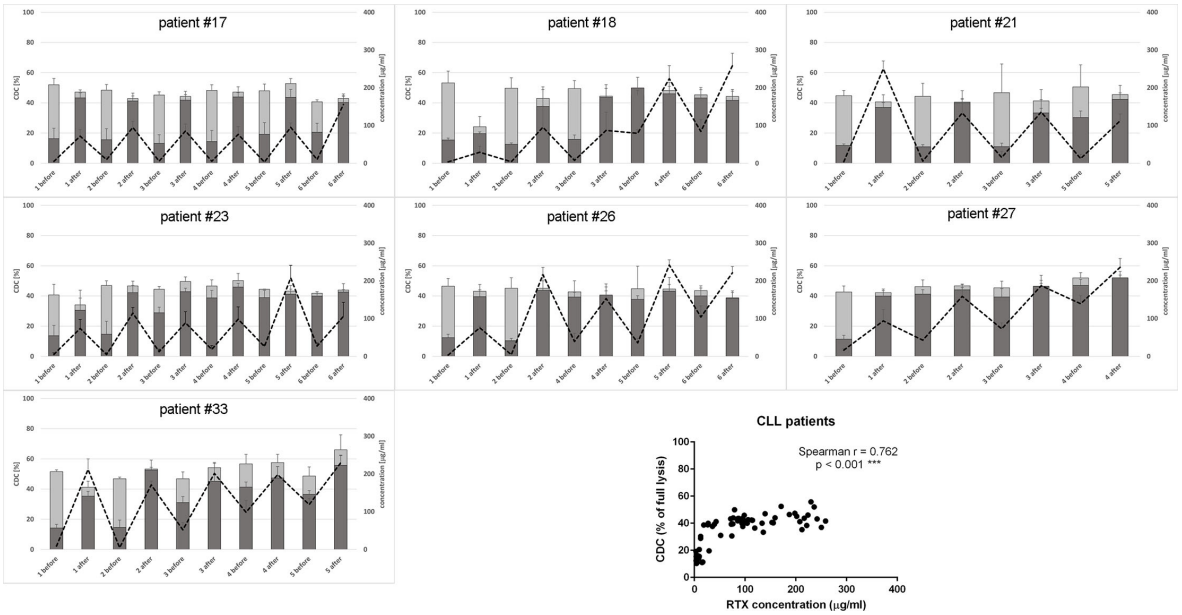

FIGURE 1 | CDC potential and rituximab concentration in serum samples collected from CLL patients. CDC potential was assessed in calcein release assay performed using Raji cells incubated with 10% patient's serum. Dark bars represent CDC levels of patients' sera non-supplemented with extra rituximab, grey bars represent CDC levels when sera were supplemented with 50 µg/ml of rituximab. Dotted line represents rituximab concentration (right Y axis). Each serum was tested in three independent experiments, error bars indicate standard deviation.

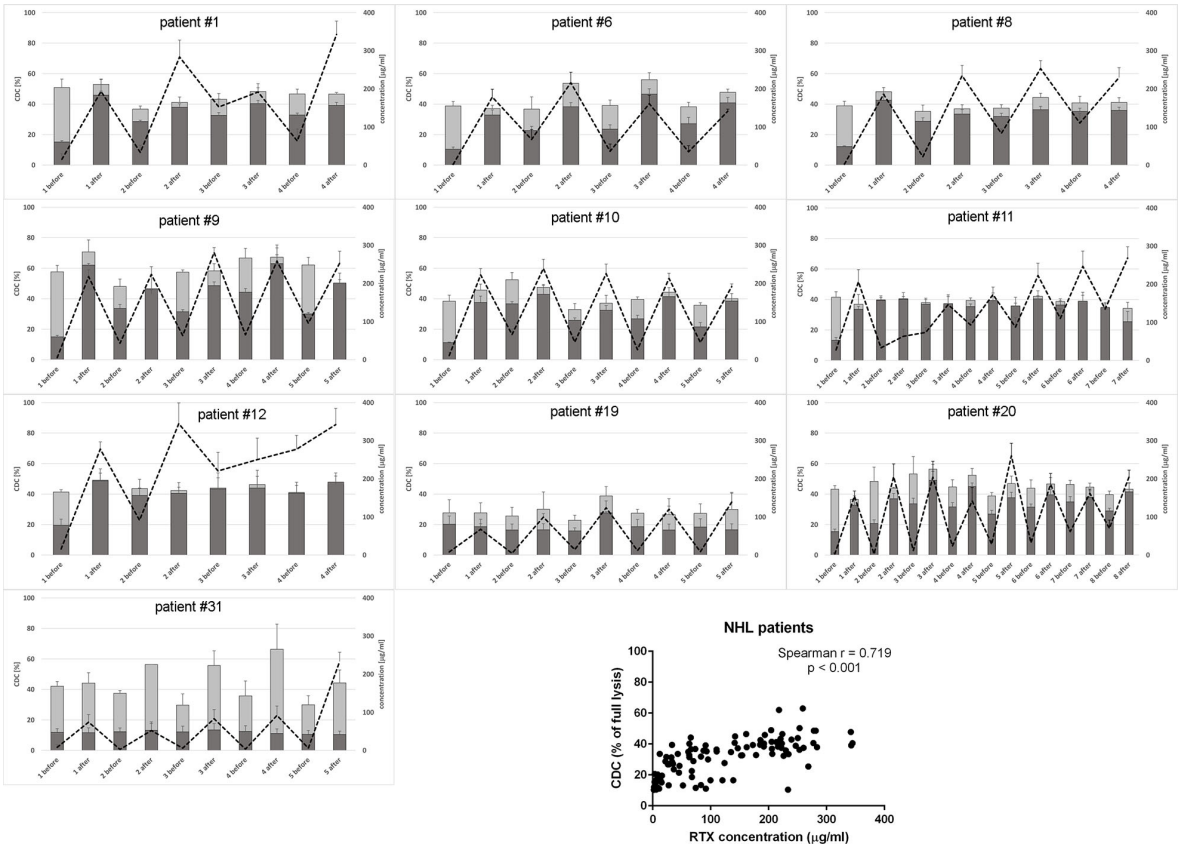

FIGURE 2 | CDC potential and rituximab concentration in serum samples collected from NHL patients. CDC potential was assessed in calcein release assay performed using Raji cells incubated with 10% patient's serum. Dark bars represent CDC level of patients' sera non-supplemented with extra rituximab, grey bars represent CDC level when sera were supplemented with 50 µg/ml of rituximab. Dotted line represents rituximab concentration (right Y axis). Each serum was tested in three independent experiments, error bars indicate standard deviation.

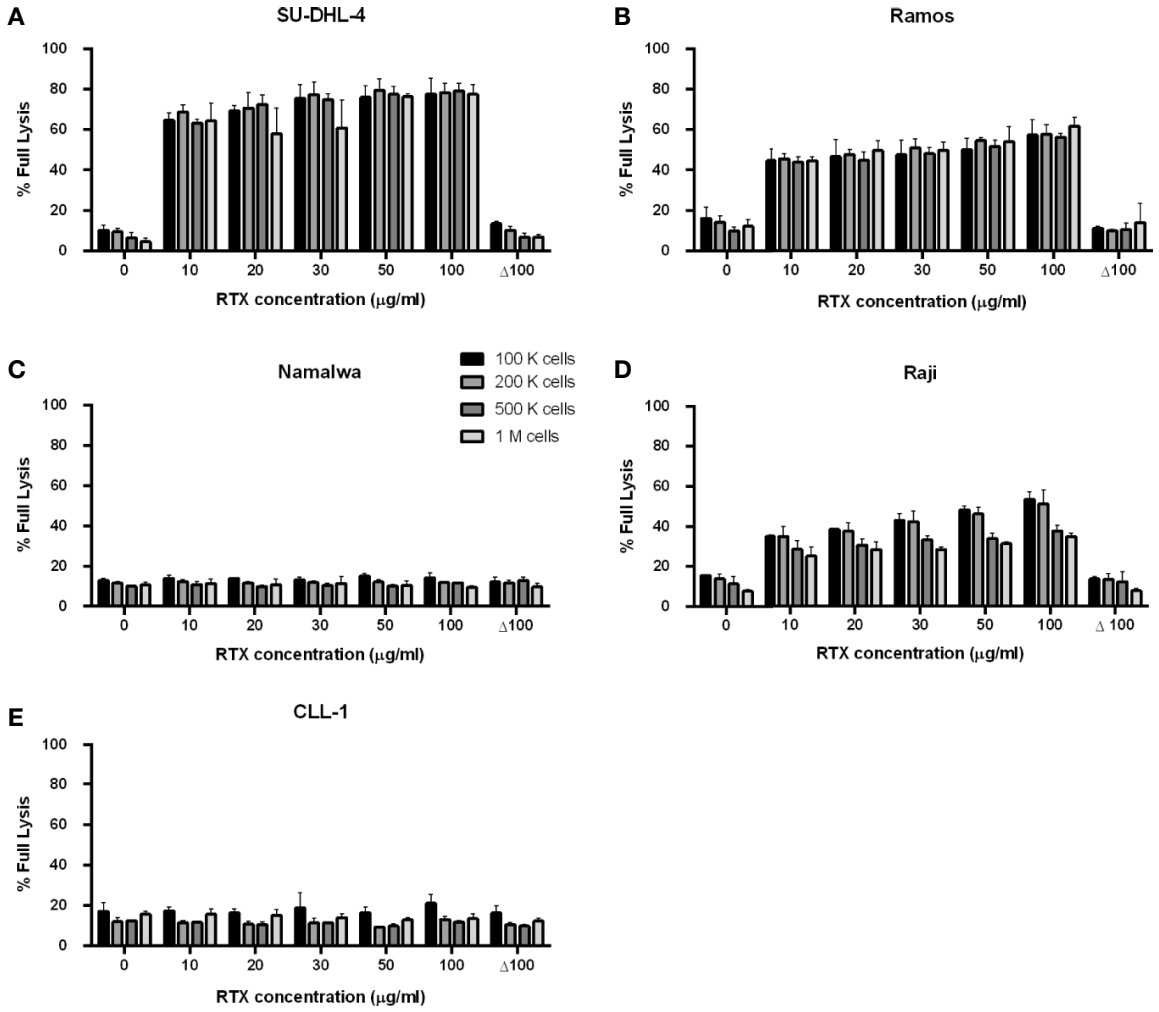

FIGURE 3 | CDC exerted in 50% normal human serum by rituximab at concentration range 10-100 μg/ml. CDC was examined on four CD20-positive cell lines: SU-DHL-4 **(A)**, Ramos **(B)**, Namalwa **(C)**, Raji **(D)** and fresh culture of CLL cells **(E)**. Supernatant collected from calcein-labelled cells lysed with 30% DMSO diluted in PBS served as the indicator of 100% (full) lysis. Readout obtained for heat-inactivated serum (Δ NHS) served as negative control, i.e. background lysis independent on complement activation. Cells were tested at quantities 1, 2, 5, and 10 × 10^5 cells/50 μl, Δ 100 group represents heat-inactivated normal human serum supplemented with 100 μg/ml of rituximab. Data were collected from three independent experiments, error bars indicate standard deviation.

two other studies presented contradictory results (52, 53). A strong argument for the complement role in CLL immunotherapy is the observation that clinical response to rituximab improved after supplementation with fresh-frozen plasma (31, 32). On the other hand, up to 40% of CLL patients may have deficiencies or low levels of circulating complement proteins (54). Therefore the first question we asked in the current study is whether the CDC activity of sera collected from the patients receiving rituximab is sufficient to lyse a model CD20-positive Raji cells. The functional assay we performed to answer this question is much more informative than measurements of the main complement components, whose physiological concentration range varies substantially (e.g., 0.6–1.4 g/L for C3 and 0.1–0.33 g/L for C4) (55). Notably, even C3 concentration as low as 0.18 g/L was reported sufficient to maintain a proper complement function (56).

Raji cell line is characterized as a moderately sensitive to rituximab compared to other B-cell lymphomas, thus enabling observation of either depressed or higher than average complement activity in CDC assays (43). Previously we demonstrated the utility of this model for the mirroring of the anti-CD20 antibody-driven complement consumption and found superior sensitivity of the assay when 10% instead of 50% serum was used (46). Importantly, 10% serum is a surrogate of the complement content in lymph or extravascular fluids, a natural microenvironment of lymphoma (13, 57, 58). However, an increase of NHS concentration from 10% to 50% did not result in a significant increase of CDC in Raji cells, as demonstrated in (43). In the current study, the readout of CDC assay at 10% patients' serum that contained saturating concentration of rituximab (**Figures 1** and **2**) in most of the cases

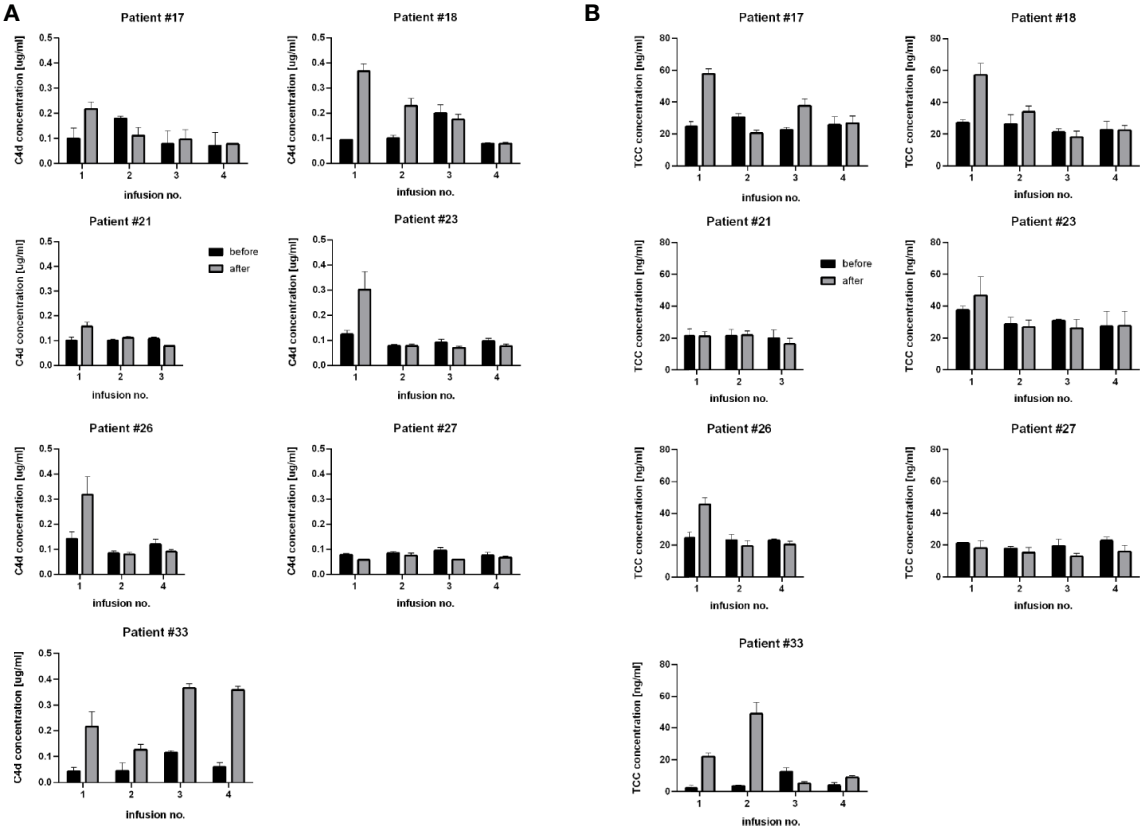

FIGURE 4 | Determination of levels of C4d and TCC, complement activation markers. Graphs show C4d concentration **(A)** and TCC concentration **(B)** in sera collected before (black bars) and after (grey bars) consecutive rituximab infusions in CLL patients. Data were collected from three independent measurements, error bars represent standard deviation.

was not significantly different from the readout obtained at 50% NHS (see **Figure 3D**, bar for the concentration of 50 μg/ml and 100 k cells). Nonetheless, there were few exceptions from this rule. All post-infusion samples of patients #19 and #31 had low CDC activity. Supplementation with additional 50 μg/ml of rituximab markedly improved the CDC readout in patient #31.

We did not study the complement activity of serum over several hours after infusion as others did (41, 59) but found only one patient (#18) who showed signs of complement exhaustion immediately after infusion. Importantly, such exhaustion did not overlap with the next infusion indicating that a four-week interval is enough for the restoration of the complement pool. These results are in agreement with another study, which analyzed the effect of ofatumumab, a stronger CDC-activating anti-CD20 antibody (60), applied in a 2-week interval (46).

CDC potential of pre-infusion serum samples (without addition of rituximab) correlated with the amount of accumulated rituximab in both CLL and NHL patients (inlets in **Figures 1** and **2**). The study by Berinstein et al. evaluated pharmacokinetics of rituximab in 137 non-Hodgkin's lymphoma patients, who received the 375 mg/m^2 dose once weekly for four injections (61). The median difference in rituximab concentration between post- and pre-infusion serum was approx. 250-270 μg/ml, whereas the median level of rituximab in pre-infusion samples was 63 μg/ml, 124 μg/ml, and 186 μg/ml at second, third, and fourth administration, respectively. Significantly higher accumulation of rituximab was noticed in responders to the therapy before the second and fourth infusion. Accumulation of the drug may be explained by a decreased number of accessible tumor cells in responders, but further studies also suggest the loss of target antigen due to internalization and trogocytic removal as a possible explanation (3, 62–64). In our cohort, the differences between post- and pre-infusion levels of serum rituximab were from 25 to 246 μg/ml in CLL patients and from 30 to 279 μg/ml in NHL patients. NHL patients who gradually accumulated rituximab throughout all infusions achieved complete response (#1, #8, and #12), partial response (#11, #20) or progression (#9). Part of the NHL patients with no gradual accumulation of rituximab had progressive disease (#6, #19), but the other part (#10 and #31) showed complete response, so there was no clear segregation into responders and non-responders in terms of rituximab accumulation. These results, opposite to the previous study, can be explained by a four-week instead of one-week interval in rituximab dosing. However, our results show that even at a four-week interval, there are patients (#1, #8, #11, and #12), which accumulate the amounts rituximab comparable to these delivered at the first infusion. Excessively administrated rituximab provides a risk for the selection of a CD20-low population of tumor cells (62). On the other hand, the bioavailability of rituximab in lymph nodes and other extravascular sites is lower than in serum (65). Such a high accumulation of rituximab and concomitant saturation of CDC potential in pre-infusion sera imposes a question if the additional dosing is necessary or counterproductive. Thus, a biopsy of lymphoma cells stained for either cell-bound rituximab or free antigenic CD20 sites will give a valuable hint on whether the therapeutic schedule should be modified or the therapy should be changed to type-II anti-CD20 antibodies such as obinutuzumab, which is superior for the killing of tumor cells *via* ADCC and direct mechanisms (37).

Circulating CD20-positive cells in CLL patients are much more accessible for complement than NHL cells in extravascular locations. Therefore, complement activation by rituximab on circulating CLL cells should be immediately mirrored by the appearance of complement activation markers such as C4d and TCC. C4d is a marker of early stages of the classical complement pathway activation, which leads to opsonization (and complement-dependent phagocytosis) and anaphylaxis. Soluble TCC is formed upon assembly of membrane attack complex (MAC) and indicates CDC. Previously we validated C4d and TCC assays on the cohort of 31 CLL patients and found that increase of TCC in the post-infusion samples took place when an increase of C4d was also observed (50). Nonetheless, the formation of C4d and TCC must depend on the expression level of either CD20 or complement inhibitors present on tumor cells and in patients' sera. We characterized numerous CD20-positive cell lines (including these presented in **Supplementary Figure 1**) and fresh CLL cultures for their expression of CD20 and endogenous complement inhibitors (43). As substantial differences were found in these cells, we assume similar variability in patients. Therefore, the concentration of detected markers cannot be directly associated with CDC intensity, as shown in **Supplementary Figure 1**, and directly compared between individuals. The appearance of C4d and TCC markers indicates whether the complement activation took place and whether it proceeded up to the terminal stages, respectively. The highest increase of complement activation markers should be expected after the first infusion when a high number of CD20-positive tumor cells is present. Indeed, most CLL patients had increased levels of C4d and TCC after the first infusion with a tendency to flatten the differences at consecutive infusions. Except for patient #17, who received rituximab as monotherapy and except for patient #33, the drop in absolute lymphocyte count after the first rituximab infusion in CLL patients was greater than 90% (**Table 1**). Patient #17 achieved a partial response and showed neither gradual accumulation of rituximab nor saturation of CDC serum activity in any of the pre-infusion samples (**Figure 2**). Two CLL patients showed a marginal (#21) or no increase (#27) in C4d. Accordingly, both patients showed no increase in TCC (**Figure 4**). Notably, patient #21 achieved a complete response, unlike patient #27, who responded partially and showed accumulated rituximab throughout all infusions and saturated serum CDC potential already before the second infusion (**Figure 1**).

Our analyses of the complement system competence accompanied by the measurements of rituximab concentration in serum during consecutive infusions performed in the group of 17 patients with heterologous B-cell malignancies are not sufficient to answer the question about the role of complement in the therapeutic effect of rituximab. However, there are two important observations from our study. Irrespectively of serum and drug concentration, rituximab could not exert CDC in freshly isolated CLL cultures (**Figure 3E**) and in Namalwa cells (**Figure 3C**), which express the relative levels of CD20 and complement inhibitors comparable to these observed in CLL cultures (33, 40, 43). These results are in line with our previous publication showing the inability of rituximab to

lyse CLL cells isolated from six patients (43). We conclude that CDC cannot be a sole killing mechanism of CLL cells *in vivo* when rituximab is applied as a monotherapy (as in patient #17), however, concomitant chemotherapy may additionally sensitize tumor cells for CDC, and complement receptor-driven phagocytosis cannot be ruled out. The second issue worth underlining is the fact that even in such a small group of heterologous patients treated with a standard rituximab dose, there were examples of individuals, who deserved a personalized approach. These examples were patient #27 who accumulated a high concentration of rituximab in serum and had fully functional complement but presented no increase of complement activation markers, patients #19 and #31 who had depressed or non-functional complement, and patients #1, #8, #11, and #12 who showed substantial accumulation of rituximab and additionally (#11 and #12) saturated CDC potential of their sera. Monitoring of the complement status and concentration of cell-free rituximab may suggest to clinicians that the ongoing therapy should be continued with type II anti-CD20 antibodies, impose the re-evaluation of a molecular target for the drug, or a delay of further infusions, respectively.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Local Bioethical Committee at Medical University of Gdańsk. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF, AU, KJ, AJ, and GS performed the experiments and/or optimized assays used in the study. MT, AM, and JZ diagnosed the patients and collected clinical material. AB, MO, and JZ wrote the manuscript. MO conceived the idea of the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Science Centre Poland grant no. 2014/14/E/NZ6/00182 and Cancerfonden.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.584509/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table 1
List of patients, who significantly decreased CDC activity in their post-infusion serum samples
* - p < 0.05, *** - p<0.001, according to Sidak's multiple comparison test. All blood samples were collected immediately before and after each rituximab infusions.
grey shedding – no infusion / missing sample

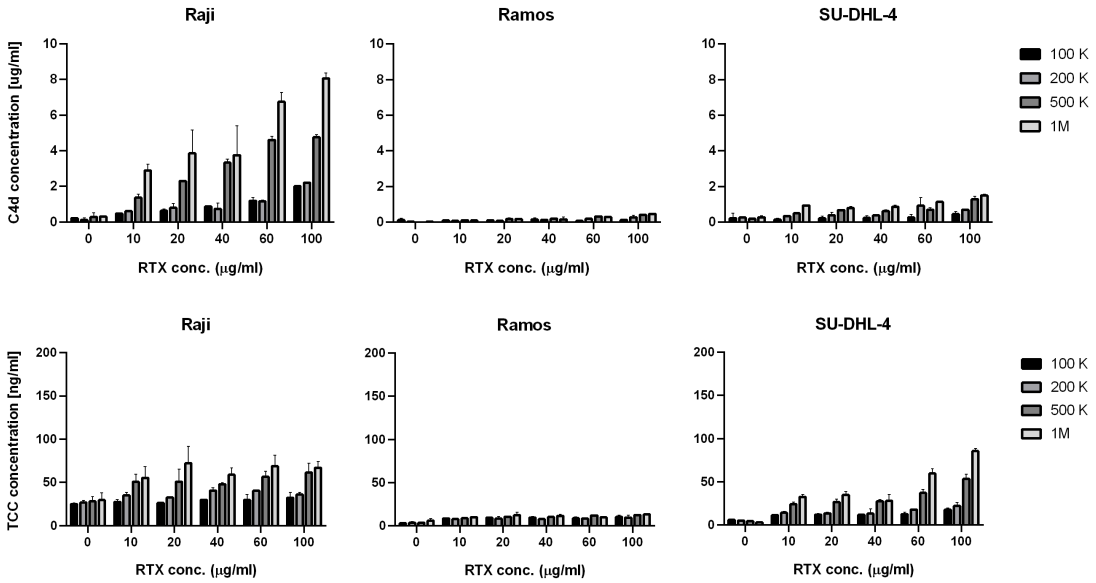
Patient #	infusion						
	2nd	3rd	4th	5th	6th	7th	8th
1							
6							
8							
9				*			
10							
11							
12							
19							
20							
31							
17							
18	***						
21							
23							
26							
27							
33							

Supplementary Table 2
List of CLL patients, who significantly increases C4d levels (top section) and TCC (bottom section) in their serum samples after administration of rituximab
** - p < 0.01, *** - p<0.001, according to Sidak's multiple comparison test
Grey shedding – missing sample

Patient #	Infusion			
	1st	2nd	3rd	4th
17	**			
18	***	***		
21	***			
23	***			
26	***			
27				
33	***	**	***	***

Patient #	Infusion			
	1st	2nd	3rd	4th
17	***		***	
18	***			
21				
23				
26	***			
27				
33	***	***		

Supplementary
Fig. 1



Levels of C4d (top panel) and TCC (bottom panel) markers detected upon incubation of Raji, Ramos and SU-DHL-4 cells with rituximab in 50% normal human serum for 30 minutes. Experiments were carried out in duplicates and results were obtained by ELISA assays, according to description in Methods section.

4 Publikacja nr 3

Stasiłojć G, Felberg A, Okrój M.

"Parameters critical for the effector mechanism of anti-CD20 antibodies revisited"

British Journal of Haematology, Vol. 180, Issue 6, 777-779, 2018

Wkład autorów:

Wszyscy autorzy byli zaangażowani w zbieranie materiałów i przygotowanie manuskryptu.

bjh editorial comment

Parameters critical for the effector mechanism of anti-CD20 antibodies revisited

Grzegorz Stasiłojć, Anna Felberg and Marcin Okrój

Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland

Keywords: anti-CD20, antibodies, hybridoma formation.

The hybridoma formation was first described in 1975 and since then, the concept of monoclonal antibodies (mAbs) as therapeutics in the treatment of B-cell lymphoma has developed. Finally, in 1997, the first antitumour anti-CD20 mAb, rituximab, was approved for clinical use in patients with non-Hodgkin lymphoma (NHL) (Salles *et al*, 2017). This prototypic immunotherapeutic, when applied as monotherapy or in combination with chemotherapeutics, contributed to the significant prolongation of patient survival and time to disease progression (Salles *et al*, 2017). Rituximab not only established a new class of anticancer drugs but was further accepted as a standard therapy for follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukaemia (CLL). Studies to improve rituximab therapy are continuing, and recently, a novel subcutaneous route of administration was positively evaluated. Despite the proven efficacy of rituximab, there are patients that show no, or only a partial, clinical response to initial therapy. Also, disease relapse in responders is common. Therefore, numerous attempts have aimed to exploit CD20 as a target molecule, including novel therapeutic schedules/strategies (Stasilojc *et al*, 2016; Bobrowicz *et al*, 2017) and novel compounds undergoing clinical trials (Bonavida, 2014; Perez-Callejo *et al*, 2015). Importantly, further improvement demands understanding of principles of *in vivo* anti-CD20 cytotoxicity, which is still a subject of ongoing debate (Okroj *et al*, 2013).

CD20 is the surface molecule found in most developmental stages of B cells. Its function is not fully recognized, except for links to B cell maturation and calcium signalling (Zhang, 2009; Okroj *et al*, 2013). However, CD20 is considered to be a good molecular target in B cell malignancies due to its relatively high content and limited shedding in comparison with other pan-B markers as well as the lack of the soluble form, which would compete for antibody binding (Zhang, 2009). There are only two extracellular fragments of CD20 molecule: residues 72–80 (small loop) and residues 140-186 (larger loop). Limited extracellular portions restrict the number of potential epitopes. In spite of the fact that the binding sites of most of the known anti-CD20 mAbs overlap, the effector mechanisms of these antibodies may be different. In some cases, antibodies can exert their cytotoxic function directly by triggering signalling pathways leading to programmed cell death (PCD). Alternatively, they utilize host immune system components, such as the complement system or cells bearing Fc receptor which are capable to induce the cytotoxic response (antibody-dependent cellular cytotoxicity, ADCC exerted by, e.g. Natural Killer cells). Anti-CD20 antibodies are classified into two types: type I, which efficiently activate complement but poorly induce PCD, and type II, with opposite characteristics. Importantly, both classes are capable of inducing ADCC, however, deposition of early complement components causes steric hindrance for immunoglobulin - Fc receptor interaction (Wang *et al*, 2008). In practice, these two effector mechanisms may cooperate, because ADCC occurs efficiently at low CD20 expression level not suitable for complement activation (van Meerten *et al*, 2006). Nonetheless, indirect mechanisms acting via the host immune system possess certain limitations. The activity of the complement system is restricted by endogenous inhibitors, which are often overexpressed by tumour cells (Okroj *et al*, 2013) or hijacked from serum (Stasilojc *et al*, 2016). Furthermore, complement is an exhaustible mechanism vulnerable to depletion by excessive mAbs or high tumour burden (Beurskens *et al*, 2012). The efficacy of ADCC depends on the ratio of effector to target cells and polymorphisms of Fc receptors. Efficacy-limiting factors common for both types of anti-CD20 mAbs include loss of CD20 by transcriptional downregulation, shedding, trogocytic removal or internalization, with the latter mechanism seeming to involve more type I than type II anti-CD20 mAbs (Vaughan *et al*, 2015). There are very few anti-CD20 mAbs classified as type III, which share type I and type II characteristics (Li *et al*, 2009; Bornstein *et al*, 2010; Nishida *et al*, 2011). Such specimens are highly desirable because they enable a complete repertoire of cytocidal instruments to be

Correspondence: Dr Marcin Okrój, Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, Dębinki 1 Street, 80-211 Gdańsk, Poland
E-mail: marcin.okroj@gumed.edu.pl

First published online 7 February 2018
doi: 10.1111/bjh.15130

used. It is highly possible that the optimal killing mechanism changes in relation to cellular (e.g. target molecule density) or supracellular factors (e.g. tumour burden, circulating vs. non-circulating tumours) therefore type III mAbs would be universal, in contrast to mAbs showing type I or type II features only. However, a key for their rationale design is the recognition of the attributes of both types. In this issue of the journal, Meyer *et al* (2018) shed a light on this topic.

The majority of anti-CD20 mAbs show type I characteristics, however, they differ in potency to induce complement-dependent cytotoxicity (CDC). Based on comparison of two popular type I anti-CD20 mAbs, rituximab and ofatumumab, preliminary conclusions were as follows: efficient CDC stems from the combination of several factors, such as dissociation rate, density of target molecule (a derivative of the ability to relocate target molecule to lipid rafts by given mAb), mutual interactions between Fc fragments of antibodies and distance of target epitope from the cell membrane (Teeling *et al*, 2006). For example, ofatumumab, which is a better CDC inducer, recognizes an epitope located more proximally to the cell membrane and dissociates slower than rituximab. Later studies showed that type I and type II specimens recognize overlapping epitopes (Mossner *et al*, 2010) so this criterion was not considered as crucial in the differentiation between those two types. Conversely, the processes of chimerisation or humanisation of the original murine mAbs may change type I/II characteristics, suggesting that manipulating the primary sequences of heavy and light chains outside the complementary determining regions (CDRs) is of particular importance (Uchiyama *et al*, 2010). The results obtained by *Meyer et al* (2018) are in agreement with this theory. The authors developed 10 anti-CD20 mAbs that recognized epitopes overlapping with rituximab and exhibited type I features but of different CDC activity. Two original mouse mAbs were expressed as mouse/human chimeric IgG1 variants and as a result, one such chimerized mAb additionally gained an ability to induce PCD. The type III characteristic was lost upon mutation of leucine to valine at position 11 (Kabat position 11) of the variable heavy chain. Mutation in this position is known to influence the elbow hinge angle (Stanfield *et al*, 2006) and interestingly, reverse V11L mutation in the type II mAb, obinutuzumab, leads to loss of PCD function (Mossner *et al*, 2010). These data indicate that conformation of the antibody, determined by its framework regions, governs the effector mechanism. Probably Kabat position 11 is not the only, although important, hot-spot, and further efforts should aim to identify other residues or certain combinations crucial for modulation of antibody effect. The general conclusion is that we may already have good prototypic antibodies in hand and further directions should not include identification of new, better clones but improvement of existing ones by engineering the non-CDR regions.

Acknowledgment

The authors are supported by National Science Centre Poland, grant no. 2014/14/E/NZ6/00182.

Author contributions

All authors were engaged in drafting the manuscript, MO wrote the final version.

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5 Publikacja nr 4

A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój

"Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics."

Cancer Immunology,Immunotherapy, Vol. 68, 587–598, 2019

Wkład autorów:

A. Felberg, A. Urban, A. Borowska, G. Stasiłojć , M. Okrój przeprowadzili eksperymenty. M. Okrój zaprojektował badanie. A.M. Blom i G. Stasiłojć dostarczyli niezbędne odczynniki i narzędzia. M. Taszner, A. Hellmann koordynowali zbieranie materiału klinicznego A. Felberg, A. Urban, A. Borowska, A.M. Blom, M. Okrój byli zaangażowani w pisanie manuskryptu.

Cancer Immunology, Immunotherapy (2019) 68:587–598
https://doi.org/10.1007/s00262-019-02304-0

ORIGINAL ARTICLE

Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics

Anna Felberg[1] · Aleksandra Urban[1] · Anna Borowska[1] · Grzegorz Stasiłojć[1] · Michał Taszner[2] · Andrzej Hellmann[2] · Anna Maria Blom[3] · Marcin Okrój[1]

Received: 6 July 2018 / Accepted: 17 January 2019 / Published online: 6 February 2019

Abstract

Anti-CD20 monoclonal antibodies (mAbs) rituximab and ofatumumab are potent activators of the classical complement pathway, and have been approved for the treatment of B-cell malignancies. However, complement exhaustion and overexpression of complement inhibitors by cancer cells diminish their therapeutic potential. The strategies of targeting membrane complement inhibitors by function-blocking antibodies and the supplementation with fresh frozen plasma have been proposed to overcome tumour cell resistance. We present a novel approach, which utilizes gain-of-function variants of complement factor B (FB), a component of alternative C3/C5 convertases, which augment mAb-activated reactions through a positive feedback mechanism called an amplification loop. If complement concentration is limited, an addition of quadruple gain-of-function FB mutant p.D279G p.F286L p.K323E p.Y363A (or selected single mutants) results in significantly increased complement-mediated lysis of ofatumumab-resistant tumour cells, as well as the complete lysis of moderately sensitive cells. Importantly, this effect cannot be achieved by further increasing ofatumumab concentration. Potentiation of cytotoxic effect towards moderately sensitive cells was less apparent at physiological serum concentration. However, an addition of hyperactive FB could compensate the loss of cytotoxic potential of serum collected from the NHL and CLL patients after infusion of rituximab. Residual levels of rituximab in such sera, in combination with added FB, were able to efficiently lyse tumour cells. We suggest that the administration of gain-of-function variants of FB can restore cytotoxic potential of complement-exhausted serum and maximize the therapeutic effect of circulating anti-CD20 mAbs.

Keywords Complement · Immunotherapy · Rituximab · Ofatumumab · Chronic lymphocytic leukemia

Abbreviations

aHUS	Atypical hemolytic uremic syndrome
C3G	C3 glomerulopathy
CDC	Complement dependent cytotoxicity
DLBCL	Diffuse large B-cell lymphoma
FB	Complement factor B
FH	Complement factor H
MAC	Membrane attack complex
NHS	Normal human serum
PNH	Paroxysmal nocturnal hemoglobinuria
ΔFB	FB-depleted serum

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-019-02304-0) contains supplementary material, which is available to authorized users.

✉ Marcin Okrój
marcin.okroj@gumed.edu.pl

[1] Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Dębinki 1 Street, 80-211 Gdańsk, Poland

[2] Department of Hematology and Transplantology, Medical University of Gdańsk, Gdańsk, Poland

[3] Department of Translational Medicine, Lund University, Malmö, Sweden

Introduction

Rituximab, an anti-CD20 antibody, was the first antitumour mAb approved for clinical use. Although it is considered as a milestone in the treatment of B-cell lymphomas [1], there are reports of minimal response in patients, and thus many efforts aim to develop more potent anti-CD20 mAbs. Ofatumumab, the fully human anti-CD20 mAb, is already used in

clinics, and a number of other candidates are passing clinical trials [2]. Ofatumumab, similar to rituximab, is classified as a type I mAb, which is a potent activator of the complement system [3]. Infusion of either rituximab [4] or ofatumumab [5] into CLL patients results in the rapid decrease of the classical complement pathway activity (CH50). However, post-infusion complement consumption does not automatically implicate tumour cell death, as numerous factors limit the efficacy of complement activation as an effector mechanism. The resistance of tumour cells to complement-dependent cytotoxicity (CDC) stems from overexpression [6, 7] and hijacking [8] of inhibitors normally protecting host cells from misguided complement attack. This makes the use of excessive concentrations of mAbs to overcome arrested CDC a suboptimal strategy. The first two components of the classical route, C1 and C2, are the ones with the lowest molar concentration in this pathway [9]. Given that most of the complement inhibitors target the mid-stages of the cascade, depletion of C1 and C2 by excessive mAbs may not only be unproductive in terms of decreased CDC but may also limit the effectiveness of further consecutive infusions [10]. Indirect evidence confirming complement exhaustion in vivo comes from observation in patients, in whom an addition of fresh frozen plasma to rituximab improved therapeutic effect [4, 11]. Moreover, high doses of anti-CD20 mAbs promote trogocytic removal of CD20 [12, 13] and produce population of $CD20^{low}$ tumour cells. Therefore, maximizing the CDC potential of anti-CD20 mAbs by neutralizing complement inhibitors [7] and supplementation with exhaustible complement components [4] is a more rational strategy.

Ofatumumab (originally designed as 2F2) was first reported in 2004 as a fully human anti-CD20 antibody, which outperformed rituximab in CDC activation [14]. These results were later confirmed by Beurskens et al., who tested both antibodies within a wide range of tumour cell load and serum concentration [10] and also by our studies, in which in vitro susceptibility to CDC under limited availability of complement of eighteen CD20 + cell lines and fresh CLL cultures was assessed [15]. We found that ratio of the target (CD20) to complement inhibitor CD55 distinguished cells highly sensitive to both anti-CD20 mAbs from those of moderate sensitivity, which were killed more efficiently by ofatumumab. CD55 enhances dissociation of both classical and alternative complement convertases, nodal points in the complement cascade. Our results were in agreement with the work of Takei et al., who found that loss of CD20 and the concurrent increase of CD55 expression are attributes of acquired resistance to rituximab [16] and also in agreement with studies showing that CD20-positive cells of similar CD20 expression undergo CDC induced by rituximab to an extent dependent on their CD55 levels [17]. Neutralization of CD55 as a concept for increasing the efficacy of therapeutic mAbs was recently exploited by Macor et al., who tested bispecific antibodies targeting CD20 and CD55 in vitro and in mouse xenograft models of Burkitt lymphoma [18]. This concept was also further demonstrated when Mamidi et al. performed siRNA-mediated silencing of membrane complement inhibitors [19]. Notably, expression of complement inhibitors is not limited to tumour cells, but is ubiquitous within the human body. Therefore, there is a need for specific delivery of siRNA for this purpose. The affinity of bi-specific antibodies is restricted to only one type of membrane complement inhibitor, and does not cover the activity of other redundant inhibitors (e.g. factor H (FH)), which is present in serum in micromolar concentration and, when hijacked by tumour cells, contributes to anti-CD20 mAb resistance [8]. Being aware of these limitations, we propose a novel strategy, which combines supplementation with an exhaustible complement component while avoiding complement inhibitors. Instead of blocking inhibitors' function, we utilized gain-of-function mutants of complement factor B (FB), which is a component of the alternative C3 and C5 convertases. Such mutants were identified in patients with atypical hemolytic uremic syndrome (aHUS) [20] and C3 glomerulopathies (C3G) [21] or designed in silico and shown experimentally [22] to form convertases insensitive to decay by multiple complement inhibitors.

Materials and methods

Protein expression and purification

Wild-type FB cDNA sequence (accession number NM_001710) additionally containing six histidine codons at 3′ terminus, as well as sequences for D279G, F286L, K323E, Y363A variants, and the quadruple mutant containing all aforementioned substitutions 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 [23]. Briefly, vector DNA was transfected into HEK293 Freestyle cells using Freestyle Max reagent (Thermo Fisher). Conditioned FreeStyle 293 expression medium (Thermo Fisher) was collected at days 2, 4 and 7 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 imidazole gradient.

In vitro culture of CD20-positive cells

All cell lines were cultured in RPMI 1640 medium with L-glutamine (Mediatech) supplemented with 10% foetal bovine serum (PANBiotech) at 37 °C and humidified 5% CO_2 atmosphere.

Clinical material (serum and erythrocytes)

Serum samples were collected from five 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. Patients #1 and #3 were diagnosed with diffuse large B-cell lymphoma (DLBCL), patients #2 and #4 with follicular lymphoma and patient #5 with CLL. Blood was collected into Vacutainer tubes with clot activator (BD Biosciences) before and after the first intra-venal infusion of standard rituximab dose (375 mg per 1 m^2 of body surface). Isolated blood was left in room temperature until clot formation (around 20 min), then centrifuged at 700 × *g* for 12 min at 4 °C, 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 [24]. For human erythrocytes, blood was collected into K_2EDTA Vacutainer tube (BD Biosciences), then loaded onto a gradient of Histopaque-1077 (Sigma) and centrifuged. The erythrocyte-containing fraction was collected, washed 5 × with PBS buffer, suspended 1:1 in Alsever's solution, and kept at 4 °C until the experiment.

Functional assays

Hemolytic assay assessing the ability of factor B mutants to enhance classical complement pathway was performed as described [25]. In some of the assays, factor B-depleted serum (Δ FB, Complement Technologies) was used instead of NHS. Two-step convertase assays measuring convertase activity over the time were performed as in [25]. Briefly, rabbit erythrocytes (Centre of Experimental Medicine, Silesian Medical University, Poland) were subjected to 5% normal human serum supplemented with wild-type or mutated factor B and C5 blocker (OmCI) for the indicated period of time. Cells were then washed and guinea pig serum (Harlan Laboratories) diluted 1:40 v:v in 40 mM EDTA-GVB (gelatin veronal buffer) buffer was added to develop lytic sites from convertases preformed in the first step of the experiment. Hemolysis was proportional to convertases' activity at given time point. A hemolytic assay measuring bystander lysis of human erythrocytes was performed by co-incubation of 1×10^5 ofatumumab-sensitized Raji cells in 10% or 50% NHS, optionally supplemented with 20 µg/ml of wild-type or mutated FB. The amount of erythrocytes was adjusted in a way that full lysis sample (10 µl of erythrocyte solution + 90 µl of water) gave absorbance readout of 2.0 AU at 405 nm. Quantification of released haemoglobin was assessed after 30 min.

CDC assay

CD20-positive cells were harvested, suspended in complete medium to yield 10^6 cells/ml and calcein-AM (Sigma) was added to the final concentration of 1 µg/ml. After 30 min incubation at standard culture conditions, cells were washed with PBS buffer with Ca^{2+}/Mg^{2+} (Biowest), loaded into the V-shape wells of 96-well microplate (Nunc) at 10^5 cells (or more, as indicated separately in the text) per well and pelleted. Pellets were overlaid with PBS w. Ca^{2+}/Mg^{2+} containing desired concentration of ofatumumab (GlaxoSmithKline) and NHS, in a total volume of 50 µl. Microplates were incubated for 30 min. at 37 °C and shaken at 650 rpm, then overlaid with another 50 µl of PBS buffer and centrifuged. Eighty microliter of the supernatant was transferred into flat-bottom plate and fluorescence 485/515 nm was measured in Synergy H1 (Biotek) reader. Fluorescence readout obtained for cells loaded with calcein-AM and lysed with 2% NP40 (Sigma) was considered as full lysis.

Assays measuring complement consumption/complement activity restoration

The concept of complement consumption assay was similar to that originally described by Beurskens et al. [10]. One hundred thousand cells of the selected CD20-positive cell lines (Daudi and Raji) were harvested and suspended in PBS solution with Ca^{2+}/Mg^{2+}-containing NHS (5% for Daudi, 10% for Raji cells) and ofatumumab (50 µg/ml). Some solutions were additionally supplemented with their physiological concentration of recombinant wild-type or quadruple FB mutant. Cells were incubated at 37 °C and 50 µl of the sample was pelleted after selected time points (0.5, 1, 2, 4, 24 h). Supernatants were collected and used in CDC assay (performed as described above) instead of an aliquot of fresh NHS. In alternative versions of the assay aimed to assess the restoration of cytotoxic potential by mutated FB, the first step was carried out in the presence of 5×10^5 Raji cells and 50% of NHS but without addition of FB. Then supernatant was transferred to the new portion of calcein-AM-labelled Raji cells (1×10^5) and FB variants were added.

Results

Alternative pathway plays a role in anti-CD20 mAb-mediated complement activation

While antitumour antibodies initiate classical complement pathway, augmentation of the cascade is achieved by the amplification loop, which engages alternative convertases and FB (Fig. 1). To confirm that alternative complement pathway is relevant to the amplification of anti-CD20

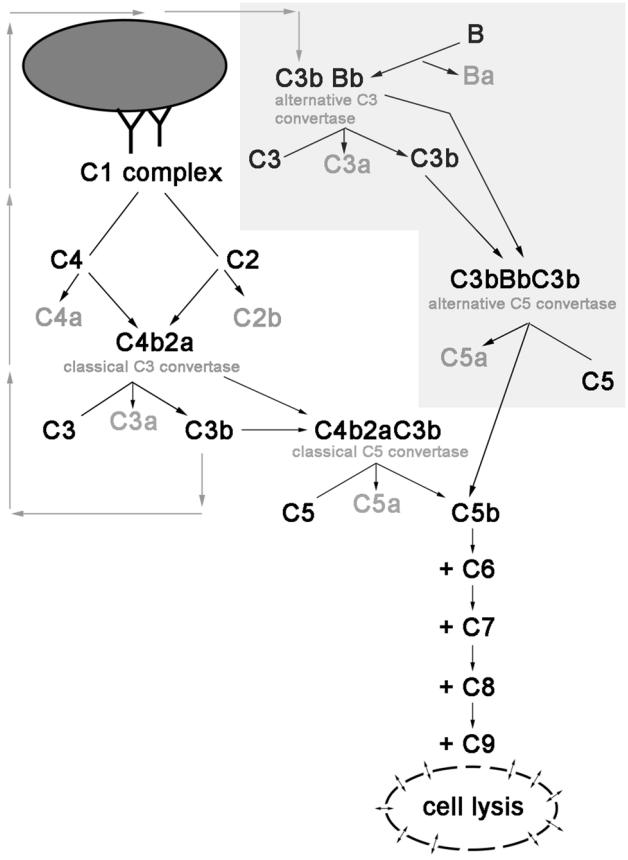


Fig. 1 Scheme of mAb-initiated complement activation. Binding of antibodies onto the surface of target cell initiates the classical complement pathway. Classical C3 convertase cleaves C3 molecule into C3a and C3b fragments. Binding of C3b to the parental C3 convertase complex switches its substrate specificity to C5, hence forming C5 classical convertase. Alternatively, binding of C3b directly to the cell surface launches so-called amplification loop (shaded polygon), whereby interaction with factor B leads to the formation of alternative C3 and C5 convertases. Cleavage of C5 by either classical or alternative C5 convertase initiates common, terminal pathway eventually leading to osmotic lysis of target cell

mAb-mediated complement activation, we performed CDC assay in serum with or without FB. Previously, we found Daudi cells to be highly sensitive to ofatumumab at low serum concentrations [15] and now we tested the same cells at two different, suboptimal concentrations of ofatumumab to better visualize the importance of alternative pathway. In parallel with increasing serum concentration, there was a trend towards higher CDC in FB-reconstituted serum. Differences reached the twofold level and statistical significance ($p < 0.01$) at 10% serum (Fig. 2) confirming that amplification of the classical complement pathway via formation of alternative convertases plays an important role even in case of cells highly sensitive to ofatumumab.

Gain-of-function mutants of factor B enhance cytotoxic effect of anti-CD20 mAbs by supporting activity of complement convertases

We successfully expressed and purified wild-type and D279G, K323E, Y363A single mutants but we could not express the F286L mutant, in spite of three independent attempts. Notably, we obtained quadruple mutant, which embraced D279G, K323E, Y363A and F286L substitutions (supplementary Fig. 1). Aforementioned mutations in factor B were previously characterized as providing resistance to numerous complement inhibitors (FH, CR1 and CD55), enhanced C3 turnover and binding affinity of FB to C3b fragment [20, 22, 26]. Next, we tested whether these FB mutants have potential to enhance CDC. To do so, we performed a preliminary study on antibody-sensitized sheep erythrocytes, a common model for assessment of the classical complement pathway [25]. The experiment was performed in FB-depleted serum reconstituted with increasing concentrations of given FB variant. Addition of all recombinant FB mutants but Y363A caused significantly more intense hemolysis, comparing to addition of wild-type (WT) FB (supplementary Fig. 2).

The hemolytic assay shows the overall effect of complement activation but provides no information about the efficiency of particular steps of the cascade. Therefore, we ran an experiment to find out whether an addition of FB mutants to NHS results in formation of alternative complement convertases of elevated activity or extended stability. This experiment was performed on the surface of rabbit erythrocytes, which spontaneously activate alternative complement pathway [25]. Serum supplemented with FB variants D279G and K323E formed alternative convertases of either higher activity (as visualized by more intense hemolysis at T_{max} point = 20 min) or extended stability (as the decay of convertase activity is slower than upon addition of wild-type FB) (Fig. 3). Convertases formed in the presence of D279G and Y363A mutants reached their high activity significantly faster; however, in case of Y363A mutation such effect was visible only at single time point of 10 min. The addition of quadruple mutant resulted in the formation of convertases of extended stability, as decay rate was significantly slower.

Erythrocytes are a widely used model for complement system activation analysis, but it may not reflect all physiologically important issues since the lack of human complement inhibitors may lead to underestimation of some relevant effects. To get full insight into consequences of each mutation on mAb-mediated killing of tumour cells, CDC assay employing anti-CD20 mAb (ofatumumab) was performed. Based on our previous work, we selected three cell lines of high (BJAB), moderate (Raji) and low (Namalwa) sensitivity to ofatumumab-mediated CDC [15]. Eighty percent of BJAB cells lysed already at 5% serum and 50 µg/

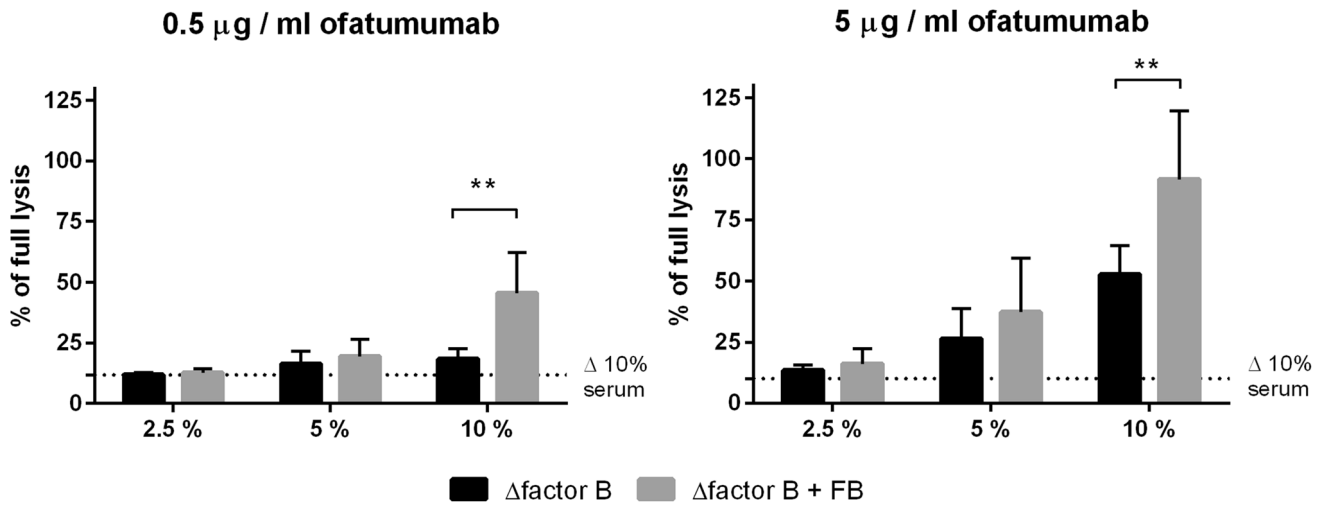
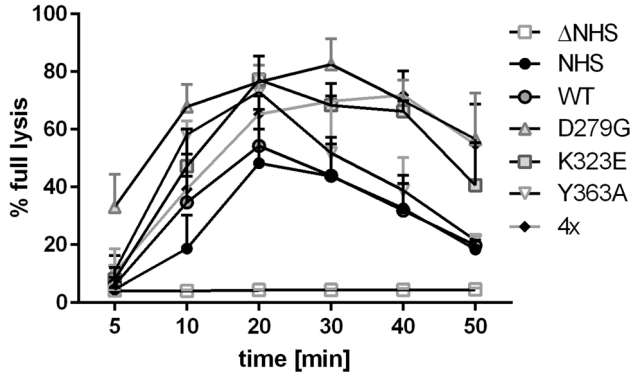


Fig. 2 CDC exerted by ofatumumab in serum ± FB. Calcein-AM-labelled Daudi cells were treated with ofatumumab (0.5 µg/ml or 5 µg/ml, left and right panel, respectively) and 2.5%, 5% and 10% of FB-depleted serum or the same serum reconstituted with recombinant wild-type FB. The readout of cells lysed with 2% NP40 was considered as maximal (100%) lysis whereas readout obtained in 10% of heat-inactivated normal human serum (Δ 10%) was considered as a background, complement independent lysis and depicted by dotted line. The graphs present data from three independent experiments and error bars represent standard deviation. **Statistical significance between FB-deficient and sufficient serum at p level < 0.01 according to Sidak's multiple comparison test for paired data (GraphPad Prism)



significance vs. wild-type FB:	*5*	*10*	*20*	*30*	*40*	*50 min*
D279G:	**	***	*	***	***	***
K323E:	-	-	*	**	***	*
Y363A:	-	**	-	-	-	-
4x:	-	-	-	**	***	***

Fig. 3 Activity of alternative complement convertases formed in serum supplemented with mutated FB variants. Assay was performed with 5% NHS supplemented with 10 µg/ml of wild-type (WT) or single (D279G, K323E or Y363A) or quadruple (4×) gain-of-function FB mutant. Heat-inactivated normal human serum (Δ NHS) was used as a negative control. Data are collected from three independent experiments and error bars show standard deviation. Statistical significance was assessed at $p < 0.05$*, $p < 0.01$** or $p < 0.001$*** according to Dunnett's multiple comparison test (GraphPad Prism)

ml ofatumumab. Addition of D279G mutant significantly increased CDC ($p < 0.05$) and addition of quadruple mutant increased lysis to maximal level ($p < 0.01$). Importantly, this effect could not be achieved by further increase of mAb concentration (Fig. 4, upper panel). CDC of Raji cells at 10% serum and 50 µg/ml ofatumumab oscillated around 45% of full lysis and further addition of ofatumumab slightly but not statistically significantly increased CDC (Fig. 4, middle panel). However, supplementation with D279G, Y363A and quadruple FB mutants resulted in significant increase of CDC and the two latter proteins brought CDC to maximal level. Notably, as little as 5 µg/ml of quadruple mutant (corresponding to c.a. 25% of wild-type FB content in 10% serum) caused more than double increase of CDC. The supportive effect of D279G and quadruple mutant was also observed in case of Namalwa cells normally resistant to ofatumumab and increased CDC from 36 to 55% of full lysis in 20% serum ($p < 0.05$, Fig. 4, bottom panel).

Experiments presented in Fig. 4 were performed at serum concentration lower than physiological (50%), thus modelling conditions of limited complement availability. We checked whether the same CDC-enhancing effect of quadruple mutant could be observed at physiological serum concentration. Further, CDC assays were performed using a higher number of tumour cells (2×10^5, $5 \times 10^5$and 1×10^6 per well) in a range of serum concentration between 5% and 50% (Fig. 5). Next to previously analysed Raji and Namalwa cells, we also incorporated two other cell lines: moderately sensitive (similar to Raji) WSU-NHL cells and resistant (similar to Namalwa) SU-DHL-8 cells. As expected, overall CDC decreased with the increase of cell number. Importantly, CDC-enhancing effect of quadruple mutant on moderately sensitive cells

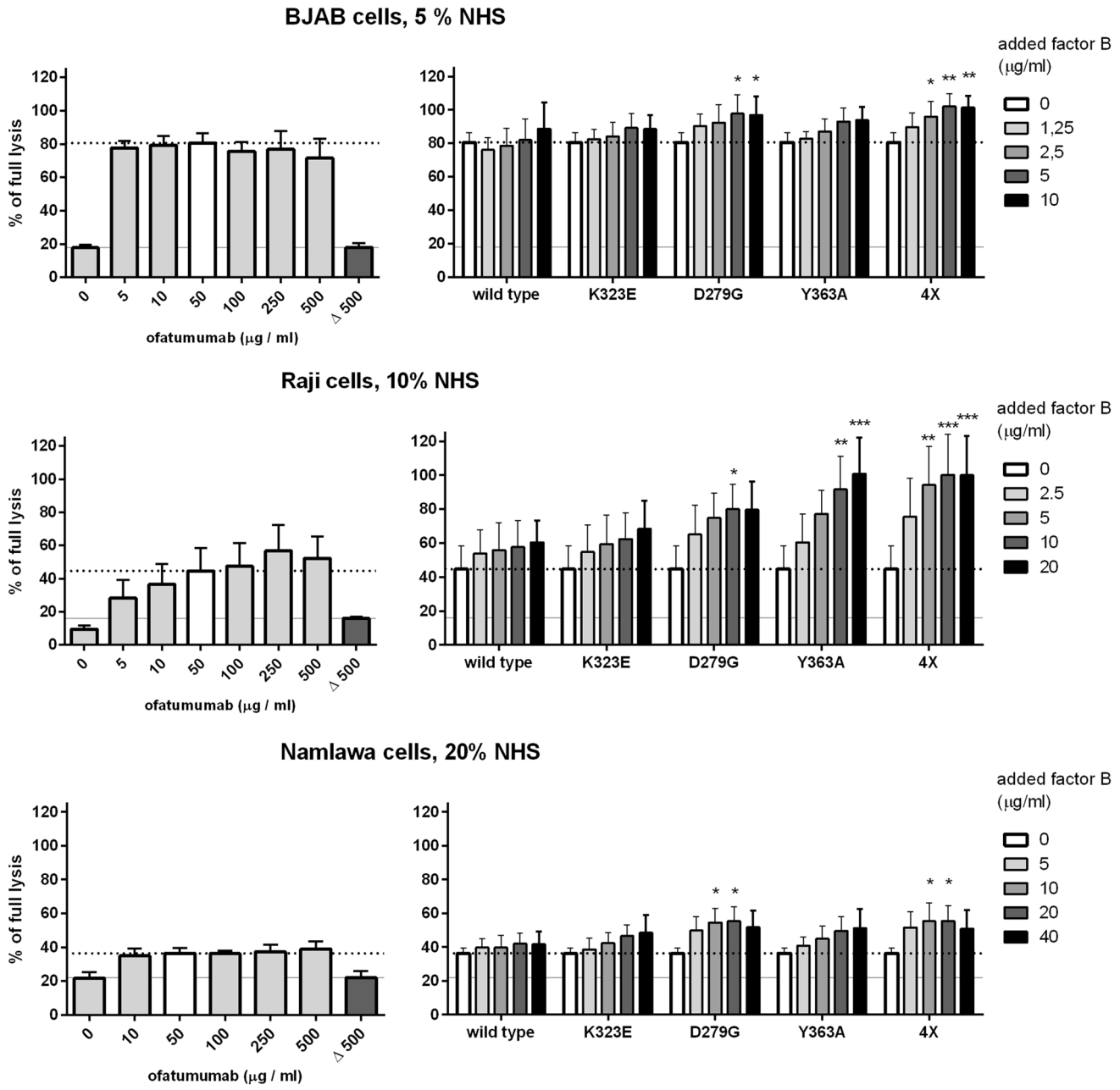


Fig. 4 CDC of ofatumumab supplemented with gain-of-function variants of FB in CD20 + cells. The left panel shows CDC exerted by ofatumumab in BJAB, Raji and Namalwa cells. The last, dark grey bar (Δ 500) and grey solid line show the readout obtained in heat-inactivated serum, thus considered as a background, complement-independent lysis. The bar for 50 µg/ml is indicated with white colour since this concentration was applied in experiments shown in right panel (and this reference CDC level is further indicated with dotted line). Cells were treated with ofatumumab, serum (at the concentration indicated for each cell line) and supplemented with increasing concentrations of wild-type or gain-of-function single or quadruple FB mutant. Statistical significance at p level < 0.05*, $p < 0.01$** and $p < 0.001$*** in comparison to CDC without any additional FB (white bar) is calculated according to Dunnett's multiple comparison test (GraphPad Prism). Graphs present data from three or four (BJAB cells) independent experiments and error bars show standard deviation

was only detected at lower (5% and 10%) serum concentrations while diminished at physiological serum concentration (Fig. 5, upper two panels). In contrast, the effect on resistant cell lines was statistically significant upon physiological serum concentration but probably of limited biological relevance, as the overall percentage of lysis did not exceed 25% and 15% of tumour cells, respectively (Fig. 5, bottom panels).

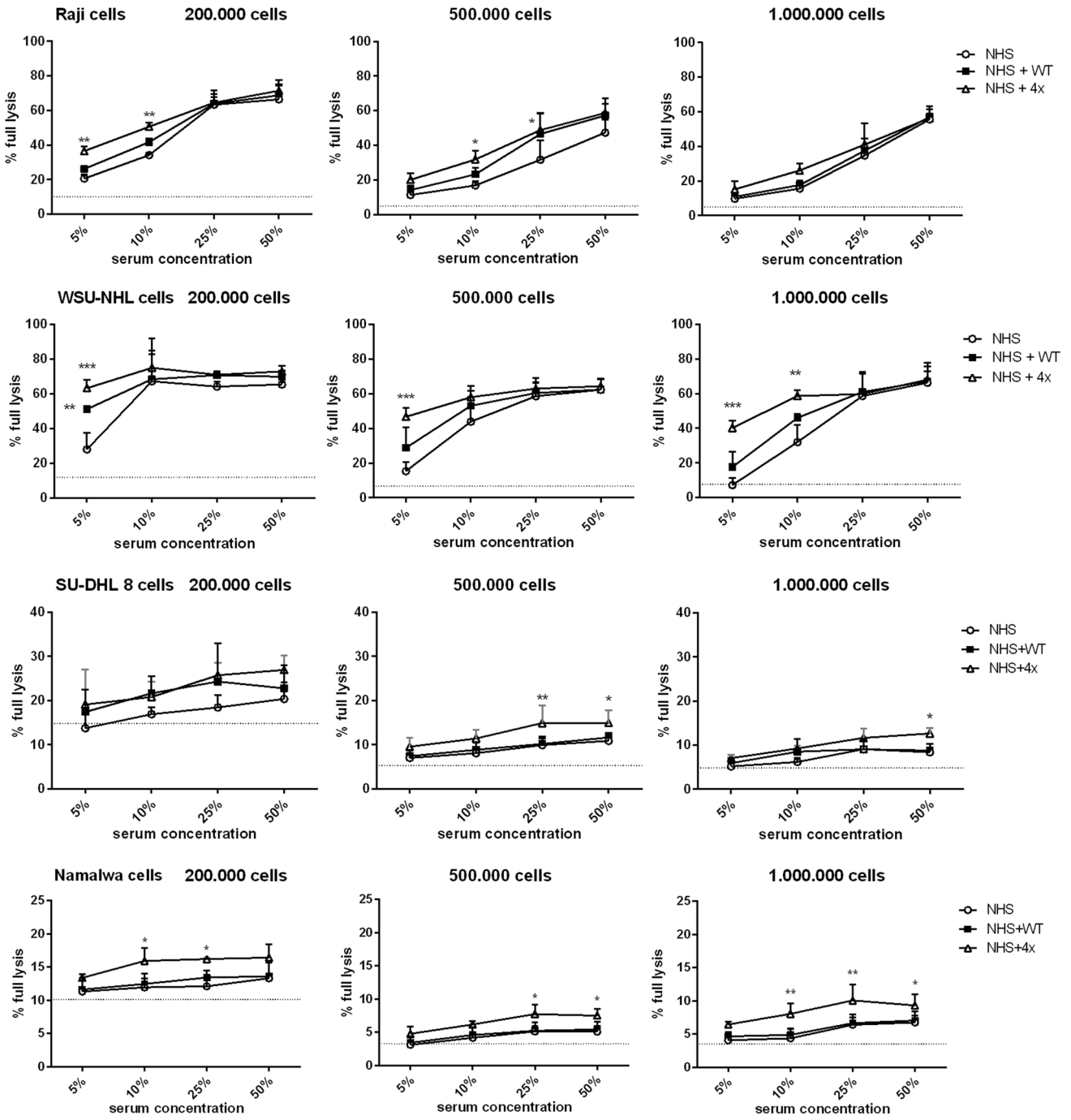


Fig. 5 CDC mediated by ofatumumab supplemented with quadruple FB mutant in CD20+cells—titration of serum and cell number. The graph shows CDC mediated by ofatumumab in WSU-NHL, Raji, Namalwa and SU-DHL-8 cells upon different serum concentrations and number of cells per well. The quadruple gain-of-function mutant or the wild-type FB was added to respective samples at a concentration of 20 µg/ml. Grey solid line shows the readout obtained in 50% heat-inactivated serum, thus considered as a background, complement-independent lysis. Statistical significance at p level < 0.05*, $p < 0.01$** and in comparison to CDC without any additional FB is calculated according to Dunnett's multiple comparison test (GraphPad Prism). Graphs present data from three independent experiments and error bars show standard deviation

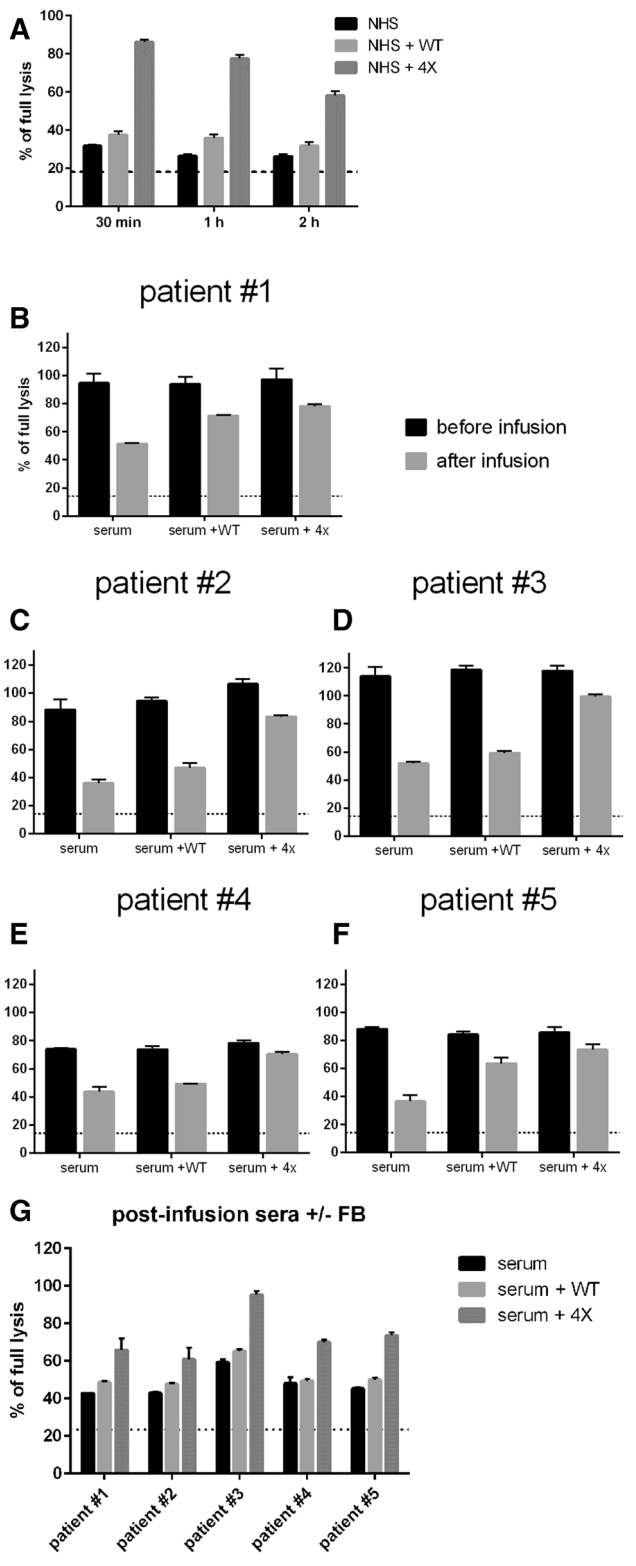


Fig. 6 Effect of quadruple FB mutant on CDC potential of complement-depleted sera. Panel **a** 50% NHS was incubated with ofatumumab-sensitized Raji cells (5×10^5) for 30, 60 or 120 min and thereafter supernatant was transferred to the new portion on calcein-AM-labelled Raji cells (1×10^5). 20 µg/ml of FB variants was added at this point and CDC was measured. The graph shows one representative (out of two performed) experiment, each analysed in triplicates. Panels **b**–**f** show CDC readouts of patients' sera collected before and after the first infusion of rituximab. Sera were supplemented with 50 µg/ml of ofatumumab and optionally with 20 µg/ml of FB variants. Panel **g** shows the CDC of post-infusion sera with no addition of ofatumumab and optionally supplemented with 20 µg/ml of FB variants. Experiments shown in panels b–f were run in triplicates

Gain-of-function FB mutants can restore cytotoxic potential of serum after complement consumption

It is known that administration of anti-CD20 mAbs induces rapid depletion of complement and possibly leaves the body devoid of effector mechanism capable of further killing of tumour cells [4–6, 10]. Therefore, we decided to model hypothetical conditions of exhausted complement in patient's serum. Two-step CDC assay was performed [10]. In the first step, complement in 50% serum was subjected to consumption by ofatumumab-sensitized cells and then in the second step, the supernatant was transferred to the fresh portion of tumour cells labelled with calcein-AM. Results showed that an addition of quadruple FB mutant at the second step but not wild-type FB can rescue cytotoxic activity of serum (Fig. 6a). The same was proven for patients' sera collected before and after infusion of rituximab. Addition of quadruple FB mutant to 50% pre-infusion serum supplemented with ofatumumab had little effect on CDC but substantially improved CDC potential of post-infusion sera, which otherwise lost half of their activity (Fig. 6b–f). Notably, the same result was obtained for sera from all five patients with different diagnoses of B-cell malignancy. Finally, we showed that quadruple mutant can even boost the CDC potential of post-infusion sera without addition of extra ofatumumab, i.e. making use of rituximab remaining in these sera (Fig. 6g) thus showing that not the availability of antitumour mAb but availability of complement is a factor limiting effective CDC of tumour cells in patients' sera.

Complement consumption and bystander lysis upon the addition of gain-of-function variants of FB

We showed that gain-of-function components of alternative convertases can enhance cytotoxic effect of antitumour antibodies. However, due to the nature of alternative pathway, which is constantly active at low level, these proteins boost spontaneous C3 activation and may deplete the available pool of exhaustible complement components. Accordingly, hypocomplementemia is often the hallmark of mutations leading to overactive phenotype of alternative convertases [27, 28]. Therefore, we performed functional assay comparing the ratio of CDC decay in serum without any supplements and sera supplemented with wild-type FB or quadruple gain-of-function FB mutant (which best supported CDC in cytotoxic assays). Concentrations of

added FB corresponded to maximal concentrations used in CDC assay shown in Fig. 4. Raji or Daudi cells were incubated with ofatumumab and serum ± supplements for time periods varying from 30 min to 24 h. Putative complement consumption influences cytotoxic capacity of serum, which was evaluated in the next step of the experiment. Supernatants were collected and used instead of serum in another round of CDC assay performed on calcein-AM-loaded cells. CDC dropped gradually from time point of 30 min and reached the lowest level after 2 h (Raji) or 4 h (Daudi). Importantly, in our experimental conditions, we did not notice any significant differences in CDC decay between normal human serum and sera with supplements (Fig. 7a, b). Thus, it is theoretically possible to adjust concentration of FB mutants in a way, which would enable enhancement of type I mAb-mediated cytotoxic effect but eliminate excessive depletion of complement in the longer period of time. However, we have evidence that such problem may exist when the most active variants of FB dominate in the sample. Our pilot hemolytic and convertase assays were performed in FB-depleted serum reconstituted with recombinant FB mutants. In such conditions, hemolytic activity of quadruple mutant was negligible (supplementary Fig. 3) and convertase activity (supplementary Fig. 4) was diminished in terms of amplitude and delayed in terms of T_{max} point. Also, we have tittered the concentration of quadruple FB mutant at constant serum concentration and observed that the increase of FB beyond 20 µg/ml did not cause more efficient CDC whereas 100 µg/ml concentration decreased CDC (data not shown). Together with the decrease of CDC-enhancing potential of quadruple mutant when moderately sensitive cells were incubated at high serum concentration (Fig. 5), these data suggest unproductive complement consumption in the fluid phase when either too many molecules of hyperactive FB are present or too many lytic sites are available.

Another hypothetic unwanted side effect of complement activation is bystander lysis, a phenomenon caused by soluble, initial components of the terminal complement pathway, i.e. C5b6 complex. This intermediate can be inserted into the membrane of virtually all cells, not necessarily the cell, which initiated complement activation and enables the formation of Membrane Attack Complex (MAC). Extensive complement activation is a condition favourable for such unspecific cell damage [29]. We tested whether bystander lysis can be provoked by the co-incubation of ofatumumab-sensitized Raji cells and human erythrocytes with either 10% or 50% serum in the presence of wild-type or mutated FB (Fig. 7c). We observed very little hemolysis comparable to the level obtained for sample incubated with heat-inactivated serum (negative control). There were no differences between wild-type, mutated FB (added at the concentration optimal for enhancement of complement-mediated lysis) and serum sample without additions (Fig. 7c).

Discussion

Our results provide a proof of concept that hyperactive variants of convertase forming proteins capable of bypassing multiple complement inhibitors can act as supporters of type I anti-CD20 mAbs. Notably, CDC assays performed on CD20 + cells revealed that results of hemolytic assays and convertase assays performed on erythrocytes do not always correspond to the results of experiments performed on human tumour cells. For example, Y363A mutation, described as the one resulting in the formation of convertases resistant to the inhibitory effect of CD55 [22] caused no increase in hemolysis of sheep erythrocytes (supplementary Fig. 2) and only moderate effect on alternative convertases (Fig. 3). Conversely, when used as a supplement to ofatumumab in CDC assay, it showed extraordinary

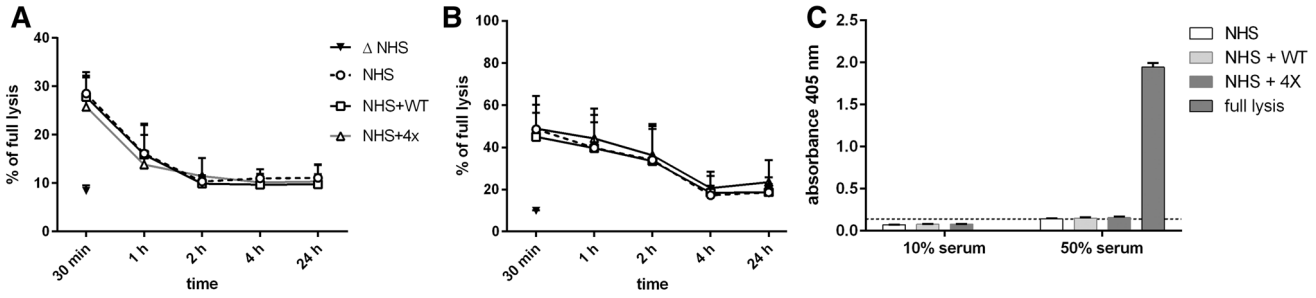


Fig. 7 Evaluation of unwanted side effects upon serum supplementation with wild-type FB and quadruple gain-of-function mutant. Raji **a** and Daudi **b** cells were treated with ofatumumab and NHS for the indicated time period. Some serum samples were supplemented with wild-type FB (WT) or quadruple FB mutant (4×). Afterwards, supernatants were collected and used instead of fresh serum sample in regular CDC assay performed on calcein-AM-loaded cells. The readout obtained for sample of heat-inactivated serum incubated for 30 min is shown as negative control. Panel **c** Raji cells were co-incubated with human erythrocytes, NHS, ofatumumab and optionally, wild-type or quadruple FB mutant. Absorbance of sample where water was added instead of serum solution was considered as positive control (full lysis). Data are collected from three independent experiments and error bars show standard deviation

enhancement of complement-mediated killing of Raji cells (Fig. 4) upon limited availability of complement. One possible explanation is that sheep erythrocytes are not equipped with human complement inhibitors but their homologues. Another explanation involves cell line-specific expression of target molecule (CD20) and complement inhibitors (e.g. CD46, CD55, CD59). Previously, we showed that BJAB, Raji and Namalwa cells used in our model experiments are characterized by different ratios of these surface proteins, which influence their sensitivity to anti-CD20 mAbs [15]. This is why we tested quadruple mutant embracing several gain-of-function mutations. Such protein should be more universally useful towards different types of tumour cells. Indeed, quadruple mutant performed better than individual single mutants in most of the CDC assays and its effect was visible at lower concentrations. However, this conclusion is limited by inability to produce single F286L mutant in our expression system.

We revealed that the quadruple gain-of-function mutant may exert different effects on CDC, depending on target cell sensitivity to ofatumumab and serum concentration. It can significantly (but still at low overall level) enhance CDC of ofatumumab-resistant cells under physiological serum concentration but the same effect on moderately sensitive cells is detectable only under low serum concentration. Nonetheless, we postulate that such a model is still relevant to the field and adequate for a situation where complement is quickly exhausted following the administration of antitumour antibodies to the bloodstream of patients with a high tumour burden. As evidenced by our experiments using clinical samples collected from DLBCL, CLL and follicular lymphoma patients treated with rituximab, an addition of quadruple gain-of-function FB mutant rescues cytotoxic capacity of their post-infusion sera (Fig. 6b–f) and helps to efficiently utilize remaining rituximab for killing tumour cells (Fig. 6g). Possibly, supplementation with gain-of-function FB mutants can be considered as a way to combat tumour cells, which escaped the first wave of CDC following administration of anti-CD20 mAbs. Also, in the same context it is worth mentioning that around 40% of patients with CLL have diminished baseline complement activity [30]. Another group reported that ex vivo experiments with CLL cells show significantly lower anti-CD20 mAb-mediated lysis in the presence of autologous patients' serum than that with normal human serum [31].

Under physiological conditions, complement convertases decay rapidly in intrinsic and extrinsic processes. On the one hand, higher and longer activity of convertases creates more lytic sites on the surface of target cells. On the other hand, increase of convertase half-life raises a chance for misguided deposition of active complement components on self-cells and tissues. Indeed, some carriers of gain-of-function mutations in alternative convertase constituents develop autoimmune diseases [20, 21] and, therefore, use of hyperactive FB may bring safety concerns. However, these FB mutations show incomplete penetrance [20] and, in contrast to autoimmune patients constantly exposed to etiologic factors, suggested supplementation with gain-of-function mutants would be temporary and coordinated with anti-CD20 mAb infusions. Administration of type I anti-CD20 antibodies into, e.g. CLL patients, who typically carry from 3×10^4 to 3×10^5 malignant B cells per microliter [32], causes massive complement engagement. With such a high tumour burden, hyperactive convertase components should incorporate into enzymatic complexes formed on target cells rather than those formed due to incidental, misguided complement activation. We showed that in vitro supplementation of NHS with quadruple FB mutant (up to the concentration of 20 µg/ml, which was effective in enhancement of CDC) does not provoke excessive and unproductive complement consumption, which normally is one of the unwanted side effects of gain-of-function components of alternative convertases. We also did not observe any bystander lysis of human erythrocytes co-incubated with ofatumumab-sensitized Raji cells and quadruple FB mutant added at the optimal concentration for enhancement of CDC. Our choice of erythrocytes as a model was due to erythrocytes being the most abundant component of blood compartments, and their relatively high vulnerability to osmotic lysis in comparison to nucleated cells. Moreover, intravascular hemolysis is a crucial event of diseases such as paroxysmal nocturnal hemoglobinuria (PNH) [33] or atypical hemolytic uremic syndrome (aHUS) [34], which are caused by malfunctions in the regulation of complement convertases. It is important to note that the risk of the development of complement-related diseases should be addressed in in vivo experiments in the animal model of B-cell malignancy, which we did not perform in this study. Another potential problem with proposed strategy is that patients receiving hyperactive FB variants may develop an immune response to these proteins and thus limit their efficacy. A rare character of such mutations and diseases related to them can explain the lack of reports on antibody response to altered FB molecules; however, the phenomenon is known for certain tumour-specific antigens, e.g. p53 mutants [35]. Nevertheless, by analogy to anti-p53 seropositive patients, efficient presentation of putative antigenic fragments of FB mutants to B lymphocytes would demand appropriate HLA class II alleles, thus limiting the chances for such unfavourable scenario to carriers of certain HLA haplotypes [35]. Although we acknowledge that unspecified risk related to an application of gain-of-function FB variants may exist, our aim was to provide a proof of concept for a novel strategy maximizing the efficacy of complement-mediated immunotherapeutics. Our experiments showed that application of gain-of-function FB mutants can compensate complement exhaustion in patients treated with rituximab. Moreover,

addition of such proteins to post-infusion serum exploits the cytotoxic potential of the drug still present in patient's bloodstream.

Author contributions MO designed the study; AMB and GS provided essential reagents and tools; MT and AH coordinated collection of clinical material and confirmed inclusion criteria of the patients; AF, AB, AU, GS and MO performed the experiments; AF, AU, GS, AMB and MO contributed to writing of the manuscript.

Funding This work was supported by two Grants from National Science Centre Poland (2015/18/M/NZ6/00334 and 2014/14/E/NZ6/00182) and Cancerfonden (Sweden).

Compliance with ethical standards

Conflict of interest Anna Felberg, Aleksandra Urban and Marcin Okrój are named as inventors in patent application: World Intellectual Property Organization (WIPO) ST 10/C PL425133 "Point mutations in complement C2 protein and complement factor B enhancing the cytotoxic activity of antitumour antibodies, their pharmaceutical composition and application". The other authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Local Bioethical Committee at Medical University of Gdańsk and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The Local Bioethical Committee at Medical University of Gdańsk granted ethical approval for this study (Approval number: Agreement no. NKBBN/500/2016).

Informed consent Written informed consent was obtained from all individual participants included in the study. Every participant was provided with an information sheet and consent form to be signed both by the participant and the person taking consent.

Cell line authentication Ramos, Daudi, SU-DHL-8 and WSU-NHL cells were obtained from German Collection of Microorganisms and Cell Cultures. Namalwa and BJAB cells were obtained from the American Type Culture Collection. All cell lines were aliquoted and cryopreserved after the first few passages. Cells used for experiments were grown from such stock aliquots, routinely checked for *Mycoplasma* infection when cultured and never kept in continuous culture for more than ten passages.

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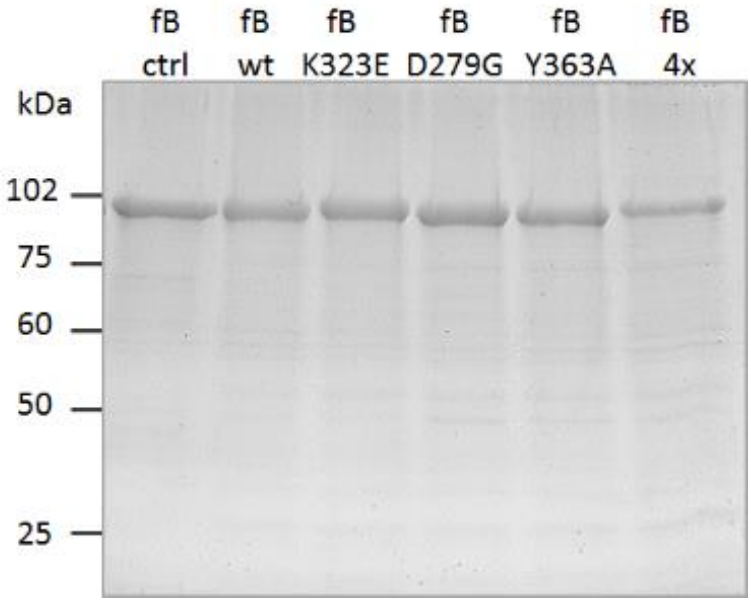
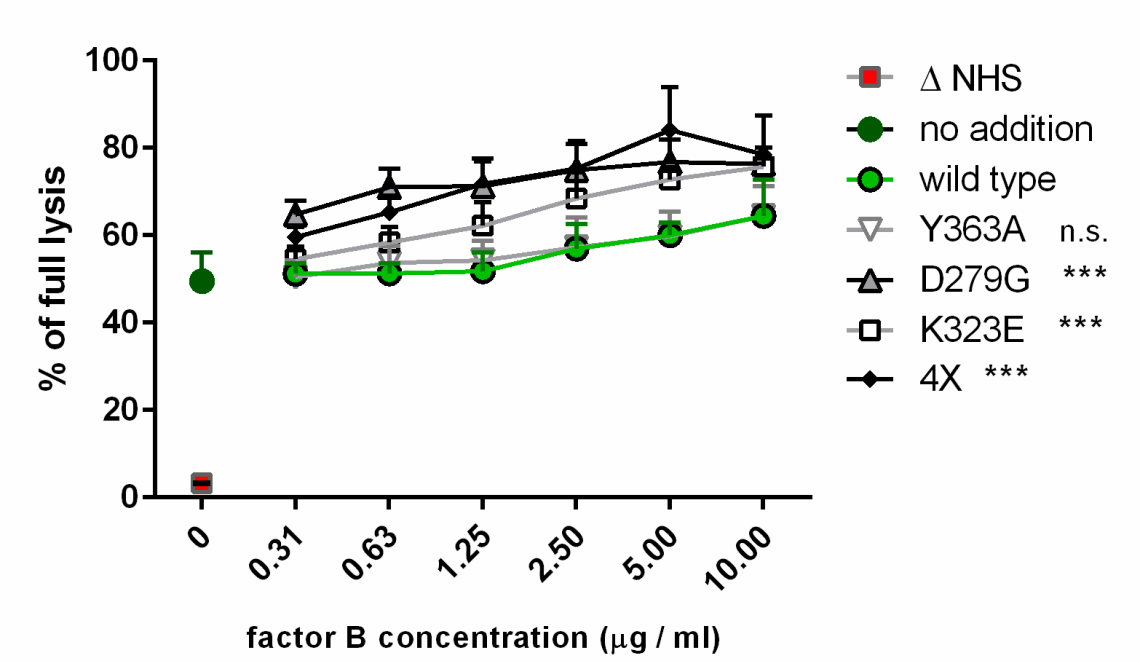
Supplementary Fig. 1



Plasma-purified FB (ctrl), recombinant wild type FB (wt), single (K323E, D279G, Y363A) and quadruple (4x) mutant proteins were electrophoresized on SDS-PAGE gel and stained with Commassie. Lanes were purposely overloaded with 10 μg/ml of protein in order to visualize impurities.

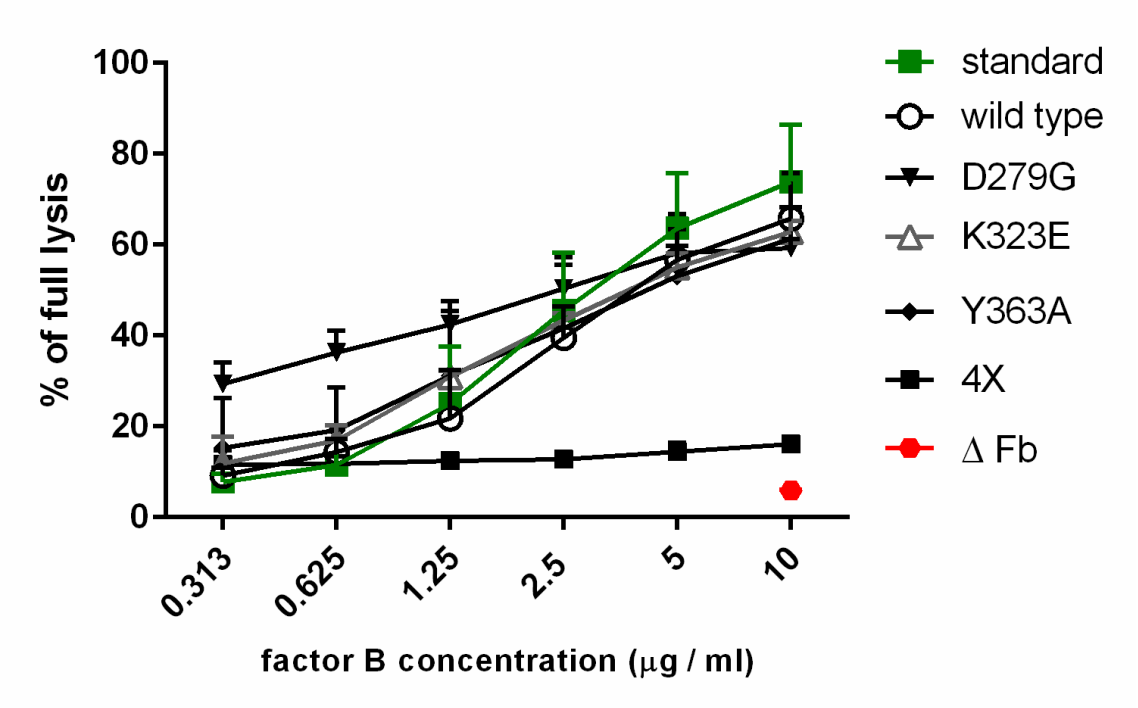
Supplementary Fig. 2



Addition of factor B augments hemolysis of sheep erythrocytes initiated by the classical complement pathway.

Sensitized sheep erythrocytes were mixed with 5% factor B-depleted serum supplemented with recombinant wild type factor B or its single (Y363A, D279G, K323E) or quadruple (4X: Y363A, D279G, K323E, F286L) mutants. Hemolysis was assessed after 30 minutes. The readout obtained for heat-inactivated normal human serum (Δ NHS) represents background lysis. Dunnett's multiple comparison test was applied for classification of differences between lytic curves of particular mutant compared to wild type factor B as: n.s. – not significant or *** - significant at p level < 0.001. Graph shows data from three independent experiments and error bars represent standard deviations.

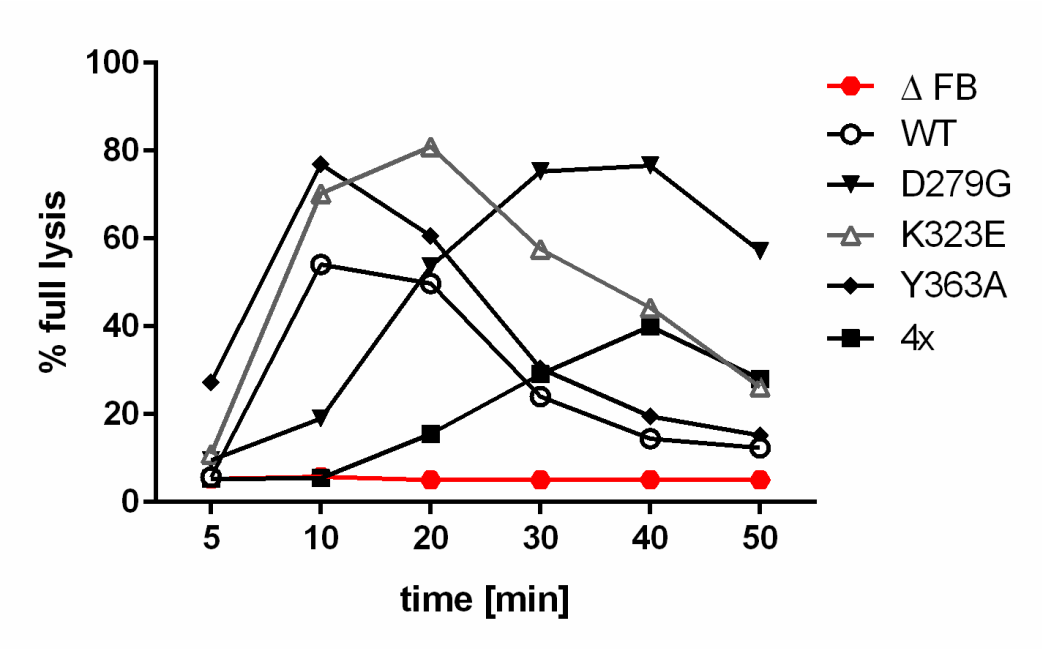
Supplementary Fig. 3



Hemolysis mediated by the alternative complement pathway.

Rabbit erythrocytes were incubated with 5% FB-depleted serum supplemented with increasing concentrations of recombinant wild type or mutated factor B. Plasma purified FB (Complement Technologies) was used as positive control (standard) and FB-depleted serum with no supplementation (Δ FB) served as negative control.

Supplementary Fig. 4



Convertase functional assay performed in FB-depleted serum

Single experiments showing convertase activity in 5% FB-depleted serum (Δ FB) + / - recombinant wild type (WT) or mutated FB variants supplemented at final concentration of 10 μg / ml.

6 Manuskrypt nr 5

A. Felberg, M. Bieńkowski, T. Stokowy, F. Mohlin, S. Nilsson, I. Jongerius, R. Spaapen, M. Mesa-Guzman, L.M. Montuenga, A.M. Blom, R. Pio, M. Okrój

"Elevated expression of complement factor I in lung cancer cells associates with shorter progression-free survival and disease-specific survival"

Manuskrypt złożony do czasopisma

Wkład autorów:

A. Felberg, M. Bieńkowski przeprowadzili eksperymenty. I. Jongerius, R. Spaapen dostarczyli niezbędne odczynniki i narzędzia. T. Stokowy przeprowadził analizę danych. L. M. Montuenga, M. Mesa-Guzman i R. Pio udostępnili oraz opisali materiał kliniczny do badań. A. Felberg i M. Okrój stworzyli manuskrypt.

Elevated expression of complement factor I in lung cancer cells is associated with shorter progression-free survival and disease-specific survival.

Anna Felberg [1], Michał Bieńkowski [2], Tomasz Stokowy [1,3], Frida Mohlin [4], Sara Nilsson [4], , Ilse Jongerius [5,6], Robbert Spaapen [6], Miguel Mesa-Guzman[7] , Luis M. Montuenga [,8,9, 10, 11], Anna M. Blom [4], Ruben Pio [,9,10, 11, 12], Marcin Okrój [1#]

[1] Department of Cell Biology and Immunology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Poland
[2] Department of Pathomorphology, Medical University of Gdańsk, Poland
[3] Department of Clinical Science, University of Bergen, Norway
[4] Department of Translational Medicine, Lund University, Sweden
[5] Department of Immunopathology, Sanquin Research, Amsterdam and Landsteiner Laboratory, Amsterdam University Medical Center, University of Amsterdam, The Netherlands
[6] Emma Children's Hospital, Department of Pediatric Immunology, Rheumatology and Infectious Diseases, Amsterdam University Medical Center, The Netherlands.
[8] Department of Pathology, Anatomy and Physiology, School of Medicine, University of Navarra, Pamplona, Spain
[9] Program in Solid Tumors, Cima-University of Navarra, Navarra's Health Research Institute (IDISNA), Pamplona, Spain
[10] Centro de Investigación Biomédica en Red Cáncer (CIBERONC), Madrid, Spain
[11] Instituto de Investigación Sanitaria de Navarra (IdisNa), Pamplona, Spain
[12] Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona, Spain

Abstract

Soluble complement inhibitors are rarely produced in locations other than the liver. Previously, we reported the expression of a set of such inhibitors: complement factor I (FI), complement factor H (FH), and C4b-binding protein (C4BP) in non-small cell lung cancer (NSCLC) cell lines. Herein, we examined the associations of FI expression in lung adenocarcinoma and squamous cell carcinoma cells with clinicopathological characteristics of the patients and clinical outcome. FI immunohistochemical staining intensity was not correlated with age, smoking status and the number of pack-years, tumor size, grade, stage, the appearance of immune infiltrates, or mutational burden. However, we noticed significant associations with progression-free survival (PFS) and cancer-specific survival (CSS). Multivariate Cox analysis of high vs. low expression of FI revealed HR 0.55, 95% CI 0.33-0.95, p=0.031 for PFS and HR 0.35, 95% CI 0.15-0.78, p=0.011 for CSS. To approach the question of whether expression of FI by tumor cells was aimed to protect tumor cells from host innate immunity, we analyzed the deposition of C4d - the end degradation product of FI-supported inactivation of active complement component C4b - and found only negligible staining in cancer tissue. On the other hand, RNA sequencing revealed differences in signaling pathway activities between FI knockout and wild-type NSCLC H2087 cells. *In vitro* assays with FI-deficient and FI-sufficient H2087 cells showed differences in migration and size of colonies. We postulate that a non-canonical activity of FI influences cellular physiology, which may contribute to the poor prognosis of lung cancer patients.

Introduction

The complement cascade is part of the innate immune system. Besides the direct killing of invading pathogens, it is considered an important mediator of inflammation and contributes to the regulation of immune response. Moreover, complement participates in the removal of immune complexes as well as apoptotic/necrotic cells and guides adaptive immunity [1]. Complement pattern recognition molecules (PRM) can also recognize danger moieties on modified host surfaces such as

damaged tissues and cancer cells [2, 3]. However, tumor cells possess means to actively oppose complement activation on their surface, including the over-expression of complement inhibitors, internalization, and/or shedding of membrane-bound complement components [4, 5, 6]. The existence of such defense mechanisms and the results of *in vivo* experiments, in which cancer cells with downregulated expression of complement inhibitors formed smaller tumors [7], imposed a theory that complement system supports host but not malignant cells. However, new reports showed that under certain circumstances complement activation may promote a pro-tumor state by fueling proangiogenic events [8] and mobilizing suppressor immune cells [9]. Complement components are now considered as an important modulator of the tumor microenvironment, which is important for tumor progression, recurrence, metastasis, and activity of tumor-infiltrating immune cells [10, 11, 12, 13]. Interestingly, the same type of tumor cells produce both activators [13] and soluble complement inhibitors [14, 15] thus suggesting that a dynamic regulation rather than constitutive inhibition/activation of the complement system is a hallmark of cancer.

Our previous studies on non-small cell lung cancer (NSCLC) revealed that many human NSCLC cell lines secrete factor H (FH) – an inhibitor of the alternative complement pathway [14]. Additionally, some of these cell lines secreted functionally active C4b-binding protein (C4BP) and factor I (FI). FI is a serine protease that inactivates C3b and C4b components that form enzymatic complexes (convertases) essential for the amplification of the complement cascade [15]. Its serum concentration of 35 μg/ml and the systemic supply is mainly supported by production in the liver [16]. Only a few extrahepatic sources of FI have been identified so far in humans [17] including cancerous breast and cutaneous tissue, where a high expression of FI associates with aggressive phenotype [18, 19]. Herein, we analyzed the associations of FI immunohistochemical staining in 94 adenocarcinoma and 69 squamous cell carcinoma patients with clinicopathological characteristics and the outcome of the disease. Moreover, in recent years a series of discoveries has brought evidence that the function of complement proteins is not confined to the extracellular space but involves also the intracellular compartment, as exemplified by modulation of tumor cell physiology and migration by a lysosomal pool of FH [20], non-canonical function on C1s in renal cancer cells [21], chromatin rearrangement by C3 protein trafficking into B cell nuclei [22], intracellular C3's role in metabolic reprogramming of immune cells [23], regulation of autophagy in pancreatic cells [24], and protection of airway epithelial cells from stress-induced injury [25]. There is an opinion that complement proteins were first engaged in the intracellular signaling pathways and acquired their acknowledged canonical function later on during the evolution [26]. Such a hypothesis would explain the complex role of complement C3 and impose similar, divergent nature of other complement components. Therefore, in our study we approached the question about the intracellular role of FI by performing RNA sequencing and experiments assessing the migration and colony formation capacity of FI-knock out and wild-type NSCLC cells.

Materials and Methods

Patients

The patients included in this study were recruited at the Clínica Universidad de Navarra (CUN). Lung primary tumors were fixed in 10% buffered formalin after surgical removal and paraffin-embedded using standard procedures. Tumors were classified according to the World Health Organization 2004 classification and the 8th Edition of the International System for Staging Lung Cancer [27]. For survival analysis, recurrence-free survival (RFS), overall survival (OS), and cancer-specific survival (CSS) were calculated from the date of surgery to the date of recurrence or death according to the clinical information. The protocol was approved by the Research Ethics Committee of the University of Navarra (Reference 108-2013) and all patients gave written informed consent. Demographic and clinicopathological characteristics of patients are provided in Table 1.

Cell Culture

The human NSCLC cell line H2087 (ATCC) was used in the study. Cells were cultured in RPMI 1640 medium with L-glutamine (ATCC) supplemented with 10% fetal bovine serum (ATCC) at 37°C and humidified 5% CO_2 atmosphere. Cells were routinely checked for Mycoplasma contamination by DAPI staining and never kept in continuous culture for more than 10 passages.

Western Blot analysis

Medium conditioned by lung cancer cells was concentrated 10-fold. Samples were separated on 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes using a semi-dry blotting system. Membranes were incubated overnight with the primary antibodies: goat anti-human FI (Complement Technology) diluted 1:1000, then washed and incubated for 1 hour with the secondary antibody rabbit anti-goat conjugated to HRP (Dako) diluted 1:1000. Blots were developed using 3,3'-Diaminobenzidine (DAB) Liquid Substrate System (Sigma).

CRISPR/Cas 9 approach to target complement Factor I in H2087 lung cancer cells.

Lentiviral constructs containing guide RNAs targeting FI (Forward: CACCGATATGAACATTGTCTTATCT, Reverse: AAACAGATAAGACAATGTTCATATC) were transfected into HEK293T cells with the packaging enzymes psPAX2, pVSVg, pAdVAntage using polyethyleneimine (PEI; Polyscience) for virus production. Filtered viral supernatants were used for the transduction of H2087 cells by spinoculation in the presence of 8 mg/mL protamine sulfate. After transduction, cells were selected based on the expression of GFP. Then, single cell-derived clonal lines were generated. To confirm the KO phenotype of the cells, firstly conventional Sanger sequencing and subsequently Western Blot analysis and ELISA were performed.

Formation of three-dimensional (3D) cell cultures and spherule diameter measurement

For 3D spheroid culture, cells were grown in Matrigel (Corning). Cells were suspended in Matrigel, thus forming a cell medium-matrigel mixture. Cells in the mixture were seeded in 20 μl drops (3500 cells per drop) in 24 well plates. Matrigel matrix was then polymerized at 37°C for half an hour. Then, 2 ml of complete medium was layered on top of the Matrigel drops. The cells were incubated at 37 °C in a humidified incubator at 5% CO_2 for 7 days. The medium was changed every 2-3 days. Post 7 days, an inverted light microscope was used to image and count the number and diameter of spherules formed in each well. The data were subjected to software-assisted image analysis (ImageJ). All the experiments were repeated thrice.

Trans well migration assay (Boyden chamber assay)

Trans-well chambers (Falcon) with a pore size of 8 μm were used to assess the migratory capacity of the cells. 25 000 cells were added to 500 μL RPMI medium (not supplemented with serum) and seeded into the upper chamber. Medium supplemented with 10% FBS as the chemoattractant was added into the lower chamber. After incubation for 24 h, the non-invading cells on the top surface of the filters were swabbed with a cotton swab, and the cells adhering to the lower surface of the filters were fixed with 4% paraformaldehyde and stained with Hoechst solution (Sigma). An inverted light microscope was used to image and count the number of migrated cells. The data were subjected to software-assisted image analysis (ImageJ).

Immunohistochemistry (IHC) and scoring of immunostaining in tissue microarrays.

Formalin-fixed and paraffin-embedded (FFPE) pellets of lung cancer cells non-expressing and expressing FI (as determined by RT-PCR and Western Blotting) were used for setting up staining parameters for FI. The staining procedure was performed as described in [18] but since a different batch of affinity-purified rabbit polyclonal antibodies were used, we re-evaluated the dilution of the primary antibody, which was finally set up at 0.75 μg/ml. For C4d immunohistochemistry, the determination of staining specificity was performed with FFPE pellets of rituximab-sensitized Raji cells (ATCC) incubated in 5% FI-depleted serum (Complement Technology) and the same cells incubated with FI-depleted serum reconstituted with plasma-purified FI (Complement Technology). The first conditions promote deposition of C4b but exclude its degradation to C4d fragment whereas the latter are permissive for C4d formation. The primary antibody was in-house raised rabbit polyclonal anti-C4d prepared as described in [28], with specificity restricted to C-terminal linear neoepitope accessible only after C4d fragment excision from C4b. Once the optimal conditions for antigen retrieval (EnVision FLEX Target Retrieval Solution, High pH, Dako, using the PT Link station, Dako), incubation time/temperature (overnight at 4°C), and antibody dilution (0.7 μg/ml) were set up, the same parameters were used for staining of tissue microarrays collected from FFPE lung cancer tissue. Staining intensity was presented as an H-score that captures both the intensity and the proportion of the biomarker of interest from the immunohistochemical image. H-score comprises values between 0 and 300, where 0 value refers to 100% negative (-) staining, value 100 refers to 100% positive (+), 200 to 100% very positive (++) and 300 to 100% of highly positive (+++) staining. Examples of each category are given in Fig. 1 C-F.
$CD4^+$, $CD8^+$ tumor-infiltrating lymphocyte (TIL) density, FOXP3, and PD-L1 expression were evaluated by standard immunohistochemistry setup by the by CIMA LAB Diagnostics.

RNA sequencing and data analysis

The RNA of Wild Type H2087 cell line and H2087 FI KO cell lines was sequenced in the framework of service provided by the Macrogen company. First, the quality of the data was checked using FastQC and MultiQC reporting tools. After quality check raw reads were aligned to human genome GRCh38.p10 using hisat 2.0.5 with Gencode v26 transcriptome reference (https://www.gencodegenes.org/ human/release_26.html). Aligned reads were sorted and compressed using Samtools. Furthermore, reads aligned in the coding regions of the genome were counted using Feature Counts. Read counts were normalized using DESeq2 1.30.0, then normalized expression values were subjected to differential analysis (fold changes) in R/Bioconductor programming environment. Results of differential analysis were saved in tab separated text files and Excel sheets for delivery. For gene lists of interest (fold change >3 and <0.33), a functional analysis was performed using CPDB server using over-representation analysis tool Consensus Path DB available online (http://cpdb.molgen.mpg.de/CPDB). Similar analysis approaches were published previously [29].

Results

FI is present in lung cancer tissue.

Previously we have shown that FI is produced by several NSCLC cell lines [15]. In the present study, we aimed to examine with immunohistochemical staining whether FI localizes in lung cancer tissue *in vivo*. To validate the method, the staining parameters were set on FFPE pellets of lung cancer cell lines non-expressing (Fig.1 A) and expressing (Fig. 1B) FI. The same staining conditions were used for the staining of tissue microarrays collected from FFPE lung cancer tissue. Pictures C-F (Figure 1) show examples of lung cancer tissue scored as negative (-), positive (+), strongly positive (++), and extremely positive (+++). We stained tissue microarrays from 163 lung cancer patients and found cytosolic staining of different intensities. Results were further evaluated by the pathologist and presented in a semiquantitative approach (H-score).

Associations of FI staining with clinicopathological parameters

Out of 175 patients suffering from various forms of lung cancer, we selected 163 individuals with two prevalent types: adenocarcinoma and squamous cell carcinoma for further statistical analysis. Patients with less than 1 month to progression or survival were filtered out for the prognostic analyses. Follow-up data were censored at 120 months. The cut-off for dichotomization of factor I expression was established using the median as a low factor I H-score ≤ 60 and a high factor I H-score > 60, respectively. High FI signal was not associated with gender, age (> 65 y.o.), smoking status, pack-years value, tumor size (> 3 cm), differentiation grade and tumor stage (Table 1)

Factor I presence in cancer cells has a negative impact on progression free survival, overall survival and cancer-specific survival

Kaplan-Meier analyses of progression free survival (PFS), overall survival (OS) and cancer-specific survival (CSS) revealed statistically significant differences between groups with high and low factor I H-score *(p* values 0.013, 0.052 and 0.055 respectively) (Fig. 2). Univariate Cox analysis revealed FI score and pack-years value significantly affecting PFS (Table S1), FI score and tumor size significantly affecting CSS (Table S2), and age, pack-years and tumor size significantly affecting OS (Table S3). Variables with a p value lower than 0.1 in the univariate analysis were included in the multivariate analysis. Multivariate Cox analysis of high vs. low FI score revealed HR 0.55, 95% CI 0.33 - 0.95, p = 0.031 for PFS (Fig. 2A) and HR 0.35, 95% CI 0.15 - 0.78, p=-0.011 for CSS (Fig. 2C). Significance of FI score (HR 0.55, 95% CI 0.29 - 1.03, p = 0.061) was not reached for OS (Fig. 2B). There was no correlation between factor I H-Score and immune cell infiltration, FOXP3 and PD-LI expression (Fig. S1).

Deposition of C4d in tumor tissue does not correlate with FI presence

We analyzed the deposition of C4d in the same tissue microarrays used for the assessment of FI presence in tumor tissue. C4d fragment is a product of FI-supported degradation of C4b that takes place in the presence of either soluble (C4BP) or membrane-bound cofactors (CD46, CD35) thus reflecting the degree of evading complement attack by tumor cells [30]. The specificity of staining was first ensured by examination of FFPE pellets of the antibody-sensitized cells incubated in FI-depleted (Fig. 3A) and FI-reconstituted (Fig. 3B) serum. Additionally, we examined kidney transplant tissue showing antibody-mediated rejection and observed C4d staining in peritubular capillaries, as expected [31] (Fig. 3D). Examination of normal lung tissue revealed C4d inside of alveolar macrophages, a pattern that suggests phagocytosis of C4d-containing material. The same evidence was observed when tissue microarrays from lung cancer patients were examined (Fig. 3F). However, this was a case only in a few samples from a small number of patients, and the vast majority of tumor tissue was stained negatively, as presented in Fig. 3E. Globally, no correlation between FI presence and C4d deposition in cancer tissue was observed.

RNA sequencing analysis in FI-knock-out and wild type H2087 cells

The absence of association between FI and C4d suggests that the role of this complement inhibitor in tumor cells is beyond the scope of regulating immune evasion. Therefore, we decided to analyze the differences in transcription profiles of FI-deficient and FI-sufficient lung cancer cells. For this purpose we chose the NSCLC cell line H2087 that was previously confirmed to produce FI by Western Blotting [15] and now re-evaluated by qPCR (Fig. S2). Factor I was knocked-out in H2087 cells by CRISPR/Cas9 technology using gRNAs targeting the first exon of the gene. After transfection with appropriate constructs, serial dilutions of cells were performed to select single cell-derived clones, which were then subcultured and examined by either conventional Sanger sequencing for deletion, and by Western Blotting for protein expression. From >100 colonies, only one FI-negative clone (clone 1) was finally isolated and subcultured. (Fig S3). Sequencing of RNA isolated from wild-type and clone 1 FI-KO cells

revealed substantial differences in their transcriptional patterns. Comparative analysis disclosed 26 up-regulated genes with fold change > 3 and 32 down-regulated with fold change < 0.3 (Table 6). Using CPDB over-representation algorithm (http://cpdb.molgen.mpg.de) these differences were translated into differences in the activity of specific signaling pathways such as senescence and autophagy in cancer pathway (Table 3). Enriched gene-ontology sets pointed out differences in, among others, cell adhesion and motility (Table 4).

Functional assays on FI knock-out NSCLC cells

To investigate the impact of factor I expression on clonogenic and metastatic potential and to experimentally confirm the gene ontology- based analysis of RNA sequencing results, we performed functional assays on FI-KO and wild-type H2087 cells. In the meantime we obtained another FI-KO clone (clone 9), which was included in these analyses. *In vitro* colony formation assay assesses the ability of a single cell to grow into a colony. This method had been shown to reliably reflect a balance between tumor cell death and proliferation in cancerous foci [32]. Results of the 3D matrigel colony-forming assay showed that both FI-KO clones formed smaller colonies compared to WT cells (Fig. A), which suggests that factor I might facilitate growth of NSCLC tumors. Transcriptomic analyzes of FI-deficient and sufficient cells prognosed differences in cell motility. The trans-well cell migration assay showed that FI knockout cells had a higher migration capacity compare to WT cells, which is one more evidence for non-immune function of FI. (Figure 4B).

Discussion

Expression of soluble complement inhibitors like FI, FH, and C4BP was previously reported in NSCLC cell lines [14, 15]. These three proteins form a functional set consisting of a serine protease (FI) and its cofactors (FH, C4BP) dedicated to the release of FI proteolytic activity towards the alternative or classical/lectin components, respectively. The effect of FH on lung cancer growth *in vivo* was examined in the mouse xenograft model, which revealed that NSCLC cells with silenced expression of this inhibitor formed smaller tumors than their mock-transfected counterparts [7]. We hypothesized that the underlying mechanism involves a lower level of protection from cytocidal complement activity. Our supposes were ensured by the fact that explanted tumor xenografts from mice inoculated with FH-silenced NSCLC cells showed enhanced deposition of C3. Additionally, the growth of engrafted FH-silenced NSCLC cells was restored when mice were depleted from complement by cobra venom factor (CVF) before and during experiment [7]. Notably, the complement inhibition by FH seems to be more robust than that by FI. In contrast to FI, FH exerts its regulatory action by two different mechanisms: i) by providing cofactor activity (CA) for FI-mediated cleavage of C3b and ii) by a decay-acceleration activity (DAA) that speeds us irreversible dissociation of alternative complement convertases [30]. Since the alternative complement pathway serves as an amplification loop for two other pathways [30, 33], FH controls not only the alternative route but the overall complement cascade. Moreover, tumor cells can hijack FH from serum and expose it on their surface, thereby mimicking the membrane-bound complement inhibitors [34]. There were no functional studies on the role of other soluble complement inhibitors produced by NSCLC on *in vivo* tumor growth.

Previously, we reported local expression of FI in breast cancer tissue and found elevated FI levels in tumor cells as a negative prognostic marker for patients' survival and tumor recurrence [18] but did not investigate the underlying mechanism responsible for such effect. Herein, we observed the association between FI presence in cancer cells and poor prognosis in another tumor type, namely lung cancer. At the same time, we did not observe any association between FI presence in tumor cells and C4d deposition, which was hardly detectable in patients' material and, if any, restricted to the intracellular compartment of alveolar macrophages (Fig. 3). Such a result was unexpected in the spotlight of our previous studies showing that C1q binds directly to lung cancer cell lines and activates the classical complement pathway [35]. In the same study, we showed C4d deposition in cancer tissue and correlation of C4d released to plasma with shorter survival [35]. However, in the present study, we

used an anti-C4d antibody specific for C-terminal linear neoepitope that appears only upon proteolytic degradation of C4b to C4d and therefore not reactive to unprocessed C4b fragment [28]. Previously applied anti-C4d antibody does not guarantee the lack of such cross-reactivity and theoretically could detect C4b portion. Another explanation is that lung cancer cells undergo trogocytic removal of their complement-containing membrane patches at the contact with phagocytes [36], a process that coincided with C4b to C4d transition. Evidence of C4d deposits inside macrophages makes such a scenario plausible. Also, there is a possibility that FI from plasma but not FI endogenously produced by NSCLC cells is responsible for C4b processing on cancer cells, which would explain the lack of correlation between FI presence and C4d deposition on the cell surface. Nonetheless, this result, and growing evidence of recently reported non-canonical functions of complement components in cancer cells [20, 21], inspired us to elucidate the similar role of FI in this particular model. As the H2087 cell line naturally produces FI, the construction of FI knock-out in these cells enabled us to elucidate the direct effects of gene expression on cellular physiology. Surprisingly, we experienced problems with the selection of H2087 clones devoid of FI expression, which may suggest an important role of this gene / protein for cell growth and survival. Sequencing of RNA from FI-deficient and sufficient cells showed significant differences in transcriptional activity: 26 genes were up-regulated in the KO clone and 32 genes were down-regulated at fold change >3 or <0.3, respectively (supplementary file_1).

Genes that were upregulated in FI-deficient H2087 cells encode metalloproteinases 13 and 14 (MMP13, MMP14), (PLAT), and serpins (SERPINB4, SERPINB3, SERPINB2), which are engaged in degradation of extracellular matrix, regulation of fibrinolytic activity and migratory capacity of tumor cells [37, 38, 39]. FI-sufficient H2087 cells expressed more transcripts coding for, among others, non-receptor tyrosine kinase FYN engaged in tumor cell proliferation and migration [40], transforming growth factor-alpha (TGFα) associated with growth factor independence of tumor cells [41], nucleoproteins (AHNAK, AHNAK2) involved in proliferation and positive regulation of phosphatidylinositol 3-kinase (PI3K) signaling pathway [42], transcription factors SOX9, HES1, and FosB, as well as FGF binding protein 1 (FGFBP1) that mobilizes fibroblasts growth factor (FGF) proteins deposited in extracellular matrix and delivers them to their receptor [43] and non-receptor tyrosine phosphatase PTEN, an acknowledged tumor suppressor with recently discovered additional role in regulation of migratory capacity of the cells [44]. The functions of abovementioned genes and analysis of gene ontology (Table 7, 8) suggest differences in cell motility, adhesion, modulation of extracellular space, cell activation and cellular developmental processes between cells expressing and non-expressing FI. We performed two functional assays, i.e. colony formation assay and migration assay, which confirmed alterations dependent on FI expression status. Importantly, we succeeded in obtaining one more clone of FI-deficient H2087 cells, which presented the same phenotype as originally isolated FI-KO clone (Fig. 4).

One of the limitations of our study is incorporation of single FI-expressing NSCLC cell line into transcriptomic and functional analyses. These results must be verified in a number of other lung cancer cell lines to ensure the impact of FI on the activity of particular genes and cellular physiology. Nonetheless, we showed for the first time that FI expression status in NSCLC cells influences cell functionality independently on the immune system as such. We also demonstrated that high expression of FI in lung adenocarcinoma and squamous cell carcinoma is associated with poor prognosis.

Tables

Table 1 Clinicopathological characteristic of patients enrolled in the study and associations with factor I immunostaining in tumors

	n	Factor I - H score median (25th to 75th percentiles)	p value
Sex			0.682
Female	30	55 (18-94)	
Male	133	60 (20-100)	

Age (years)			0.992
≤ 65	84	60 (20-100)	
> 65	79	63 (22-92)	
Smoking status			0.372
Never smoker	17	67 (10-97)	
Former smoker	112	60 (20-96)	
Current smoker	34	63 (50-104)	
Pack-years			0.211
≤ 45	81	55 (20-95)	
> 45	79	63 (20-103)	
unknown	3	80 (75-88)	
Tumor size (cm)			0.286
≤ 3	93	60 (20-97)	
> 3	67	70 (37-103)	
unknown	3	30 (20-40)	
Histology			0.186
Adenocarcinoma	94	56 (17-99)	
Squamous cell carcinoma	69	70 (40-100)	
Grade			0.947
Well differentiated	19	63 (32-98)	
Moderately differentiated	71	60 (18-104)	
Poorly differentiated	52	67 (20-100)	
Unknown	21	50 (23-70)	
Stage			0.306
I	97	60 (20-90)	
II	41	67 (30-103)	
III	20	88 (50-101)	
IV	5	95 (50-163)	

p value was calculated according to Kruskal-Wallis test

Table 2. Genes with the strongest expression changes in the dataset (10 up- and 10 down-regulated).

EnsemblGeneName	GeneName	FI-KO clone	wild-type	fold_changes	Gene function/product
ENSG00000023839.10	ABCC2	11,7756188	8,38132541	10,5143913	multidrug resistance protein 2 (MRP2)
ENSG00000206073.10	SERPINB4	10,2926338	7,07346021	9,31253287	serpin B4, protease inhibitor
ENSG00000057149.15	SERPINB3	9,48595858	6,69832778	6,90494922	serpin B3, protease inhibitor
ENSG00000104368.17	PLAT	14,1228483	11,3611274	6,7820476	tissue-type plasminogen activator
ENSG00000157227.12	MMP14	12,9234874	10,5631996	5,134728	matrix metallopeptidase 14
ENSG00000137727.12	ARHGAP20	9,5573213	7,31037403	4,74677371	rho GTPase Activating Protein 20
ENSG00000169594.13	BNC1	11,1825446	9,04267398	4,40722533	zinc finger protein basonuclin-1

ENSG00000237729.2	AC002075.4	8,20639897	6,1952715	4,03097116	ribosomal protein S27, pseudogene
ENSG00000101955.14	SRPX	9,32229944	7,35548428	3,90904221	sushi repeat-containing protein SRPX
ENSG00000176046.8	NUPR1	9,06755515	7,11784148	3,86297856	nuclear protein 1, transcription regulator.
ENSG00000181577.15	C6orf223	6,57106665	9,00018748	0,18567856	C6orf223, uncharacterized protein.
ENSG00000173267.13	SNCG	7,95356831	10,4121588	0,18192421	gamma-synuclein
ENSG00000117322.17	CR2	7,83994152	10,4426264	0,16463182	complement receptor type 2
ENSG00000234444.9	ZNF736	6,57106665	9,19072307	0,16270648	zinc finger protein 736
ENSG00000167601.11	AXL	7,05809486	10,0004371	0,13009683	tyrosine-protein kinase receptor
ENSG00000136244.11	IL6	7,85388695	10,8206444	0,12791368	interleukin-6
ENSG00000076706.16	MCAM	7,59882797	10,8142381	0,10766266	cell surface glycoprotein MUC18
ENSG00000092929.11	UNC13D	7,4891819	10,7265446	0,10603683	protein unc-13 homolog D
ENSG00000114315.3	HES1	8,84510281	12,1744235	0,0994889	transcription factor HES-1
ENSG00000143851.15	PTPN7	6,1952715	9,77548815	0,08360792	non-receptor tyrosine-phosphatase

Values in FI-KO clone and wild-type columns indicate normalized expression levels in FI-KO and wild-type cells, respectively. Values were filtered for genes, which expression was above the presumed level of expression (8) in at least one of the two comparator samples. A extended list of the genes, which expression change was >3 or <0.33 fold is available in supplementary file_1.

Table 3. Enriched pathway based sets for the list of 68 genes with expression changed at least 3 times (26 up- and 32 down-regulated).

Pathway Name	Set Size	Candidates Contained	p-value	q-value	Pathway Source
Senescence and Autophagy in Cancer	106	5 (4.7%)	5.73e-05	0.0112	Wikipathways
Amoebiasis – Homo sapiens (human)	96	4 (4.2%)	0.000515	0.018	KEGG
Graft-versus-host disease - Homo sapiens (human)	41	3 (7.3%)	0.000547	0.018	KEGG
Splicing factor NOVA regulated synaptic proteins	42	3 (7.1%)	0.000587	0.018	Wikipathways
Gene regulatory network modelling somitogenesis	11	2 (18.2%)	0.000812	0.018	Wikipathways

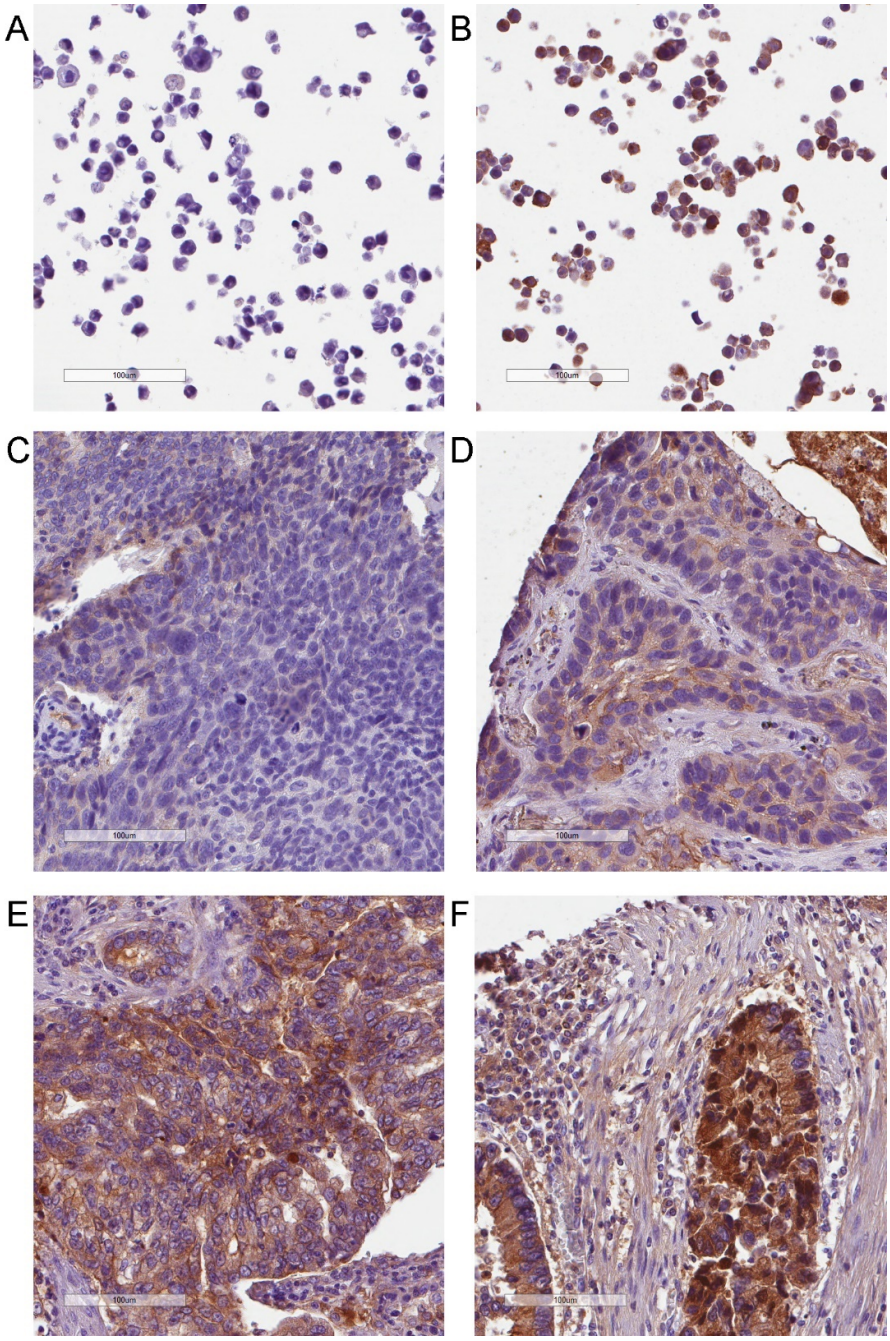
Antigen processing and presentation	12	2 (16.7%)	0.000972	0.018	BioCarta
Vitamin B12 Metabolism	51	3 (5.9%)	0.00104	0.018	Wikipathways
Epstein-Barr virus infection Homo sapiens (human)	201	5 (2.5%)	0.00109	0.018	KEGG
Spinal Cord Injury	117	4 (3.4%)	0.00113	0.018	Wikipathways
Ick and fyn tyrosine kinases in initiation of tcr activation	13	2 (15.4%)	0.00115	0.018	BioCarta

Table 4. Enriched gene ontology-based sets for the list of 68 genes with expression changed at least 3 times (26 up- and 32 down-regulated).

Gene Ontology Term	Category, Level	Set Size	Candidates Contained	p-value	q-value
GO:0007155 cell adhesion	BP 2	1389	18 (1.3%)	2.13e-07	1.39e-05
GO:0045321 leukocyte activation	BP 2	1284	17 (1.3%)	3.71e-07	1.39e-05
GO:0009653 anatomical structure morphogenesis	BP 2	2560	24 (0.9%)	4.68e-07	1.39e-05
GO:0048646 anatomical structure formation involved in morphogenesis	BP 2	1078	15 (1.4%)	1.13e-06	2.51e-05
GO:0005615 extracellular space	CC 2	3347	27 (0.8%)	1.3e-06	5.6e-05
GO:0001775 cell activation	BP 2	1435	17 (1.2%)	1.75e-06	3.12e-05
GO:0048856 anatomical structure development	BP 2	5790	35 (0.6%)	1.27e-05	0.000189
GO:0048870 cell motility	BP 2	1623	16 (1.0%)	3.79e-05	0.000421
GO:0051674 localization of cell	BP 2	1623	16 (1.0%)	3.79e-05	0.000421
GO:0048869 cellular developmental process	13	4275	28 (0.7%)	4.71e-05	0.000466

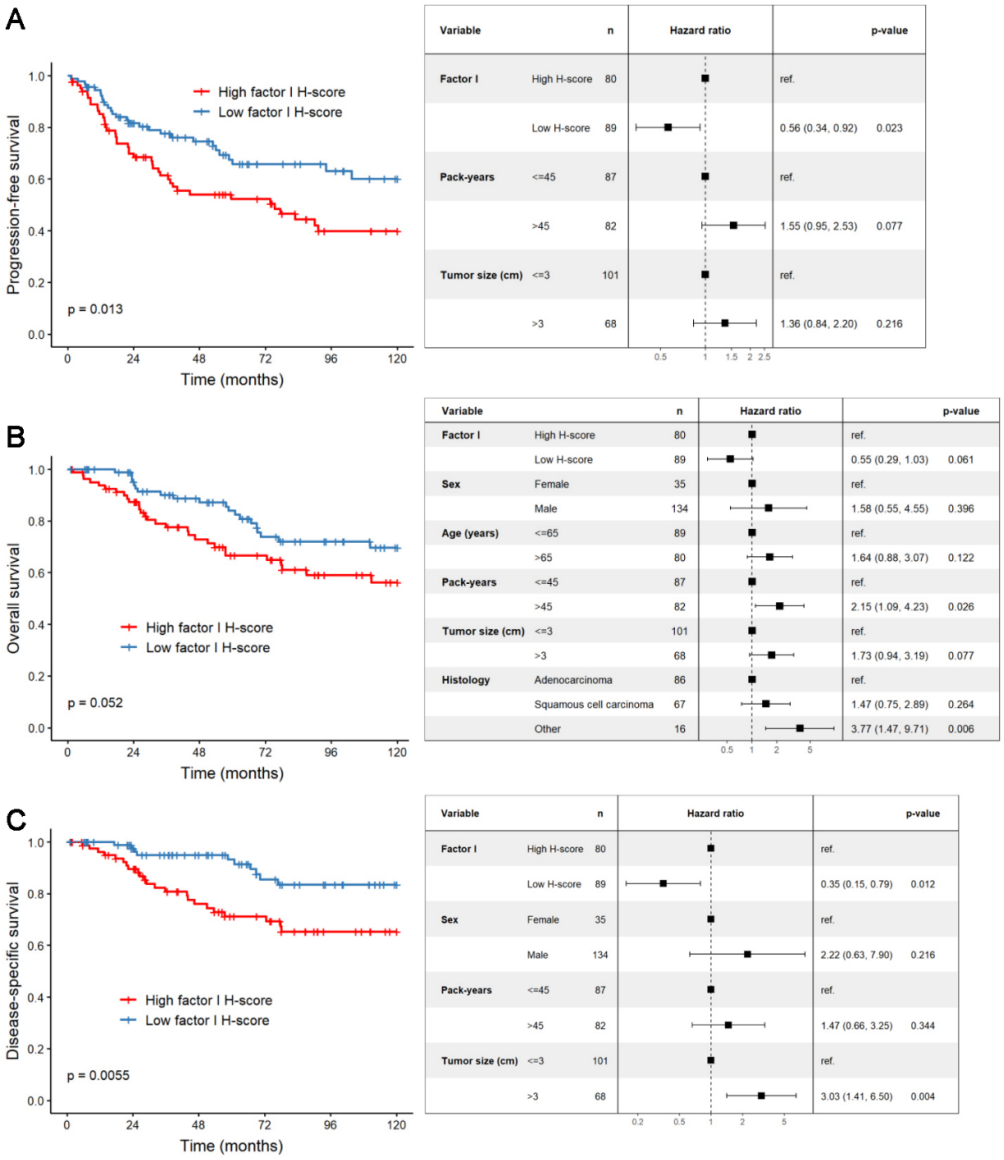
Figures and legends

Fig. 1 Immunohistochemical staining of FI in lung cancer tissue microarrays.



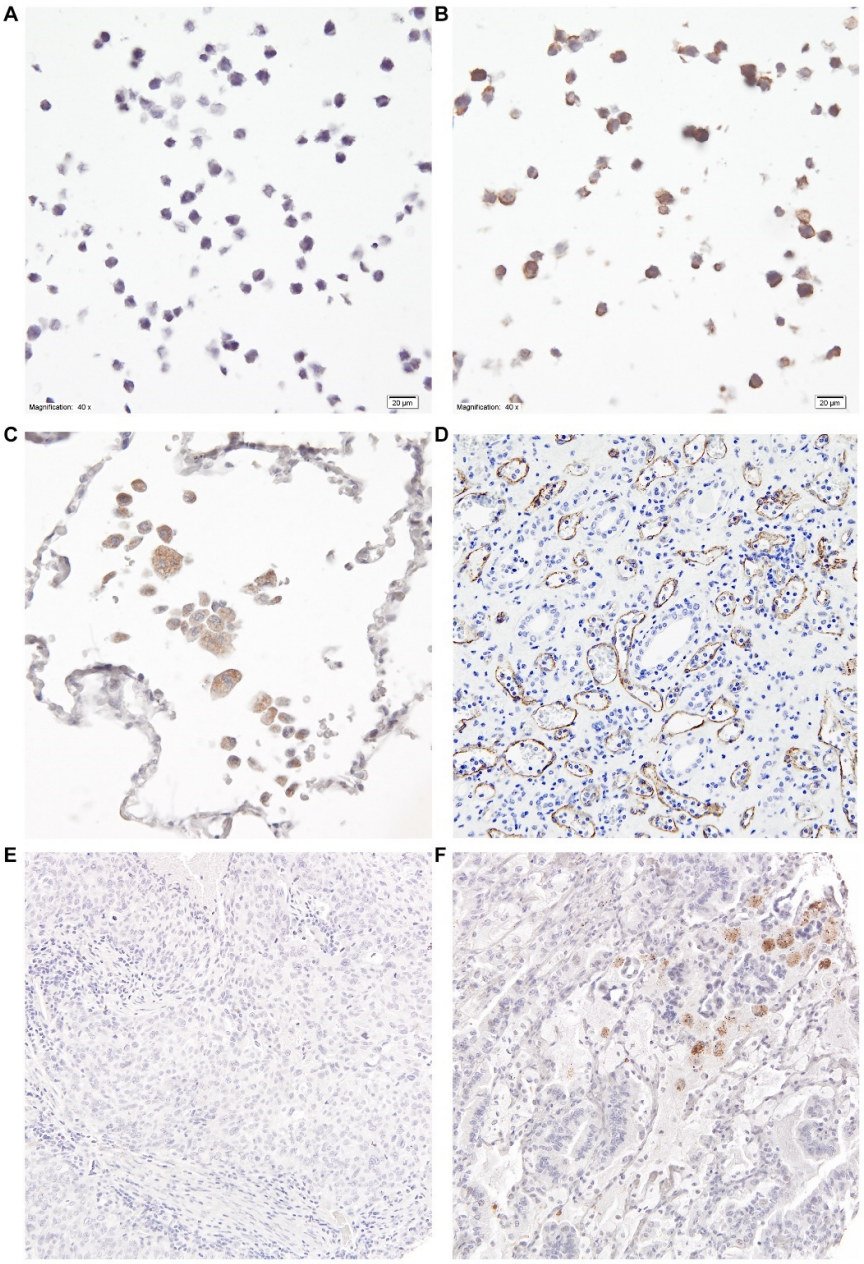
Immunohistochemical staining for FI in lung cancer tissue microarrays.
Formalin-fixed and paraffin-embedded (FFPE) pellets of lung cancer cell lines not expressing (A) and expressing (B) FI were used to setting up staining parameters that ensure obtaining of a truly positive and negative signal. Then, the same conditions were applied to the staining of tissue microarrays collected from the FFPE lung cancer tissue of the patients. Pictures C-F show examples of lung cancer tissue scored as negative (-), positive (+), strongly positive (+), and extremely positive (+++), a scale that was further implemented in the H-score assessment.

Fig. 2 Effect of FI on progression-free survival, overall survival and cancer-specific survival



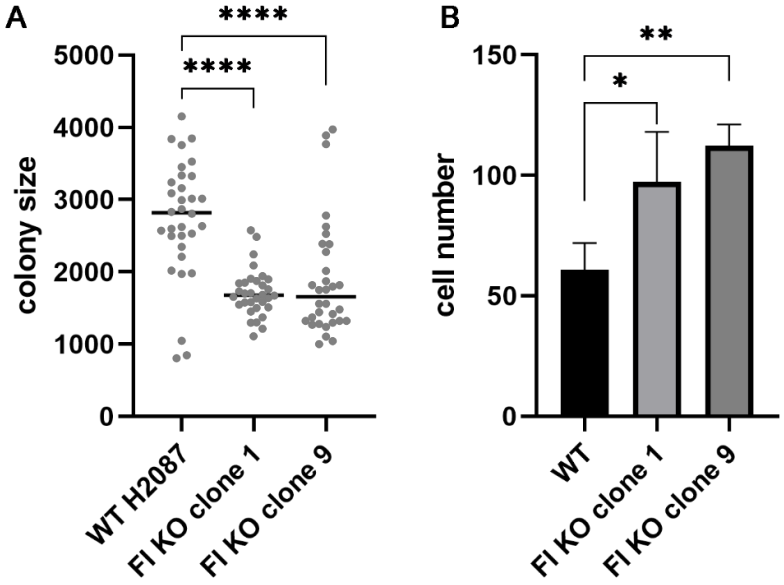
Graphs show Kaplan-Meyer analyses of progression-free survival (A), overall survival (B) and cancer-specific survival (C) of patients with high FI H-score (red line) and low FI H-score (blue line). The results of corresponding multivariate Cox analyses of the parameters that achieved p value lower than 0.1 in the univariate analysis are included.

Fig. 3 Immunohistochemical staining of C4d



The staining was first validated in FFPE pellets of Raji cells sensitized with anti-CD20 antibody rituximab in FI-depleted serum (A) and the same serum reconstituted with FI (B). Then, the same conditions were applied for the staining of normal lung tissue (C), kidney transplant tissue showing antibody-mediated rejection (D) and lung cancer issue from patients (E-F).

Fig. 4 In vitro functional assays on FI-deficient and FI-sufficient H2087 cells



3D spheroid cultures were prepared as described in methods by mixing cell suspension in culture medium with Matrigel. The number and diameter of spherules formed by each cell type/clone were recorded. Graph A shows the colony size of cells from three independent experiments. The results of the cell migration assay collected from four independent experiments are shown in B. Symbols *, ** and **** indicate statistical significance at p level <0.05, <0.01, and <0.0001 according to Dunnett's multiple comparison test, respectively.

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Table S1

Univariate analysis of progression-free survival

	HR (95% CI)	p-value
Factor I		
B B High H-score	ref.	0.014
B B Low H-score	0.55 (0.34-0.89)	
Sex		
B B Female	ref.	0.296
B B Male	1.41 (0.74-2.68)	
Age		
B B <=65	ref.	0.808
B B >65	1.06 (0.66-1.7)	
Smoking status		
B B Never smoker	ref.	0.442
B B Former smoker	0.8 (0.38-1.71)	
B B Current smoker	1.13 (0.49-2.58)	
Pack-years		
B B <=45	ref.	0.041
B B >45	1.65 (1.02-2.66)	
Tumor size (cm)		
B B <=3	ref.	0.157
B B >3	1.41 (0.88-2.27)	
Histology		
B B Adenocarcinoma	ref.	0.65
B B Squamous cell carcinoma	1.15 (0.7-1.88)	
B B Other	1.44 (0.64-3.24)	
Grade		
B B Well differentiated	ref.	0.989
B B Moderately differentiated	0.99 (0.45-2.18)	
B B Poorly differentiated	1.04 (0.46-2.33)	
Stage		
B B I	ref.	0.496
B B II-IV	0.84 (0.52-1.37)	

Table S2

Univariate analysis of disease-specific survival

	HR (95% CI)	p-value
Factor I		
B B High H-score	ref.	0.008
B B Low H-score	0.36 (0.17-0.77)	
Sex		
B B Female	ref.	0.098
B B Male	2.72 (0.83-8.92)	
Age		
B B <=65	ref.	0.544
B B >65	1.24 (0.62-2.46)	
Smoking status		
B B Never smoker	ref.	0.869
B B Former smoker	1.29 (0.39-4.3)	
B B Current smoker	1.09 (0.28-4.22)	
Pack-years		
B B <=45	ref.	0.061
B B >45	1.98 (0.97-4.06)	
Tumor size (cm)		
B B <=3	ref.	0.006
B B >3	2.83 (1.35-5.91)	
Histology		
B B Adenocarcinoma	ref.	0.23
B B Squamous cell carcinoma	1.7 (0.81-3.56)	
B B Other	2.16 (0.77-6.06)	
Grade		
B B Well differentiated	ref.	0.852
B B Moderately differentiated	1.31 (0.38-4.52)	
B B Poorly differentiated	1.44 (0.41-5.1)	
Stage		
B B I	ref.	0.135
B B II-IV	0.56 (0.26-1.2)	

Table S3

Univariate analysis of overall survival

	HR (95% CI)	p-value
Factor I		
B B High H-score	ref.	0.055
B B Low H-score	0.57 (0.32-1.01)	
Sex		
B B Female	ref.	0.063
B B Male	2.4 (0.95-6.06)	
Age		
B B <=65	ref.	0.047
B B >65	1.78 (1.01-3.14)	
Smoking status		
B B Never smoker	ref.	0.744
B B Former smoker	1.46 (0.52-4.12)	
B B Current smoker	1.27 (0.41-4.01)	
Pack-years		
B B <=45	ref.	0.006
B B >45	2.33 (1.28-4.27)	
Tumor size (cm)		
B B <=3	ref.	0.088
B B >3	1.65 (0.93-2.92)	
Histology		
B B Adenocarcinoma	ref.	0.098
B B Squamous cell carcinoma	1.79 (0.97-3.29)	
B B Other	2.12 (0.89-5.05)	
Grade		
B B Well differentiated	ref.	0.804
B B Moderately differentiated	0.83 (0.33-2.09)	
B B Poorly differentiated	1.03 (0.41-2.62)	
Stage		
B B I	ref.	0.842
B B II-IV	0.94 (0.53-1.68)	

Fig. S1 Association of FI presence in tumor tissue with infiltration of CD4+, CD8+, FOXP3+, and PD-LI+ cells.

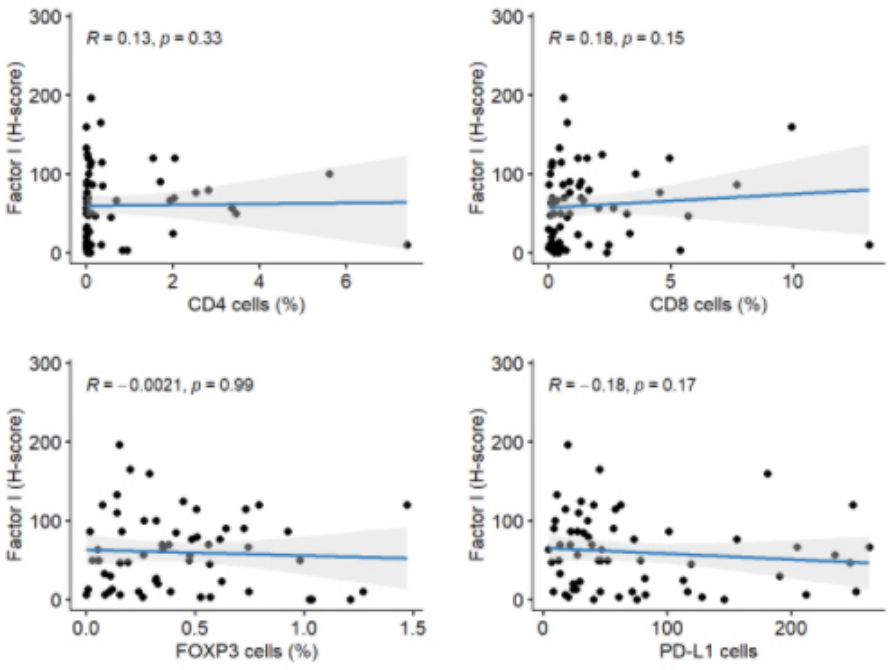


Fig.S2 qPCR on different cell lines to confirm FI production

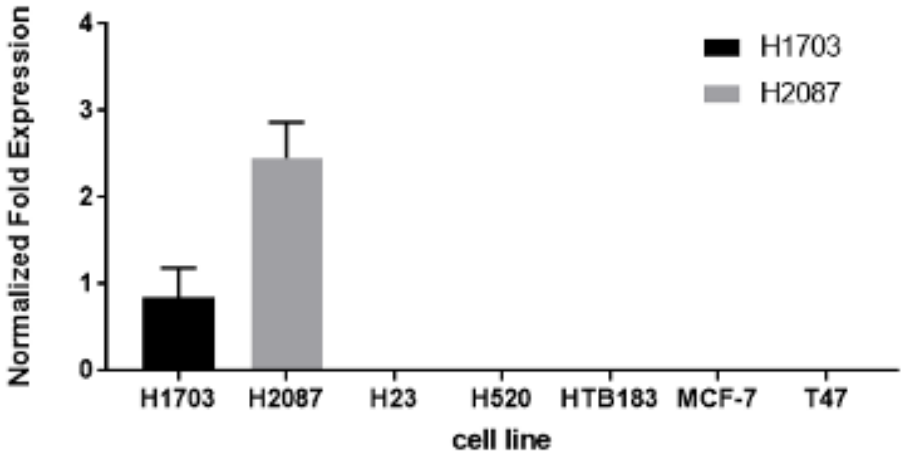
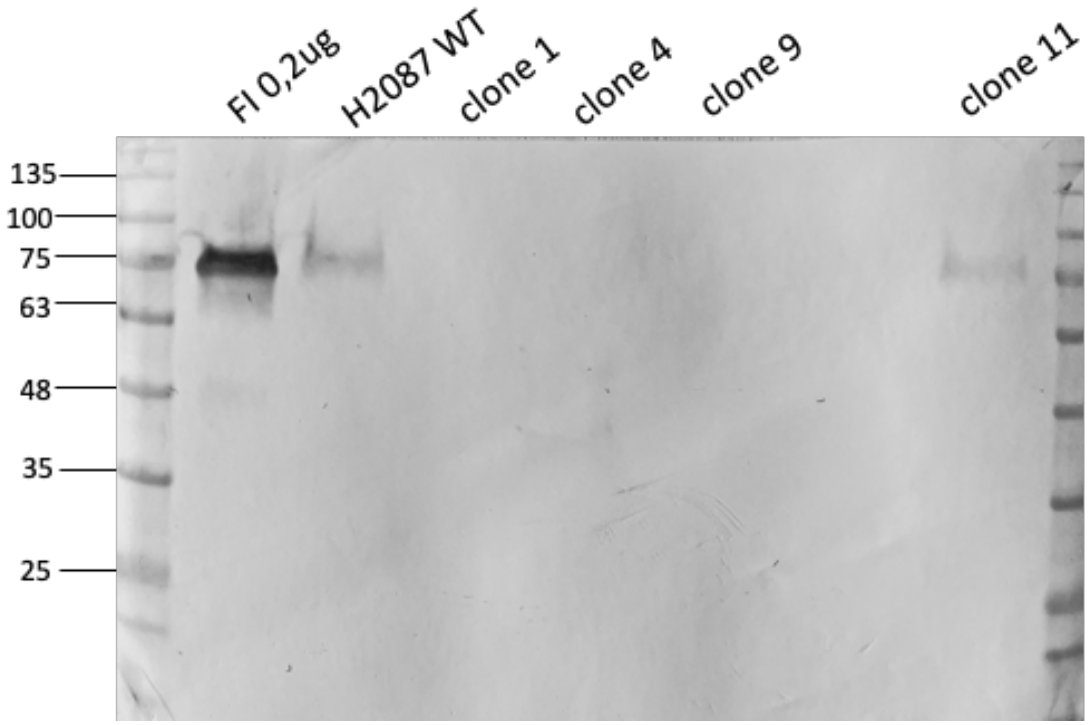


Fig. S3 Western Blott with medium conditioned by lung cancer cells for FI expression



7 Podsumowanie i dyskusja

Obecnie wiemy, że układ dopełniacza to nie tylko kaskada enzymatyczna, której aktywacja prowadzi do lizy komórki docelowej i ochrony przed patogenami, ale skomplikowany system mający znaczenie w przebiegu odpowiedzi immunologicznej, reakcji zapalnej czy zachowaniu homeostazy organizmu. Pełni również rolę w powstawaniu nowotworów i terapii przeciwnowotworowej. Chociaż początkowo działanie układu dopełniacza było postrzegane jako mechanizm przeciwnowotworowy, ostatnie badania pokazują, iż w pewnych warunkach aktywność układu dopełniacza może stanowić element wspomagający progresję nowotworu. Wykazano również, że wiele linii komórek nowotworowych produkuje nie tylko inhibitory, ale również białka aktywujące dopełniacz, co zdaje się uprawdopodabniać jego korzystny wpływ na rozwój nowotworu w pewnych warunkach.

Istnieje szereg terapeutykow, ktore aktywują układ dopełniacza i wykorzystują go jako potencjalny mechanizm działania. Przykładem są przeciwciała terapeutyczne anty-CD20 stosowane w leczeniu chorych na białaczki i chłoniaki. Niestety nie u wszystkich pacjentów występuje odpowiedź kliniczna, co więcej, cześć pacjentów okazuje się oporna na terapię. Jedną z przyczyn oporności na terapeutyki działające za pośrednictwem układu dopełniacza jest nadekspresja inhibitorów tego układu na powierzchni komórek nowotworowych oraz wyczerpanie puli białek dopełniacza zużywanych podczas aktywacji tego układu przez przeciwciała. Niewystarczająca ilość białek dopełniacza spowodowana nadmierną konsumpcją układu dopełniacza przy pierwszym podaniu leku może skutkować obniżoną efektywnością kolejnych infuzji.

W badaniach wykonanych w ramach pracy doktorskiej pokazałam aktywność układu dopełniacza w surowicach pacjentów podczas terapii przeciwciałami przeciwnowotworowymi (alemtuzumab i ofatumumab, stosowanych w terapii łączonej) [4]. Badania miały na celu walidację nowej metody określania aktywności CDC surowicy a także określenie, jak długo stan wyczerpania białek układu dopełniacza utrzymuje się po wlewie przeciwciał terapeutycznych aktywujących dopełniacz oraz czy pula białek zostaje odnowiona przed kolejnym podaniem leku. Wyniki pokazały, że podawanie samego alemtuzumab lub alemtuzumabu w połaczeniu z ofatumumabem, nawet w dużej dawce 2000 mg nie powodowało trwałego zmniejszenia dostępnej puli dopełniacza u większości pacjentów. Niestety, mając do dyspozycji jedynie próbki surowicy nie mogliśmy przeanalizować ekspresji CD20, CD55 ani depozycji markerów aktywacji układu dopełniacza, takich jak C3d i/lub C4d na komórkach białaczkowych (CLL). Niemożliwe zatem okazało się wyciągnięcie jednoznacznych wniosków czy harmonogram terapeutyczny był optymalny dla odbudowy dopełniacza przed kolejnymi dawkami leku lub czy minimalne zmniejszenie potencjału CDC obserwowane u niektórych pacjentów miało związek z niską ekspresja celu molekularnego. Niemniej jednak postulujemy, że pomiar CDC w surowicy przed podaniem immunoterapeutyków anty-CD20 typu I tudzież innych przeciwciał aktywujących dopełniacz jest ważny nie tylko w celu ustalenia, czy efektor dla danych przeciwciał jest obecny, ale także aby upewnić się, że pacjent (już pozbawiony limfocytów przez przeciwciała anty-CD20 i anty-CD52) nie jest dodatkowo pozbawiony ważnej części odporności wrodzonej. Jest to znamienne w świetle wyników innych badaczy sugerujących, że nawet 40% pacjentów cierpiących na nowotwory wywodzące się z limfocytów

B i leczonych przeciwciałami anty-CD20 typu I wykazuje obniżony poziom składowych dopełniacza w surowicy [31].

W swojej pracy doktorskiej wykorzystałam nową metodą badawczą opisaną przez nasz zespół [4] służącą do sprawdzania aktywności cytotoksycznej (CDC) surowic pacjentów. W opisanej metodzie wykorzystujemy komórki nowotworowe rozpoznawane przez identyczne przeciwciała, jak te stosowane podczas terapii. W przeciwieństwie do erytrocytów owczych (które są obecnie powszechnie wykorzystywane w testach cytotoksycznych) na powierzchni komórek nowotworowych znajdują się inhibitory dopełniacza, będące jedną z głównych przeszkód w skutecznej terapii przeciwciałami typu I. Co więcej, erytrocyty owcze wyposażone są w domniemane homologi ludzkich białek, ale o nieznanej skuteczności w stosunku do ludzkiej surowicy. Warty uwagi jest również fakt, że erytrocyty owcze są bardzo podatne na lizę nawet przy bardzo niskim stężeniu surowicy (0,25% - 1%) [32] w przeciwieństwie do większości komórek białaczkowych [3, 33]. Wykorzystanie dużych rozcieńczeń surowicy i bardzo wrażliwych komórek predysponuje do większych odchyleń między wynikami poszczególnych powtórzeń, niż w przypadku testu cytotoksycznego na komórkach nowotworowych w 50% surowicy. W innej części pracy doktorskiej, podczas badania zastosowania wariantów GOF czynnika B jako wspomagaczy immunoterapii zaobserwowałam, że dodanie tych hiperaktywnych białek do uwrażliwionych erytrocytów owczych jedynie nieznacznie zwiększa lizę. Kiedy zamiast erytrocytów wykorzystałam komórki Raji, dodatek wariantów GOF czynnika B spowodował znaczący wzrost wrażliwości na CDC [16]. Jest to kolejny przykład pokazujący, że erytrocyty owcze nie są idealnym modelem do badania aktywności cytotoksycznej ludzkich surowic w kontekście ich potencjału do zwalczania komórek nowotworowych.

W użyciu znajduje się wiele immunoterapeutyków, które mogą wykorzystywać układ dopełniacza jako mechanizm efektorowy, nimniej jednak związanie przez nie antygentu na powierzchni komórek nowotworowych może aktywować kilka innych mechanizmów, takich jak cytotoksyczność zależna od przeciwciał (ADCC) lub bezpośrednia śmierć apoptotyczna, jak również wzmożona fagocytoza. W jednej z prac wchodzących w skład niniejszego cyklu zawarłam podsumowanie na temat trzech typów przeciwciał anty-CD20 i ich mechanizmów działania [5]. Chociaż istnieją dane badawcze sugerujące role układu dopełniacza w efekcie terapeutycznym przeciwciał anty-CD20 stosowanych do leczenia białaczek i chłoniaków, świat naukowy jednak jest w tym temacie daleki od konsensusu. Badania innych zespołów pokazują z jednej strony, że wyizolowane CD20-pozytywne komórki podatne na CDC mogą być predyktorem odpowiedzi klinicznej na rytuksymab [34] z drugiej strony w dwóch innych badaniach przedstawiono przeciwne wyniki [35, 36]. Aspektem pomijanym często w rozważaniach na temat kluczowego dla terapii mechanizmu efektorowego przeciwciał anty-CD20 jest fakt, że większość doświadczeń *in vivo* sugerujących brak znaczenia układu dopełniacza przeprowadzono na modelu mysim. Większość szczepów myszy laboratoryjnych charakteryzuje się relatywnie niską aktywnością dopełniacza wyrażoną odczytami CH50 o rząd wielkości niższą od pomiarów u człowieka [37]. Wobec powyższego, wyniki uzyskane na modelu mysim, gdzie znacznie bardziej wydajne od dopełniacza mechanizmy efektorowe zdają się dominować, nie mogą być bezpośrednio ekstrapolowane na człowieka, u którego relatywna wydajność dopełniacza jest znacznie wyższa. Argumentem przemawiającym za rolą dopełniacza w immunoterapii są wyniki badań pokazujące, że odpowiedź kliniczna na rytuksymab

poprawiła się po suplementacji świeżym mrożonym osoczem będącym rezerwuarem białek dopełniacza, przy czym pozytywny efekt obserwowano nawet u pacjentów nie wykazujących uprzednio odpowiedzi na rytuksymab [38, 39].

W celu uzupełnienia wiedzy na temat roli układu dopełniacza w efekcie terapeutycznym przeciwciał anty-CD20 typu I oznaczyłam markery aktywacji układu dopełniacza, zużycie komponentów dopełniacza oraz kumulację rytuksymabu w osoczu pacjentów z przewlekłą białaczką limfocytowa (CLL) oraz chłoniakami nieziarniczymi (NHL). Monitorowanie stanu układu dopełniacza i stężenia rytuksymabu podczas terapii jest ważne z naukowego punktu widzenia, ale także w kontekście samego leczenia chorych na białaczki i chłoniaki. W zależności od wyników oznaczeń lekarz prowadzący mógłby podjąć decyzję co do konieczności podawania nowych dawek leku, co przekłada się nie tylko na względy ekonomiczne ale także na unikanie ekspozycji pacjenta na wysokie dawki przeciwciał anty-CD20, które wg. literatury naukowej sprzyjają selekcji populacji komórek nowotworowych o niskiej ekspresji celu molekularnego [40]. Badanie poziomów markerów aktywacji czy aktywności CDC surowic mogłoby być również przesłanką do zmiany leczenia z przeciwciał typu I na przeciwciała typu II u pacjentów wykazujących niską początkową aktywność dopełniacza, saturację aktywności CDC surowicy przed kolejnymi wlewami leku połączoną z brakiem odpowiedzi klinicznej, lub też wykazujących brak pojawienia się markerów aktywacji dopełniacza pomimo utrzymującego się wysokiego stężenia wolnego przeciwciała typu I w surowicy. Biorąc pod uwagę przedstawione wyniki możemy przypuszczać, że dalsze badania nad przeciwciałami anty-CD20 typu III i ich wprowadzenie do użytku również mogłoby poprawić efektywność terapii [5].

Znanym problemem terapii działającej za pośrednictwem układu dopełniacza jest nadekspresja inhibitorów tego układu na powierzchni komórek nowotworowych. W celu przeciwdziałania temu mechanizmowi oporności w pracy doktorskiej zaproponowałam nowatorskie rozwiązanie polegające na zastosowaniu zmutowanych białek układu dopełniacza wchodzących w skład konwertaz. Badania wykazały, że suplementacja surowicy wariantami GOF czynnika B może wzmocnić efekt cytotoksyczny terapeutycznych przeciwciał anty-CD20 [16]. Innowacyjność zaproponowanego rozwiązania polega na zastosowaniu potencjalnie patogennych białek jako suplementu podnoszącego aktywność cytotoksyczną immunoterapeutyków. Wykorzystanie wariantów czynnika B tworzących nadreaktywną konwertazę alternatywną w terapii przeciwnowotworowej wzbudza kontrowersje. Powodem jest m.in. fakt, że wybrane mutacje zostały pierwotnie zidentyfikowane u chorych cierpiących na schorzenia zapalne oraz autoimmunologiczne oraz na fakt, że czynnik B jest składnikiem ścieżki alternatywnej, która jest aktywowana w sposób spontaniczny. Przedstawione w tej części badania są badaniami wstępnymi, które postanowiliśmy kontynuować wykorzystując białko ścieżki klasycznej układu dopełniacza C2 będące homologiem czynnika B. Powodami kontynuowania badań z wykorzystaniem białka C2 jest między innym fakt, że jest to białko ścieżki klasycznej, której aktywacja jest zależna od wiązania przeciwciał, co z kolei zapewnia dodatkowy element kontroli. Kolejnym powodem jest fakt, że białko C2 występuje w surowicy w dużo niższym stężeniu (25 µg/ml) niż czynnik B (180 µg/ml), co może przyczynić się do ułatwienia efektywnej suplementacji pacjentów za pomocą relatywnie niskich ilości białka rekombinowanego. Białko C2 i czynnik B mają prawie identyczną strukturę i organizację domen,

dzięki temu byliśmy w stanie utworzyć warianty C2 na podstawie znanych i przedstawionych w wyżej opisanych badaniach mutacji w czynniku B. Badania z wykorzystaniem mutantów GOF białka C2 jako czynnika zwiększającego efekt terapeutyczny przeciwciał anty-CD20 zostały ostatnio opublikowane w osobnej pracy mojego współautorstwa [41].

Wcześniejsze badania wskazują, że komórki nowotowrowe mogą wytwarzać zarówno aktywatory [28], jak i rozpuszczalne inhibitory dopełniacza [29, 30]. W pracy [30] pokazano, że komórki raka płuc wydzielają szereg rozpuszczalnych inhibitorów dopełniacza m.in. czynnik I (FI). Aby poszerzyć wiedzę na temat roli czynnika I w rozwoju guzów litych sprawdziliśmy, czy podwyższona ekspresja czynnika I w komórkach raka płuc ma wpływ na czas przeżycia pacjentów z chorobą nowotworową. Zaobserwowaliśmy związek między obecnością czynnika I w komórkach nowotworowych a złym rokowaniem pacjentów. Jednocześnie nie zaobserwowaliśmy żadnego związku między obecnością FI w komórkach nowotworowych a depozycją C4d. Był to wynik nieoczekiwany, ponieważ wcześniejsze badania pokazały, że C1q wiąże się bezpośrednio z komórkami raka płuc aktywując ścieżkę klasyczną układu dopełniacza oraz że uwolnienie C4d do surowicy wiąże się z krótszym przeżyciem pacjentów [42]. Istnieje kilka prawdopodobnych wyjaśnień, które zostały opisane w pracy [Manuskrypt nr 5] niemniej jednak ten wynik w połączeniu z przeprowadzoną analizą transkryptomu komórek H2087 produkujących oraz pozbawionych FI a także rosnąca liczba doniesień na temat niekanonicznej funkcji składników dopełniacza w komórkach nowotworowych [43, 44] skłoniła nas do wniosków, że czynnik I wpływa na fizjologię komórek raka płuc niezależnie od funkcji pełnionej w ramach odporności nieswoistej, co może tłumaczyć złe rokowanie u chorych. Podsumowując otrzymane wyniki, można stwierdzić, że lepsze zrozumienie interakcji nowotwór - układ dopełniacza jest istotnie nie tylko z naukowego punktu widzenia, ale w przyszłości może nieść za sobą praktyczne korzyści dla pacjentów onkologicznych.

Projekt doktorski sfinansowany został z projektu NCN Sonata Bis dr hab. Marcina Okroja.

Ryciny przedstawione w pracy wykonano w programie PowerPoint oraz aplikacji BioRender.

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Oświadczenia współautorów dotyczące wspólnego dorobku publikacyjnego na potrzeby postępowania w sprawie nadania stopnia doktora.

Dotyczy rozprawy doktorskiej Pani mgr Anny Felberg-Miętki pod tytułem:

Znaczenie układu dopełniacza w powstawaniu nowotworów oraz w terapii przeciwnowotworowej

Promotor: dr hab. Marcin Okrój, prof. GUMed
Instytut Biotechnologii Medycznej i Okologii Doświadczalnej
Zakład Biologii Komórki i Immunologii MWB UG-GUMed

Gdańsk 2022

Dr hab. Marcin Okrój
Zakład Biologii Komórki i Immunologii
Międzyuczelniany Wydział Biotechnologii UG-GUMed
Tel. +48 583491410, e-mail: marcin.okroj@gumed.edu.pl

Gdańsk, 29 kwietnia 2022 r

Niniejszym oświadczam, że we wszystkich publikacjach włączonych do rozprawy doktorskiej mojej podopiecznej, mgr Anny Felberg-Miętki, pełniłem nadzór nad integralnością naukową zaprezentowanych wyników, opracowywałem (w porozumieniu i z udziałem doktorantki) koncepcję pracy, brałem udział w planowaniu poszczególnych doświadczeń oraz uczestniczyłem w pisaniu oraz edycji ostatecznej wersji manuskryptów.

Oświadczenie określające indywidualny wkład osoby ubiegającej się o nadanie stopnia doktora

Potwierdzam mój wkład w publikacje/manusktypty zawarte w pracy doktorskiej

Publikacja 1

G. Stasiłojć, A. Felberg*, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin , A. Österborg, M. Okrój*

"Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies"

Journal of Immunological Methods, Vol. 476, 112675, 2020

**autorzy maja równy wkład w przygotowanie publikacji*

Wkład autora: Spośród wszystkich autorów Anna Felberg-Miętka oraz Grzegorz Stasiłojć mieli największy wkład w pracę. Kandydatka wykonała badania porównawcze na próbkach uzyskanych z miejscowego szpitala w celu cross-walidacji, uczestniczyła w przygotowaniu doświadczeń oraz w pisaniu manuskryptu.

Publikacja 2

A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój

"Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab"

Frontiers in Immunology, Vol. 11, 584509, 2020

Wkład autora: Kandydatka jest wiodącym autorem. Spośród wszystkich autorów kandydatka miała największy wkład w pracę. Uczestniczyła w zbieraniu materiału klinicznego do badań, wykonała część doświadczalną, zebrała dane do części teoretycznej, uczestniczyła w pisaniu manuskryptu.

Publikacja 3

G. Stasiłojć, A. Felberg, M. Okrój

"Parameters critical for the effector mechanism of anti-CD20 antibodies revisited"

British Journal of Haematology, Vol. 180, Issue 6, 777-779, 2018

Wkład autora: Spośród wszystkich autorów kandydatka oraz Grzegorz Stasiłojć mieli największy wkład w pracę. Kandydatka wykonała przegląd literaturowy oraz uczestniczyła w pisaniu manuskryptu.

Anna Felberg-Miętka

Publikacja 4

A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój

"Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics"

Cancer Immunology, Immunotherapy, Vol. 68, 587–598, 2019

Wkład autora: Kandydatka jest wiodącym autorem. Spośród wszystkich autorów kandydatka miała największy wkład w pracę . Wykonała większość części doświadczalnej opisanej w pracy, uczestniczyła w pisaniu manuskryptu.

Dodatkowo, ustna prezentacja wyników powyższej pracy na konferencji 17th European Meeting on Complement in Human Disease (EMCHD) w dniach 13-17 września 2019 roku w Madrycie została uhonorowana wyróżnieniem przyznanym przez The European Complement Network.

Manuskrypt 5

A. Felberg, M. Bieńkowski, T. Stokowy, F. Mohlin, S. Nilsson, I. Jongerius,

R. Spaapen, L. M. Montuenga, A. M. Blom, R. Pio, M. Okrój

"Elevated expression of complement factor I in lung cancer cells is associated with shorter progression-free survival and disease-specific survival"

Wkład autora:

Kandydatka jest wiodącym autorem. Spośród wszystkich autorów kandydatka miała największy wkład w pracę. Wykonała większość części doświadczalnej opisanej w pracy, uczestniczyła w pisaniu manuskryptu.

Anna Felberg-Miętka

Anna Felberg-Miętka

LUND UNIVERSITY
Faculty of Medicine

Department of Translational Medicine
University Hospital, Malmö
Anna Blom, PhD
professor of medical protein chemistry

Malmö 22 04 28

Hereby I confirm that my contribution to the publications/manuscripts:

1) G. Stasiłojć, A. Felberg, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin, A. Österborg, M. Okrój, entitled "Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies", published in Journal of Immunological Methods in 2020

and

2) A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój, entitled "Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab", published in Frontiers in Immunology in 2020

and

3) A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój, entitled "Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics.", published in Cancer Immunology, Immunotherapy in 2019

and

4) Felberg, M. Bieńkowski, T. Stokowy, F. Mohlin, I. Jongerius, R. Spaapen, M. Mesa-Guzman, L.M. Montuenga, A.M. Blom, R. Pio, M. Okrój, entitled "Elevated expression of complement factor I in lung cancer cells associates with shorter progression-free survival and disease-specific survival" (manuscript)

involved intellectual input, drafting of the manuscripts, and their critical revision, in collaboration with other co-authors. Additionally, I have provided the necessary molecular tools that were used in publication no.3 and supervised the part of the experimental work (immunohistochemical staining of clinical material) presented in the manuscript no.4.

Sincerely,

Anna Blom, professor

Anna Blom

Lund University, Department of Translational Medicine, Section of Medical Protein Chemistry
Visiting address *Wallenberg Laboratory, Inga Marie Nilssons street 53, S-214 28 Malmö*
Phone *+46 704 15 06 82,* E-mail *Anna.Blom@med.lu.se*

Dr hab. inż. Tomasz Stokowy
Group Leader Scientific Computing
IT Division
Uniwersytet w Bergen, Norwegia
e-mail: Tomasz.Stokowy@uib.no

Bergen, 29 kwietnia 2022 r

Niniejszym oświadczam, że mój wkład w manuskrypt wchodzący w skład rozprawy doktorskiej mgr Anny Felberg-Miętki, zatytułowany “Elevated expression of complement factor I in lung cancer cells associates with shorter progression-free survival and disease-specific survival” polegał na przeprowadzeniu porównawczej analizy transkryptomu komórek H2087 poddanych modyfikacji genetycznej oraz komórek bez ww. modyfikacji.

Tomasz Stokowy

29.04.2022

Dr Aleksandra Urban
Department of Cancer Immunology
Oslo University Hospital
e-mail: aleksandra.urban@ous-research.no

Oslo, 27 kwietnia 2022

Oświadczam, iż w publikacji nr.1 (G. Stasiłojć, A. Felberg, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin,A. Österborg, M. Okrój. Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies" Journal of Immunological Methods, Vol. 476, 112675, 2020) moja praca polegała na optymalizacji testów CDC, które zostały następnie użyte w doświadczeniach zaprezentowanych w poniżej wspomnianych pracach. W publikacji nr. 2 (A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój "Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab" Frontiers in Immunology, Vol. 11, 584509, 2020, przedłożonej w ramach pracy doktorskiej mgr Anny Felberg-Miętka, mój wkład polegał na wykonaniu testów ELISA w celu pomiaru poziomu terapeutyku pozostałego w surowicach pobranych od pacjentów będących w trakcie leczenia rituksymabem. Ponadto, byłam odpowiedzialna za analizę uzyskanych wyników.

W publikacji nr. 4 (A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój "Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics." Cancer Immunology,Immunotherapy, Vol. 68, 587–598, 2019 sprawowałam bezpośredni nadzór nad magistrantką, p. Anną Borowską, z którą wspólnie zajmowałyśmy się ekspresją mutantów czynnika B w systemie eukariotycznym. Uczestniczyłam także, wraz z innymi współautorami, w przygotowaniu pierwotnej wersji manuskryptu.

Aleksandra Urban

Dr Grzegorz Stasiłojć
Zakład Biologii Komórki i Immunologii
Międzyuczelniany Wydział Biotechnologii UG-GUMed
Tel. +48 583491411, e-mail: gstasilojc@gumed.edu.pl

Gdańsk, 27 kwietnia 2022

Oświadczam, iż w publikacji nr.1 (G. Stasiłojć, A. Felberg, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin,A. Österborg, M. Okrój. Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies" Journal of Immunological Methods, Vol. 476, 112675, 2020), przedłożonej w ramach pracy doktorskiej mgr Anny Felberg mój wkład polegał na przeprowadzeniu, razem z doktorantką, testów CDC na ludzkich liniach komórek CD20+ z wykorzystaniem surowic pochodzących z badania klinicznego. Nasz wkład został uznany jako równorzędny, co jest udokumentowane w statusie równorzędnego, pierwszego autora.

W kolejnych publikacjach (nr. 2 i nr. 4) : A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój. "Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab" Frontiers in Immunology, Vol. 11, 584509, 2020 oraz A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój. "Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics." Cancer Immunology,Immunotherapy, Vol. 68, 587–598, 2019 moja rola sprowadzała się do optymalizacji części metod zastosowanych w doświadczeniach.

W publikacji nr. 3 (Stasiłojć G, Felberg A, Okrój M. "Parameters critical for the effector mechanism of anti-CD20 antibodies revisited" British Journal of Haematology, Vol. 180, Issue 6, 777-779, 2018) wraz z pozostałymi współautorami dokonałem kwerendy literaturowej w celu zebrania potrzebnych informacji omówinych w artykule.

Grzegorz Stasiłojć

Dr Michał Bieńkowski
Katedra i Zakład Patomorfologii
Gdański Uniwersytet Medyczny
e-mail: michal.bienkowski@gumed.edu.pl

Gdańsk, 28 kwietnia 2022 r

Niniejszym oświadczam, że mój wkład w manuskrypt wchodzący w skład rozprawy doktorskiej mgr Anny Felberg-Miętki, zatytułowany"Elevated expression of complement factor I in lung cancer cells associates with shorter progression-free survival and disease-specific survival" polegał na przeprowadzeniu barwień immunohistochemicznych i ocenie patomofrologicznej uzyskanych preparatów pochodzących z tkanek nowotworowych pacjentów objętych badaniem.

Michał Bieńkowski

Lek. Alan Majeranowski
Katedra i Klinika Transplantologii i Hematologii
Gdański Uniwersytet Medyczny
e-mail: alan.majeranowski@gumed.edu.pl

Gdańsk, 27 kwietnia 2022

Oświadczam, iż w publikacji nr. 2 (A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój "Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab" Frontiers in Immunology, Vol. 11, 584509, 2020), przedłożonej w ramach pracy doktorskiej mgr Anny Felberg mój wkład polegał na diagnozie pacjentów i koordynacji procesu pozyskiwania materiału klinicznego użytego następnie w doświadczeniach opisanych w pracy.

Lek. Michał Taszner
Katedra i Klinika Transplantologii i Hematologii
Gdański Uniwersytet Medyczny

Gdańsk, 27 kwietnia 2022

Oświadczam, iż w publikacji nr. 2 (A. Felberg, M. Taszner, A. Urban, A. Majeranowski, K. Jaskuła, A. Jurkiewicz, G. Stasiłojć, A. M. Blom, J. M. Zaucha, M. Okrój "Monitoring of the complement system status in patients with B-cell malignancies treated with rituximab" Frontiers in Immunology, Vol. 11, 584509, 2020), oraz w publikacji nr. 4 (A. Felberg, A. Urban, A. Borowska, G. Stasiłojć, M. Taszner, A. Hellmann, A.M. Blom, M. Okrój "Mutations resulting in the formation of hyperactive complement convertases support cytocidal effect of anti-CD20 immunotherapeutics." Cancer Immunology, Immunotherapy, Vol. 68, 587–598, 2019) wchodzących w skład rozprawy doktorskiej mgr Anny Felberg, mój wkład polegał na diagnozie pacjentów i koordynacji procesu pozyskiwania materiału klinicznego użytego następnie w doświadczeniach opisanych w pracy.

Michał Taszner
specjalista chorób wewnętrznych
hematolog
PWZ: 1893421

Mgr Daria Kowalska
Zakład Biologii Komórki i Immunologii
Międzyuczelniany Wydział Biotechnologii UG-GUMed
Tel. +48 583491438, e-mail: daria.kowalska@gumed.edu.pl

Gdańsk, 27 kwietnia 2022

Oświadczam, iż w publikacji nr.1 (G. Stasiłojć, A. Felberg, A. Urban, D. Kowalska, S. Ma, A.M. Blom, J. Lundin,A. Österborg, M. Okrój. Calcein release assay as a method for monitoring serum complement activity during monoclonal antibody therapy in patients with B-cell malignancies" Journal of Immunological Methods, Vol. 476, 112675, 2020), przedłożonej w ramach pracy doktorskiej mgr Anny Felberg mój wkład polegał na optymalizacji testów CDC, które zostały następnie użyte w doświadczeniach zaprezentowanych w pracy. Moja działalność w tym projekcie odbywała się w ramach realizacji pracy magisterskiego w Zakładzie Biologii Komórki i Immunologii.

Daria Kowalska