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Article

CD20 mAb-Mediated Complement Dependent Cytotoxicity of Tumor Cells is Enhanced by Blocking the Action of Factor I

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Abstract: The CD20 mAbs, rituximab (RTX) and ofatumumab (OFA), have been used with success in the clinic in the treatment of B cell malignancies. These mAbs can eliminate B cells only by utilizing the body's immune effector mechanisms, and there is considerable evidence that OFA is particularly effective at eliminating B cells by mediating complement dependent cytotoxicity (CDC). However, effector mechanisms such as complement can be exhausted or down-regulated. Therefore, several approaches are being investigated with the goal of increasing CDC mediated by these mAbs. We reported that when patients with chronic lymphocytic leukemia (CLL) are treated with RTX or with OFA, complement is rapidly activated on circulating, targeted CLL B cells. However, a substantial fraction of these cells escape CDC and clearance due to degradation of covalently deposited active C3b fragments to inactive fragments iC3b and C3d. This process is mediated by a plasma protease, Factor I. Therefore, a rational approach for increasing CDC would be to block this reaction by inhibiting Factor I with a neutralizing mAb. Indeed, we have demonstrated that use of neutralizing mAb A247, specific for factor I, significantly and substantially increases CD20 mAb-mediated CDC of both cell lines and of primary CLL cells *in vitro*.

Keywords: monoclonal antibody; complement; Factor I; rituximab; ofatumumab

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1. Introduction

The mechanisms of action of monoclonal antibodies (mAbs) that have been approved for, or are under consideration to be used for the immunotherapy of cancer are under active and intense investigation [1–10]. At first glance this continued investigation might be considered surprising, especially for mAbs which have already been approved by the FDA. However, it is now clear that certain tumor-specific mAbs, in particular rituximab (RTX) and ofatumumab (OFA) can promote cytotoxicity of targeted cells by several independent mechanisms, all of which require participation by the host's immune effector mechanisms [1,4,5,11–13]. These mechanisms require recognition of the Fc regions of cell-bound, aggregated IgG mAbs, by either Fcγ receptors on effector cells (NK cells or macrophages) or recognition and activation by the complement system. As has been emphasized in several recent reports, the apparent relative contribution of each of these effector mechanisms depends upon many independent factors, which may include the tumor compartment, the tumor load, and the intrinsic cytotoxic capacity of the effector systems [4,14–16]. Moreover, pre-clinical mouse model studies have revealed that the relative contribution of these effector mechanisms depends in part upon the detailed nature of the model under investigation [14,17–19]. However, these mechanisms are critical for clinical efficacy, and therefore many ongoing research efforts have been initiated with a goal of improving and enhancing mAb-based therapies, based on increasing the efficacy of specific effector mechanisms [20–34]. Killing of targeted tumor cells can be increased by either developing next generation mAbs with rationally-based differences in primary structure or degree of glycosylation, or strategies can be employed to increase the capacity/efficiency of the effector functions.

In order for mAb-opsonized nucleated cells to be killed directly by complement, it is necessary that the cell-associated immune complexed mAbs activate complement sufficiently well that large numbers of active C3b molecules are deposited on the cells, thus setting the stage for downstream assembly and attack on the cells by multiple copies of the membrane attack complex (MAC) of complement [11,35–38]. It is also likely that the deposited C3 fragments will facilitate synergistic *in vivo* elimination of cells, based on their interaction with fixed tissue macrophages that express receptors for the Fc regions of mAbs as well as for C3 fragments [39–41]. However, several complement control mechanisms maintain homeostasis in the body and prevent excessive and damaging complement activation on normal tissue and cells, and these control mechanisms may limit the efficacy by which complement can eliminate mAb-targeted tumor cells [42–48].

For example, Factor I is a plasma protease that can, in the presence of co-factors such as Factor H or CR1, degrade active C3b to an inactive form, iC3b, thus interrupting and terminating the complement cascade [47,49,50]. On this basis, a rational approach to increase the efficacy of mAb-based complement dependent cytotoxicity (CDC)-mediated tumor therapy would be to block the action of Factor H or Factor I. We describe in detail how a neutralizing mAb specific for Factor I can increase CDC of both cell lines and primary CLL B cells reacted with either RTX or OFA.

2. Results and Discussion

2.1. Complement Activation in Two Steps

We made use of a two-step paradigm, that makes use of sera depleted of C5, in order to gain insight into the relationship between C3b deposition and inactivation, and CDC of mAb-opsonized cells [51,52]. Our working hypothesis was that these initial experiments would provide key and quantitative information with respect to the role of Factor I in down-regulating mAb-mediated CDC.

In step 1, OFA-opsonized Z138 cells are first reacted at 37 °C for varying periods of time in C5-depleted normal human serum (NHS), in order to activate complement and deposit (active) C3b fragments on the cells. During this step the cells are not lysed, because, due to the absence of C5, the membrane attack complex (MAC) of complement cannot be generated. Secondary reaction of the cells in step 2 with NHS-ethylenediaminetetraacetic acid (EDTA) (which provides the "missing" C5) should promote cell lysis, because the terminal portion of the complement cascade has no metal ion requirements, and can therefore be activated, thus allowing for formation of the MAC. In addition, under these conditions no more C3b can be deposited on the cells, because Ca2+ and Mg2+, required for upstream complement activation and C3b deposition, are chelated by the EDTA. Indeed, as illustrated in Figure 1A, OFA-opsonized Z138 cells that are *briefly* reacted with C5-depleted NHS in step 1 are indeed lysed on addition of NHS-EDTA in step 2; this result strongly suggests that sufficient amounts of active C3b are deposited during the brief step 1 incubation to reach the C3b deposition threshold needed to activate the terminal phase of the complement pathway in step 2. The key "zero time" control in step 1 provides additional validation for the paradigm, because the cells in this sample (no deposited C3b) are not lysed when they are reacted in NHS-EDTA. Moreover, prolonged incubation of mAb-opsonized cells in step 1 should lead to Factor I-mediated decay of activated C3b to iC3b and then to C3d [45,46,51–54], and the results illustrated in Figure 1A reveal that after a 30 min incubation in step 1, lysis of the cells in step 2 is reduced considerably, thus indicating that a substantial fraction of the deposited C3b fragments must have been degraded to inactive iC3b or C3d.

In order to obtain a deeper understanding of C3b deposition and processing, we probed the cells with mAbs 7C12 and 1H8, specific for C3b/iC3b, and C3b/iC3b/C3d, respectively [16,55,56]. The results (Figure 1B,C) show that C3b deposition peaks within 90 s; in addition, for incubation periods in step 1 of between 90 s and 30 min, binding of the two mAbs to the cells remains quite high, and so we can conclude that the principal decay fragment generated on the cells due to the *in vitro* action of Factor I must be iC3b, because the relative amount of binding of 7C12 and 1H8 remained approximately constant for incubations of 90 s to 30 min. In contrast, however, in most cases the principal C3 breakdown fragment found covalently bound to circulating CLL cells after infusion of RTX or OFA is C3d [16,56].

We also investigated whether there might be an *upper limit* to the amount of C3b that can be deposited on mAb-opsonized cells. OFA-opsonized Z138 cells, that had been reacted for varying times with C5-depleted NHS in step 1, were washed and then reacted again (step 1A) for a full 30 min with additional C5-depleted NHS. The results of this experiment ("Double incubation", open symbols, Figure 1B,C) reveal that the high level of C3 fragment binding observed after the first 90 s incubation in step 1 appears to represent the maximum amount that can be deposited, because reaction of this sample (or other "later time" samples from step 1) for an additional 30 min with fresh C5-depleted

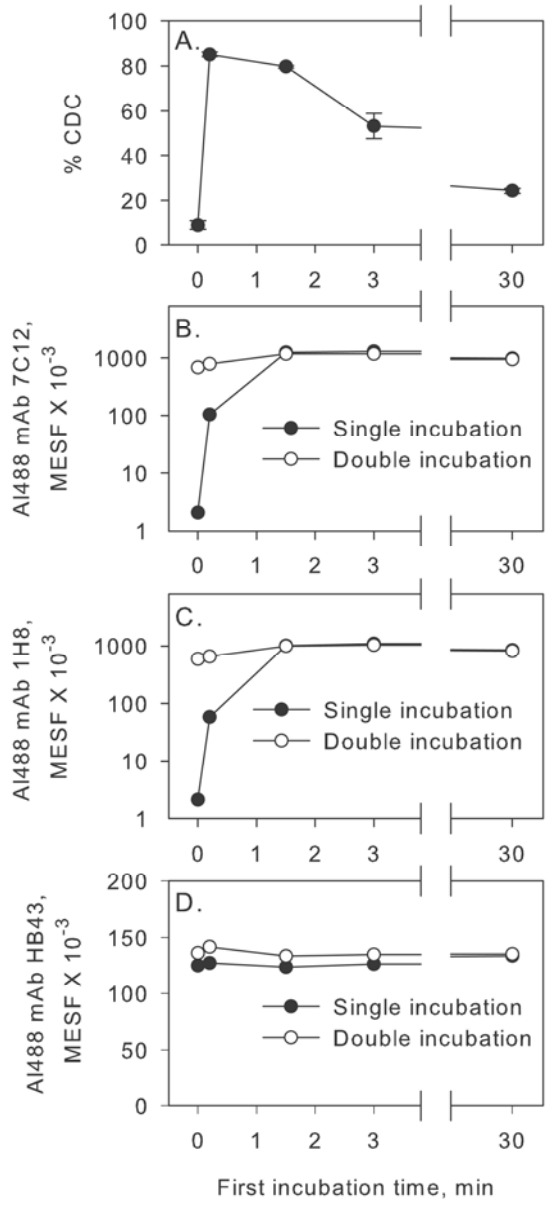
NHS does not increase C3 fragment deposition. An alternative explanation for the lack of additional C3 fragment deposition in Step 1A is that the bound OFA (opsonized on the cells at saturating conditions) might have dissociated from the cells, a condition which would preclude additional complement activation. We examined this question by probing the cells with mAb HB43, specific for the Fc region of human IgG [56]. The results (Figure 1D) clearly demonstrate that over the time periods of these experiments (cumulatively no more than 60 min at 37 °C), binding of OFA to the cells in C5-depleted NHS is quite stable, in agreement with earlier reports [57,58].

To test for generality, we examined three different cells types in the two-step assay: DB, Raji, and Z138 cells. In these experiments, after the first incubation in C5-depleted NHS, the cells were then reacted in either NHS (a source of intact complement), or in NHS-EDTA (only terminal pathway can be activated, as in Figure 1). The patterns of CDC for DB cells and Raji cells reacted in NHS-EDTA in step 2 (Figure 2 B,C) closely follow the trends observed for Z138 cells (Figures 1A and 2A). However, a different and common pattern is evident for all three cell types reacted in NHS in step 2 (open symbols, Figure 2). First, as expected, the "zero time" samples are now lysed in step 2, because NHS is able to promote CDC of the complement-naïve mAb-opsonized cells. Moreover, CDC of the cells reacted in step 1 is better preserved for longer incubation periods, presumably because the complete complement activation pathway (NHS, not NHS-EDTA) can better "outrun" complement inhibition by all soluble inhibitors. However, it is very interesting to note that even when NHS is added to the cells in step 2, CDC of cells reacted for 30–60 min with C5-depleted NHS in step 1 is still reduced. This is most likely because by these times the majority of deposited C3b fragments are degraded to the iC3b stage, and in addition, there are apparently few additional available sites on the OFA-opsonized cells to allow for deposition of additional fresh C3b. The decrease in CDC at longer times could also be due to the decay-acceleration activities of both C4b binding protein and factor H, as recently demonstrated by Okroj et al, in a similar model [51]. As noted above (Figure 1B,C), the maximum amount of C3b had already been deposited and, presumably due to the action of Factor I, the active C3b had been largely converted to iC3b after the 30 min reaction in step 1.

2.2. Inhibition of Factor I Increases CDC in the Two-Step Assay

We next used the two-step assay to test directly for the effects of Factor I by attempting to suppress its activity with neutralizing mAb A247 (Quidel) specific for Factor I (Figure 3). OFA-opsonized Z138 cells were reacted with 50% C5-depleted NHS in the presence or absence of the anti-Factor I mAb, and then either one volume of 100% NHS-EDTA or ten volumes of 50% NHS-EDTA were added to the cells. The larger volume of 50% NHS-EDTA was added to provide a secondary control to insure that: 1, all complement components were in excess; and 2, the excess NHS-EDTA would provide enough additional Factor I to overwhelm the anti-Factor I mAb used in the first incubation. The results of these experiments clearly demonstrate that for a 30 s or 3 min incubation period in step 1, the anti-Factor I mAb can indeed block the action of Factor I, because CDC is enhanced and preserved on addition of NHS-EDTA (Figure 3). However, inhibition of the action of Factor I by mAb A247 is not absolute, because CDC does decrease for a 20 min incubation time, even in the presence of the anti-Factor I mAb.

Figure 1. Kinetics of cell-killing reflect the rapid decay of C3b to iC3b. (**A**) Z138 cells opsonized with 10 μg/mL ofatumumab (OFA) were first incubated in 50% C5-depleted NHS for varying times as indicated. The second incubation in 50% NHS supplemented with 10 mM EDTA (final concentration) for 30 min allows for membrane attack complex (MAC) formation on cells with sufficient levels of C3b, but does not permit additional C3b deposition. Thus, the reduction in the % complement dependent cytotoxicity (CDC) is due to the decay of cell-associated C3b to non-functional iC3b. (**B**,**C**) The initial treatment with C5-depleted serum results in rapid covalent deposition of C3b/iC3b on almost all available sites. A second incubation with C5-depleted NHS does not increase the level of C3b/iC3b as demonstrated by interrogation with two mAbs specific for C3b/iC3b (mAb 7C12) and C3b/iC3b/C3d (mAb 1H8). (**D**) The amount of cell-bound opsonizing mAb (OFA), as detected by mAb HB43, remains constant during these incubations.



2.3. Inhibition of Factor I Increases CDC in Intact NHS

The results of the experiments using the two-step paradigm provide proof of principle that the action of Factor I can be suppressed in C5-depleted NHS, thus ultimately enhancing and better preserving CDC in this model. We made use of a single incubation paradigm to confirm the neutralizing capacity of anti-Factor I mAb A247 (Quidel) by comparing its action to that of a non-neutralizing, but IgG1 isotype-matched anti-Factor I mAb OX21 (Cedarlane). Under comparable conditions the Quidel mAb A247 (Figure 4A,B) enhanced CDC of cells that had been opsonized with RTX, but CDC was not increased (above the value for mAb-opsonized cells) in the presence of Cedarlane mAb OX21. It is also clear that addition of the anti-Factor I mAb A247 does not increase CDC to ≥90%, suggesting that other factors, including cell-associated complement control proteins [42–44], may limit OFA-mediated CDC for these cells.

Figure 2. Extended incubation with C5-depleted serum reduces the ability of intact NHS to kill cells: Comparison of NHS/EDTA with NHS in the second incubation. Cells (**A**. Z138, **B**. Raji or **C**. DB) opsonized with 10 μg/mL OFA were first incubated with 50% C5-depleted serum as in Figure 1A. The second incubation was carried out for 30 min in either 50% NHS supplemented with 10 mM EDTA (filled circles) or in 50% NHS (open triangles). At longer first incubation times, there was less killing of cells, even if intact NHS was used in the second incubation step.

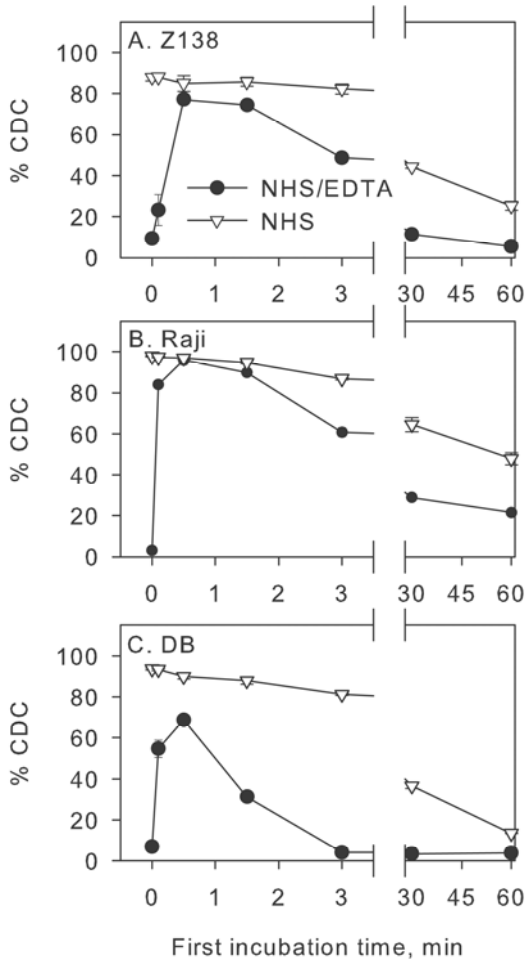


Figure 3. mAb A247 prolongs the lifetime of functional C3b. Z138 cells opsonized with 10 μg/mL OFA were subjected to the two-step treatment as in Figure 1, in the presence (open circles) or absence (filled circles) of mAb A247 specific for Factor I. The second step incubation was begun either by addition of an equal volume of 100% NHS supplemented with 20 mM EDTA (filled and open circles) or by addition of ten volumes of 50% NHS supplemented with 10 mM EDTA (open triangles).

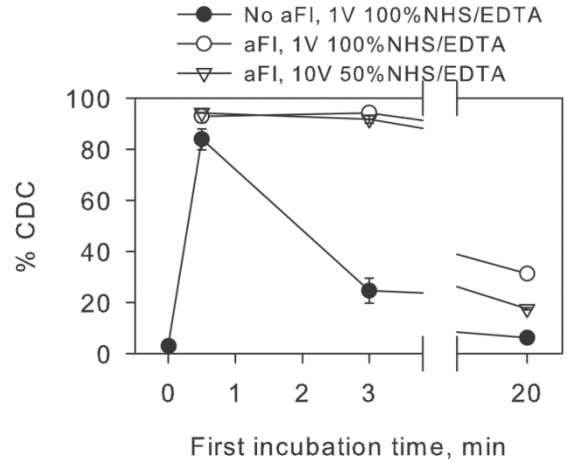
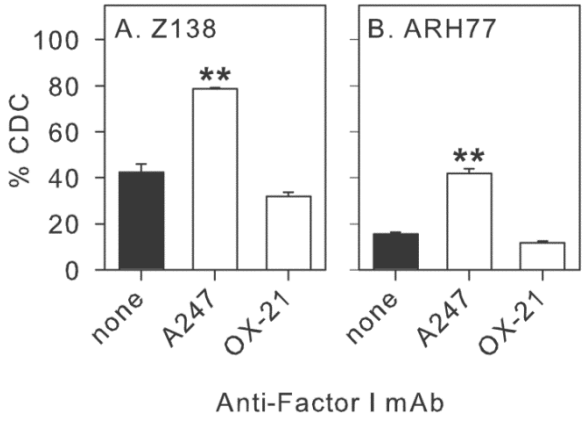


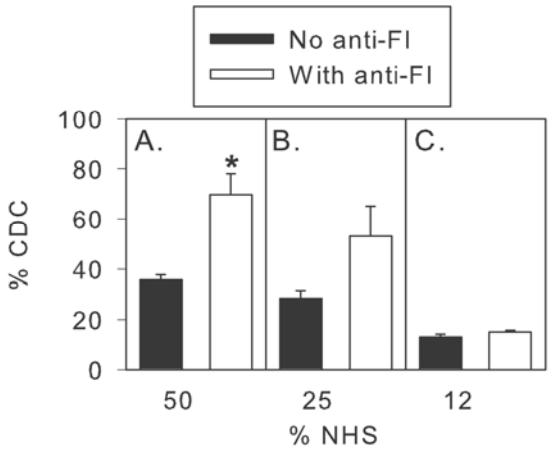
Figure 4. (**A**,**B**) *Not all mAbs* specific for Factor I increase CDC. Z138 cells (**A**) or ARH77 cells (**B**) were opsonized with 10 μg/mL rituximab (RTX), washed and then incubated in the presence or absence of 45 ug/mL mAb A247 or mAb OX-21 and 45% NHS for 15 min at 37 °C.



The experiments described thus far were all based on analyzing cells that were opsonized with mAbs *prior* to the addition of NHS and the anti-Factor I mAb, and they provide reasonable proof of principle that neutralization of factor I can increase CDC in this model. However, in any clinical scenario, the mAbs would of course be infused intravenously and then reach cells that are bathed by plasma in whole blood; there can be no opsonization step in media. Therefore, we next examined the action of the anti-Factor I mAb A247 under conditions in which Z138 cells were *first dispersed* in varying amounts of NHS, and were then combined with both RTX and the anti-Factor I mAb (Figure 5); that is, the cells were not first opsonized with RTX, but were reacted with both the CD20 mAb and anti-Factor I mAb A247 in the presence of NHS. Under these conditions mAb A247 also enhanced

RTX-mediated CDC of cells reacted in NHS, and the enhanced CDC is evident at final NHS levels of 50% and 25%, but little CDC is obtained in 12.5% NHS. This observation also reinforces the idea that the effector mechanisms at even modest cell burdens (5×10^6 cells/mL) may not promote robust cell killing if they have been even partially exhausted due for example, to reduction in complement titers [16,56].

Figure 5. mAb A247, specific for Factor I, increases CDC when added in combination with the opsonizing mAb. (**A**) CDC of Z138 cells suspended in 50% NHS was initiated by the addition of either 8 μg/mL RTX, or a combination of 8 μg/mL RTX and 42 μg/mL mAb A247. These conditions are designed to simulate the physiological situation. (**B**) mAb A247 increases CDC in 25% NHS. (**C**) In 12% NHS, the complement titer was too low to promote CDC. All incubations at 37 °C, 15 min. CDC of cells incubated in 50% NHS with anti-Factor I alone was 3%.



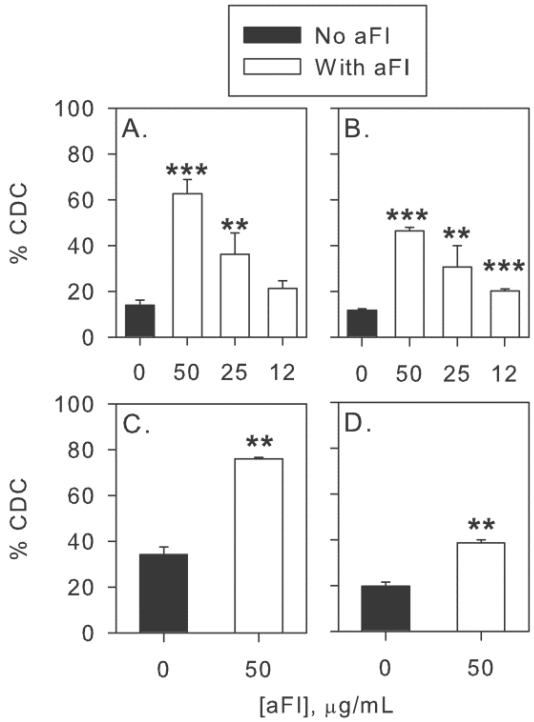
2.4. Inhibition of Factor I Increases CDC of CLL Cells in Intact NHS

We next tested mAb A247 for its ability to enhance RTX- and OFA-mediated CDC of primary malignant B cells in PBMC preparations from 6 different patients with chronic lymphocytic leukemia (CLL). In all cases, except for the washed whole blood protocol (see below), the experiments were conducted following the conditions described in Figure 5. That is, the PBMC were dispersed in 50% NHS, and then reacted simultaneously with RTX or with OFA, plus or minus anti-Factor I. We found that RTX-mediated CDC of B cells from CLL patients A and B is increased considerably in the presence of anti-Factor I mAb A247 (Figure 6A,B). The dose-response experiments indicate that reasonably high concentrations of the mAb (enough to presumably inhibit all active Factor I) are required, because CDC is reduced at lower concentrations of the mAb. mAb A247 was also able to increase OFA-mediated CDC of cells from patients A and B (Figure 6C,D). When CLL cells from patients A and B were reacted in 50% NHS, in the presence and absence of anti-Factor I mAb A247, CDC was less than 3% (not shown).

CLL cells from patient C were first examined in dose-response experiments for CDC mediated by RTX or OFA (Figure 7A). On this basis we chose to test the effects of anti-Factor I mAb A247 at final concentrations of RTX and OFA of 90 μg/mL and 10 μg/mL, respectively. We find that CDC mediated by both CD20 mAbs is enhanced in the presence of the anti-Factor I mAb and as

demonstrated in Figure 7B, the dose-response experiment again demonstrates that higher concentrations of mAb A247 are more effective at enhancing CDC mediated by both RTX and by OFA. We also note (Figure 7C, left panel) that *in the absence of RTX or OFA*, the anti-Factor I mAb alone does not promote any CLL cell killing.

Figure 6. CDC, mediated by RTX or by OFA, of primary CLL cells is increased in the presence of mAb A247, specific for Factor I. (**A**,**B**) PBMC from Patient A (**A**) and Patient B (**B**) were subjected to CDC as in Figure 5, except the final concentration of RTX was 50 μg/mL and the concentration of mAb A247 was varied as indicated. (**C**,**D**) As in A,B, except OFA was present at 1.25 μg/mL. All incubations were for 15 min at 37 °C in 42% NHS.



The CLL cells of patient D were less sensitive to mAb-mediated CDC, but we again found that mAb A247 was able to increase CDC mediated by both RTX and OFA (Figure 7D). We note that for patient E a very high concentration of OFA (95 μg/mL) was used, and although the anti-Factor I mAb increases cell killing, CDC peaked at only 60% (Figure 8A). We have made similar observations that there can be a limit to CDC, in the presence of high concentrations of OFA plus or minus anti-Factor I, for CLL cells from other patients (not shown). We also examined the B cells of patient E for C3b deposition by probing the cells with mAb 7C12, after reaction with RTX or OFA, in the presence or absence of anti-Factor I mAb A247. The results of these experiments (Figure 8B) indeed demonstrate that C3b deposition mediated by either RTX or OFA can be enhanced considerably by blocking the action of Factor I. Finally, we also compared the action of the anti-Factor I mAb in NHS (isolated PBMC), with its efficacy in whole washed blood for the CLL B cells of patient F, the later paradigm providing a more representative model for testing the action of the anti-Factor I mAb. We find an approximately comparable increase in CDC is mediated by the anti-Factor I mAb A247 for cells

reacted with OFA in either NHS or in washed whole blood (Figure 9), thus providing additional evidence that mAb A247 should enhance CD20 mAb-mediated CDC in the bloodstream.

Figure 7. CDC mediated by RTX or OFA of primary CLL cells is increased in the presence of mAb A247. (**A**) PBMC from Patient C were subjected to CDC in the presence of varying concentrations of OFA or RTX. (**B**) PBMC from Patient C were subjected to CDC in the presence of 10 μg/mL OFA or 90 μg/mL RTX, and varying concentrations of mAb A247 as indicated. (**C**) This control experiment demonstrates only background level of CDC for mAb A247 incubated with CLL cells (open bar) in the absence of opsonizing mAb (None) and also independently replicates the result observed in panel B for 42 μg/mL mAb A247. All incubations were for 15 min at 37 °C in 42% NHS. D. PBMC from CLL Patient D were tested for CDC in the presence of 7 μg/mL OFA or 83 μg/mL RTX in 42% NHS, in the presence or absence of 42 μg/mL mAb A247.

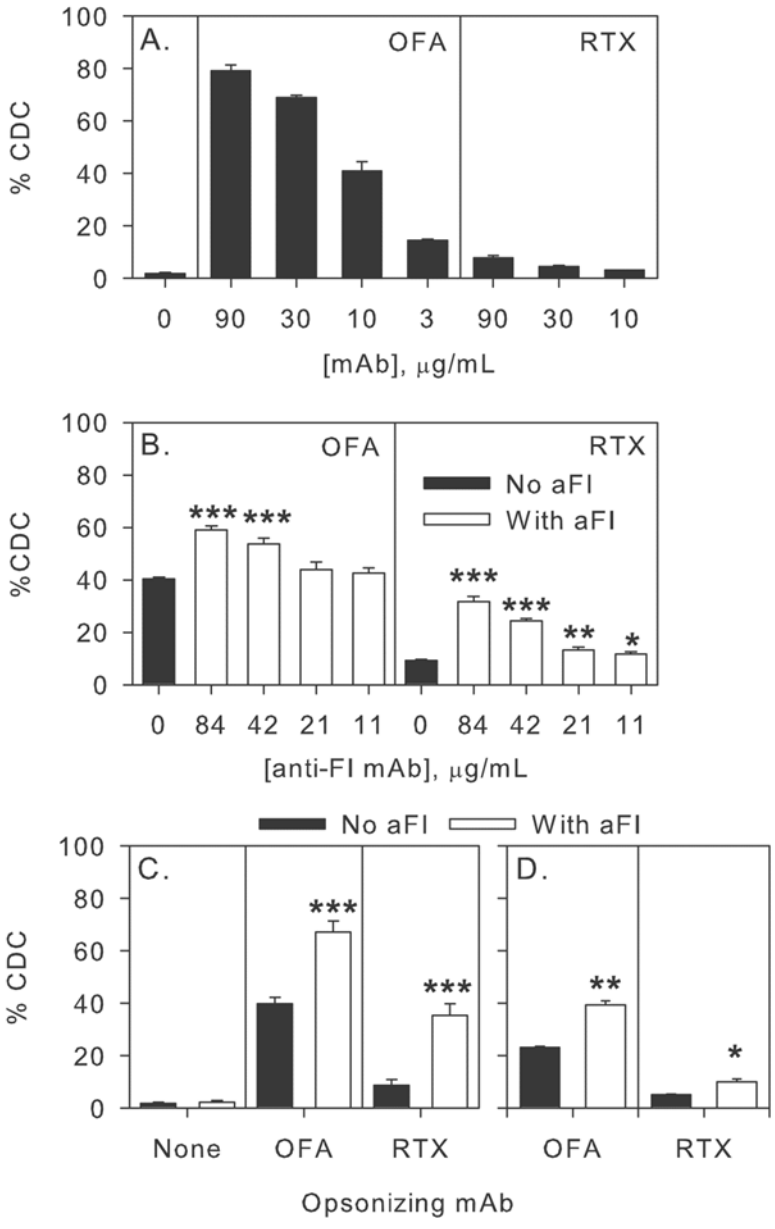


Figure 8. CDC and C3b deposition mediated by RTX or OFA of primary CLL cells are increased in the presence of mAb A247. PBMC isolated from CLL Patient E were incubated in the presence of mAb A247, 95 ug/mL OFA or RTX, and 48% NHS for 15 min at 37 °C. At the end of the incubation the samples were washed twice with ice cold BSA/PBS, resuspended in 2 mg/mL msIgG and probed with FITC mAb 7C12 at 10 µg/mL for 15 min at RT, washed once with BSA/PBS and finally resuspended in ToPro3.

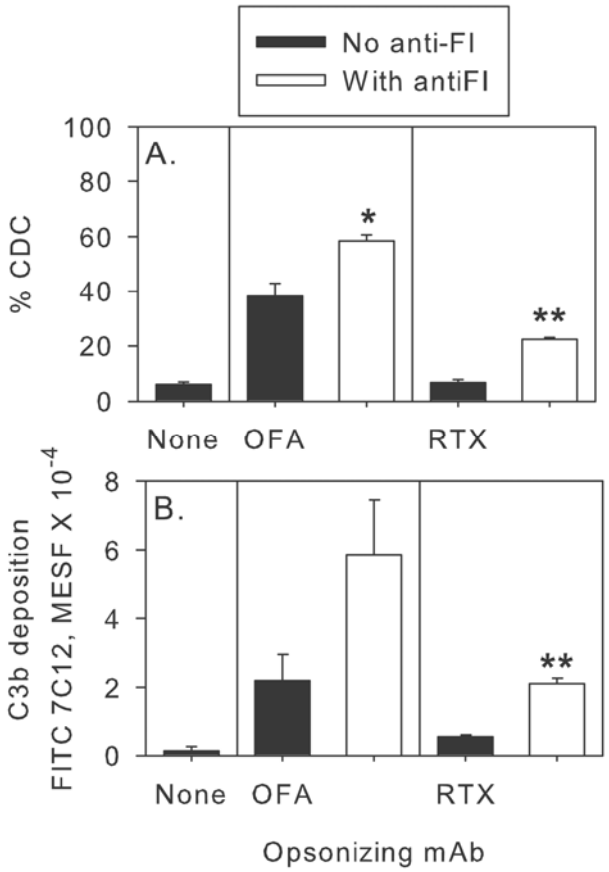
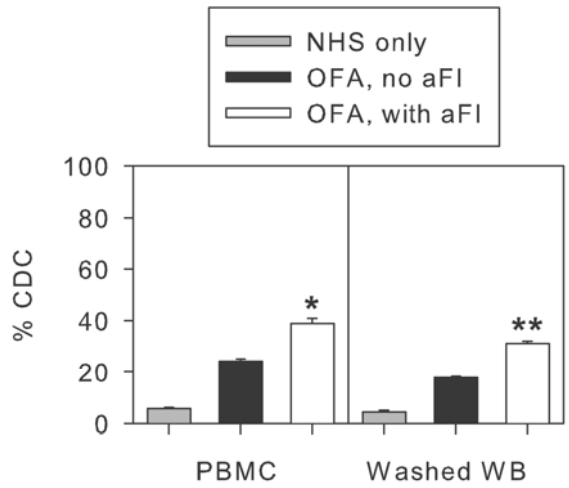


Figure 9. CDC mediated by OFA is increased in washed whole blood from a patient with CLL. CLL cells in 42% NHS or in washed whole blood were incubated with 7 µg/mL OFA in the presence or absence of 42 µg/mL mAb A247.



2.5. Modulation of Effector Functions to Increase mAb-mediated Killing of Tumor Cells

The successful use of mAbs in the immunotherapy of cancer is now very well-documented, but increasing evidence indicates that many of these unconjugated mAbs absolutely require the body's immune effector systems, including complement, to promote inflammation and cell killing [11]. Therefore, one of the limiting factors in such immunotherapies will be the body's natural anti-inflammatory and homestatic mechanisms that limit low-level antibody-mediated inflammatory reactions that could otherwise damage normal cells and tissues [17,31,43]. Indeed, the targeting mAbs must bind at sufficiently high levels so that they can overwhelm these control mechanisms, thus allowing initiation of cytotoxic programs. Even under these conditions, cell killing may not be quantitative and complete due to several factors. First, at high tumor burdens the cell killing mechanisms can be saturated/exhausted [15,16,59,60]. Indeed, we now provide evidence that C3b deposition on a mAb-targeted cell can reach a saturating level, and that if all of this C3b is decayed to iC3b, then it will not be possible to kill this cell with complement, even if the mAb is still bound to the cells (Figures 1–3). Alternatively, both soluble factors as well as the complement control proteins expressed on the tumor cells, in common with normal cells, may limit mAb-mediated killing [42,43,53,54]. Therefore, focused strategies that can mitigate or limit exhaustion, or that can block the action of these factors have the potential to increase substantially killing of tumors by mAbs already approved by the FDA [20,22,30,33,53,54,61].

With respect to complement, there is now good evidence supporting these concepts based on studies with the CD20 mAbs RTX and OFA The principal difference in activity between these mAbs appears to be due to the fact that OFA targets a site on CD20 very close to the cell membrane, thus allowing for considerably more effective CDC and C3b deposition on a wide range of B cell substrates [13,62]. However, as noted above, prototype *in vitro* experiments have in fact demonstrated that inhibition of cell-associated complement control proteins CD55 and CD59, as well as inhibition of the action of soluble Factor H can all enhance CDC mediated by both of these mAbs [20,33,53,54,61], thus indicating there is indeed room for improvement. Moreover, levels of CDC higher than 90% are difficult to achieve with RTX or with OFA, even with cell lines, and this is particular evident when a clinically important target, *i.e.*, primary CLL cells are examined [16,63].

In this report we have evaluated the role of Factor I as a potential inhibitor of RTX or OFA-mediated CDC. Our kinetic analyses in C5-depleted NHS strongly suggest that the C3b that is deposited on RTX- or OFA-opsonized cells is rapidly degraded to an inactive form, iC3b, thus limiting cell lysis when the cells are secondarily developed with NHS-EDTA (Figures 1 and 2). This finding is in good agreement with and complements (no pun intended!) the observations recently reported by Stoiber's group, who found that Factor H (the co-factor for Factor I) can promote this degradation process, and that by blocking the action of Factor H on cells, CDC of CLL cells mediated by RTX or OFA can be enhanced [53,54]. We confirmed that factor I (presumably in concert with co-factors which include Factor H) must play a key role in the degradation of active C3b to inactive iC3b, because inhibition of Factor I, with a specific neutralizing mAb preserved effective CDC for longer time periods for cells reacted in C5-depleted serum in the two-step paradigm (Figure 3). On this basis we extended the investigations and demonstrated that RTX- and OFA-mediated CDC of both cell lines as well as primary CLL B cells could be enhanced in NHS by blocking the action of factor I

(Figures 4–9). The increases in CDC, mediated by the anti-Factor I mAb for cell lines (Figure 5) and for CLL cells (Figures 6–9) were somewhat variable. However, in most cases CDC of CLL cells reacted with either RTX or with OFA were increased approximately two-fold or more by addition of the anti-Factor I mAb, which corresponded to absolute increases in CDC of approximately 20 to 50%. These results are in good agreement with the findings of Stoiber et al who reported averaged results for increases in RTX- or OFA-mediated CDC of CLL B cells based on blocking the action of Factor H [53,54]. In terms of potential translation to the clinic, targeting of Factor I for inhibition may be preferable, due its lower concentration in the bloodstream [47].

2.6. Future Directions

Although use of the anti-Factor I mAb A247 produced impressive and significant increases in cell killing, CDC rarely reached 90%. In addition, our kinetic studies indicated that neutralization of Factor I by this mAb was not absolute, because CDC in the two-step paradigm still decreased over longer step 1 incubation periods, suggesting that perhaps use of a higher affinity neutralizing mAb might be more effective. Alternatively, as proposed by Stoiber for blocking factor H [54], generation of bispecifc mAbs, with specificity for CD20 and Factor I, could afford a far more effective targeting paradigm. That is, aggregates of neutralizing function-blocking anti-Factor I would be placed directly on the opsonized cells, thus maximizing their potential to completely eliminate the inhibitory action of Factor I at the site of complement activation. In other words, the neutralizing anti-Factor I activity would be concentrated and focused at the places on the targeted cells where it would be most effective at maintaining and prolonging complement activation and CDC. This approach, combined with appropriate chemotherapy [16,64] and/or additional agents that block other complement control proteins [33], may provide direction for future and far more effective chemo-immunotherapies.

3. Experimental Section

3.1. Cell Lines and Primary Cells

ARH77, DB, Raji and Z138 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate and 2 mM glutamine as previously described [65,66]. CLL cells were obtained from blood samples of de-identified patients in accordance with protocols of the UVA Institutional Review Board. PBMC were isolated by density-gradient centrifugation on Ficoll-Paque using standard methods [65]. Washed whole blood was reconstituted in 42% ABO-matched NHS for certain CDC assays [56].

3.2. Antibodies and Reagents

RTX was obtained from the UVA pharmacy. OFA was the kind gift of Dr. Clive Zent, Mayo Clinic. Monoclonal antibodies specific for human Factor I (both mouse IgG1) were obtained from Quidel (mAb A247, neutralizing), and Cedarlane (mAb OX-21, not neutralizing). Antibodies specific for C3 fragments (mAb 7C12, 1H8) have been described previously [55,67]. mAb HB43 specific for the Fc portion of human IgG (ATCC) has been described previously [56]. mAbs were labeled with FITC (Sigma) with standard procedures or with Alexa 488 (Invitrogen) according to the manufacturer's

directions. NHS consisted of pooled sera obtained from at least four healthy donors. C5-depleted serum was obtained from Complement Technology and supplemented with 2 mM Mg+2 and 2 mM Ca+2 before use. TO-PRO-3 was obtained from Invitrogen.

3.3. mAb Opsonization and Complement Activation

Cells (typically 1–5 × 10^7 cells/mL) were incubated with saturating amounts (10 μg/mL) OFA or RTX in RPMI 1640 media/10% FBS (complete media) for 15 min at 37 °C, washed three times with BSA/PBS and resuspended in complete media.

For two-step procedures, mAb-opsonized cells were diluted with an equal volume of C5-depleted serum, with or without mAb specific for Factor I, for varying lengths of time at 37 °C. The samples were not washed, and the second incubation was initiated by addition of an equal volume of NHS/20 mM EDTA or NHS and carried out at 37 °C for 5–60 min (see Figure Legends). The reaction was quenched by 50-100-fold dilution with ice cold BSA/PBS. Pelleted cells were resuspended in 0.2 μM TOPRO-3 in PBS. TOPRO-3 positive cells were determined by flow cytometry on a Becton Dickinson FACSCalibur. In some experiments counting beads (Spherotech) were also added to the resuspended cells, to provide an alternative measure of CDC [5], and the determinations were in excellent agreement (not shown).

For one-step procedures, cells were suspended in 50% NHS and complement activation initiated by addition of opsonizing mAb with or without mAb specific for Factor I. In some experiments, C3b deposition was measured on twice washed samples by probing with FITC- or Alexa 488-labeled mAbs specific for C3b/iC3b/C3d. To allow for more exact quantitation, mean fluorescent signal intensities were converted to molecules of equivalent soluble fluorochrome (MESF) [55]. In some experiments (as noted), due to technical requirements, minor modifications of the assays were performed and CDC was measured in 42% NHS instead of in 50% NHS.

3.4. Statistical Analyses

Experiments were conducted independently two or more times and representative results are presented. Means and SD (n = 2 to 4 in all cases) are displayed and unpaired two-tailed t tests (Excel) were used. * $p < 0.05$; ** $p < 0.01$;*** $p < 0.001$.

4. Conclusions

We have demonstrated that targeted inhibition of Factor I has the potential to enhance mAb-mediated CDC of cancer cells. Translation of these findings to the clinic may best be accomplished by generation of neutralizing human mAbs specific for Factor I, which are then formulated as bispecific complexes with cancer cell-specific complement-fixing mAbs.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Glennie, M.J.; French, R.; Cragg, M.S.; Taylor, R.P. Mechanisms of killing by anti-CD20 monoclonal antibodies. *Mol. Immunol.* **2007**, *44*, 3823–3837.
2. Weiner, G.J. Rituximab: Mechanism of action. *Sem. Hematol.* **2010**, *47*, 115–123.
3. Lim, S.H.; Beers, S.A.; French, R.R.; Johnson, P.W.M.; Glennie, M.J.; Cragg, M.S. Anti-CD20 monoclonal antibodies--historical and future perspectives. *Haematologica* **2010**, *95*, 135–143.
4. Boross, P.; Leusen, J.H.W. Mechanisms of action of CD20 antibodies. *Am. J. Cancer Res.* **2012**, *2*, 676–690.
5. Golay, J.; Introna, M. Mechanism of action of therapeutic monoclonal antibodies: Promises and pitfalls of *in vitro* and *invivo* assays. *Arch. Biochem. Biophys.* **2012**, *526*, 146–153.
6. Scott, A.M.; Wolchok, J.D.; Old, L.J. Antibody therapy of cancer. *Nature* **2012**, *12*, 278–287.
7. Goswami, S.; Wang, W.; Arakawa, T.; Ohtake, S. Developments and challenges for mAb-based therapeutics. *Antibodies* **2013**, *2*, 452–500.
8. Bologna, L.; Gotti, E.; da Roit, F.; Intermesoli, T.; Rambaldi, A.; Introna, M.; Golay, J. Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy. *J. Immunol.* **2013**, *190*, 231–239.
9. Simpson, T.R.; Li, F.; Montalvo-Ortiz, W.; Sepulveda, M.A.; Bergerhoff, K.; Arce, F.; Roddie, C.; Henry, J.Y.; Yagita, H.; Wolchok, J.D.; *et al.* Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J. Exp. Med.* **2013**, *210*, 1695–1710.
10. Bulliard, Y.; Jolicoeur, R.; Windman, M.; Rue, S.M.; Ettenberg, S.; Knee, D.A.; Wilson, N.S.; Dranoff, G.; Brogdon, J.L. Activating Fc g receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *J. Exp. Med.* **2013**, *210*, 1685–1693.
11. Taylor, R.P.; Lindorfer, M.A. The role of complement in mAb-based therapies of cancer. *Methods* **2013**, doi: 10.1016/j.ymeth.2013.07.027.
12. Okroj, M.; Osterborg, A.; Blom, A.M. Effector mechanisms of anti-CD20 monoclonal antibodies in B cell malignancies. *Cancer Treat. Rev.* **2013**, *39*, 632–639.
13. Pawluczkowycz, A.W.; Beurskens, F.J.; Beum, P.V.; Lindorfer, M.A.; van de Winkel, J.G.J.; Parren, P.W.H.I.; Taylor, R.P. Binding of submaximal C1q promotes complement-dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or rituximab (RTX): Considerably higher levels of CDC are induced by OFA than by RTX. *J. Immunol.* **2009**, *183*, 749–758.
14. Gong, Q.; Ou, Q.; Ye, S.; Lee, W.P.; Cornelius, J.; Diehl, L.; Lin, W.Y.; Hu, Z.; Lu, Y.; Chen, Y.; *et al.* Importance of cellular microenvironment and circulatory dynamics in B cell immunotherapy. *J. Immunol.* **2005**, *174*, 817–826.
15. Boross, P.; Jansen, J.H.M.; de Haij, S.; Beurskens, F.J.; van der Poel, C.E.; Bevaart, L.; Nederend, M.; Golay, J.; van de Winkel, J.G.J.; Parren, P.W.H.I.; *et al.* The *in vivo* mechanism of action of CD20 monoclonal antibodies depends on local tumor burden. *Haematologica* **2011**, *96*, 1822–1830.
16. Beurskens, F.J.; Lindorfer, M.A.; Farooqui, M.; Beum, P.V.; Engelberts, P.; Mackus, W.J.M.; Parren, P.W.H.I.; Wiestner, A.; Taylor, R.P. Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J. Immunol.* **2012**, *188*, 3532–3541.

17. Clynes, R.A.; Towers, T.L.; Presta, L.G.; Ravetch, J.V. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat. Med.* **2000**, *6*, 443–446.
18. Di Gaetano, N.; Cittera, E.; Nota, R.; Vecchi, A.; Grieco, V.; Scanziani, E.; Botto, M.; Introna, M.; Golay, J. Complement activation determines the therapeutic activity of rituximab *in vivo*. *J. Immunol.* **2003**, *171*, 1581–1587.
19. Tedder, T.F.; Baras, A.; Xiu, Y. Fcg receptor-dependent effector mechanisms regulate CD19 and CD20 antibody immunotherapies for B lymphocyte malignancies and autoimmunity. *Springer Semin. Immun.* **2006**, *28*, 351–364.
20. Macor, P.; Tripodo, C.; Zorzet, S.; Piovan, E.; Bossi, F.; Marzari, R.; Amadori, A.; Tedesco, F. *In vivo* targeting of human neutralizing antibodies against CD55 and CD59 to lymphoma cells increases the antitumor activity of rituximab. *Cancer Res.* **2007**, *67*, 10556–10563.
21. Masuda, K.; Kubota, T.; Kaneko, E.; Iida, S.; Wakitani, M.; Kobayashi-Natsume, Y.; Kubota, A.; Shitara, K.; Nakamura, K. Enhanced binding affinity for FcgRIIIa of fucose-negative antibody is sufficient to induce maximal antibody-dependent cellular cytotoxicity. *Mol. Immunol.* **2007**, *44*, 3122–3131.
22. Imai, M.; Ohta, R.; Varela, J.C.; Song, H.; Tomlinson, S. Enhancement of antibody-dependent mechanisms of tumor cell lysis by a targeted activator of complement. *Cancer Res.* **2007**, *67*, 9535–9541.
23. Li, B.; Shi, S.; Qian, W.; Zhao, L.; Zhang, D.; Hou, S.; Zheng, L.; Dai, J.; Zhao, J.; Wang, H.; *et al.* Development of novel tetravalent anti-CD20 antibodies with potent antitumor activity. *Cancer Res.* **2008**, *68*, 2400–2408.
24. de Romeuf, C.; Dutertre, C.A.; Le Garff-Tavernier, M.; Fournier, N.; Gaucher, C.; Glacet, A.; Jorieux, S.; Bihoreau, N.; Behrens, C.K.; Beliard, R.; *et al.* Chronic lymphocytic leukaemia cells are efficiently killed by an anti-CD20 monoclonal antibody selected for improved engagement of FcgRIIIA/CD16. *Br. J. Haematol.* **2008**, *140*, 635–643.
25. Riaz, W.; Hernandez-Ilizaliturri, F.J.; Czuczman, M.S. Strategies to enhance rituximab anti-tumor activity in the treatment of CD20-positive B-cell neoplasms. *Immunol. Res.* **2009**, *46*, 192–205.
26. Li, B.; Zhao, L.; Guo, H.; Wang, C.; Zhang, X.; Wu, L.; Chen, L.T.Q.; Qian, W.; Wang, H.; Guo, Y. Characterization of a rituximab variant with potent antitumor activity against rituximab-resistant B-cell lymphoma. *Blood* **2009**, *114*, 5007–5015.
27. van Meerten, T.; Hagenbeek, A. Novel antibodies against follicular non-Hodgkin's lymphoma. *Best Practice Res. Clin. Haematol.* **2011**, *24*, 231–256.
28. Peipp, M.; van de Winkel, J.G.J.; Valerius, T. Molecular engineering to improve antibodies' anti-lymphoma activity. *Best Practice Res. Clin. Haematol.* **2011**, *24*, 217–229.
29. Lindorfer, M.A.; Wiestner, A.; Zent, C.S.; Taylor, R.P. Monoclonal antibody (mAb)-based cancer therapy: Is it time to reevaluate dosing strategies? *Oncoimmunology* **2012**, *1*, 959–961.
30. Elvington, M.; Huang, Y.; Morgan, B.P.; Ziao, F.; van Rooijen, N.; Atkinson, C.; Tomlinson, S. A targeted complement-dependent strategy to improve the outcome of mAb therapy, and characterization in a murine model of metastatic cancer. *Blood* **2012**, *119*, 6043–6051.
31. Nimmerjahn, F.; Ravetch, J.V. Translating basic mechanisms of IgG effector activity into next generation cancer therapies. *Cancer Immunity* **2012**, *12*, 13–20.

32. Zent, C.S.; Wu, W.; Bowen, D.A.; Hanson, C.A.; Pettinger, A.M.; Shanafelt, T.D.; Kay, N.E.; Leis, J.F.; Call, T.G. Addition of granulocyte macrophage colony stimulating factor does not improve response to early treatment of high-risk chronic lymphocytic leukemia with alemtuzumab and rituximab. *Leuk. Lymph.* **2013**, *54*, 476–482.
33. Mamidi, S.; Cinci, M.; Hasmann, M.; Fehring, V.; Kirschfink, M. Lipoplex mediated silencing of membrane regulators (CD46, CD55 and CD59) enhances complement-dependent anti-tumor activity of trastuzumab and pertuzumab. *Mol. Oncol.* **2013**, *7*, 580–594.
34. Weiskopf, K.; Ring, A.M.; Ho, C.C.M.; Volkmer, J.P.; Levin, A.M.; Volkmer, A.K.; Ozkan, E.; Fernhoff, N.B.; van de Rijn, M.; Weissman, I.L.; *et al.* Engineered SIRPa variants as immunotherapeutic adjuvants to anticancer antibodies. *Science* **2013**, *341*, 88–91.
35. Koski, C.; Ramm, L.; Hammer, C.; Mayer, M.; Shin, M. Cytolysis of nucleated cells by complement: Cell death displays multi-hit characteristics. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 3816–3820.
36. Carney, D.F.; Hammer, C.H.; Shin, M.L. Elimination of terminal complement complexes in the plasma membrane of nucleated cells: influence of extracellular Ca2+ and association with cellular Ca2+. *J. Immunol.* **1986**, *137*, 263–270.
37. Kim, S.; Carney, D.F.; Hammer, C.H.; Shin, M.L. Nucleated cell killing by complement: Effects of C5b-9 channel size and extracellular Ca-2+ on the lytic process. *J. Immunol.* **1987**, *138*, 1530–1536.
38. Reiter, Y.; Ciobotariu, A.; Jones, J.; Morgan, B.P.; Fishelson, Z. Complement membrane attack complex, perforin, and bacterial exotoxins induce in K562 cells calcium-dependent cross-protection from lysis. *J. Immunol.* **1995**, *155*, 2203–2210.
39. Helmy, K.Y.; Katschke, K.J.; Gorgani, N.N.; Kljavin, N.M.; Elliott, J.M.; Diehl, L.; Scales, S.J.; Ghilardi, N.; van Lookeren Campagne, M. CRIg: A macrophage complement receptor required for phagocytosis of circulating pathogens. *Cell* **2006**, *124*, 915–927.
40. He, J.Q.; Wiesmann, C.; van Lookeren Campagne, M. A role of macrophage complement receptor CR1g in immune clearance and inflammation. *Mol. Immunol.* **2008**, *45*, 4041–4047.
41. Lindorfer, M.A.; Kohl, J.; Taylor, R.P. Interactions between the complement system and Fcg receptors. In *Antibody Fc: Linking Adaptive and Innate Immunity*; Ackerman, M.E., Nimmerjahn, F., Eds.; Elsevier: Philadelphia, PA, USA, 2014.
42. Morgan, B.P.; Harris, C.L. *Complement Regulatory Proteins*; Academic Press: San Diego, CA, USA, 1999.
43. Fishelson, Z.; Donin, N.; Zell, S.; Schultz, S.; Kirschfink, M. Obstacles to cancer immunotherapy: Expression of membrane complement regulatory proteins (mCRPs) in tumors. *Mol. Immunol.* **2003**, *40*, 109–123.
44. Zipfel, P.F.; Skerka, C. Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* **2009**, *9*, 729–740.
45. Dunkelberger, J.R.; Song, W.C. Role and mechanism of action of complement in regulating T cell immunity. *Mol. Immunol.* **2010**, *47*, 2176–2186.
46. Ehrnthaller, C.; Ignatius, A.; Gebhard, F.; Huber-Lang, M. New insights of an old defense system: Structure, function and clinical relevance of the complement system. *Mol. Med.* **2011**, *17*, 317–329.

47. Nilsson, S.C.; Sim, R.B.; Lea, S.M.; Fremeaux-Bacchi, V.; Blom, A.M. Complement factor I in health and disease. *Mol. Immunol.* **2011**, *48*, 1611–1620.
48. Kolev, M.; Towner, L.; Donev, R. Complement in cancer and cancer immunotherapy. *Arch. Immunol. Ther. Exp.* **2011**, *59*, 407–419.
49. Nilsson, S.C.; Nita, I.; Mansson, L.; Groeneveld, T.W.L.; Trouw, L.A.; Villoutreix, B.O.; Blom, A.M. Analysis of binding sites on complement Factor I that are required for its activity. *J. Biol. Chem.* **2010**, *285*, 6235–6245.
50. Roversi, P.; Johnson, S.; Caesar, J.J.; McLean, F.; Leath, K.J.; Tsiftsoglou, S.A.; Morgan, B.P.; Harris, C.L.; Sim, R.B.; Lea, S.M. Structural basis for complement factor I control and its disease-associated sequence polymorphisms. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 12839–12844.
51. Beum, P.V.; Lindorfer, M.A.; Peek, E.M.; Stukenberg, P.T.; de Weers, M.; Beurskens, F.J.; Parren, P.W.H.I.; van de Winkel, J.G.J.; Taylor, R.P. Penetration of antibody-opsonized cells by the membrane attack complex of complement promotes Ca2+ influx and induces streamers. *Eur. J. Immunol.* **2011**, *41*, 2436–2446.
52. Okroj, M.; Holmquist, E.: King, B.C.; Blom, A.M. Functional analyses of complement convertases using C3 and C5-depleted sera. *PLoS One* **2012**, *7*, e47245
53. Horl, S.; Banki, Z.; Huber, G.; Ejaz, A.; Windisch, D.; Muellauer, B.; Willenbacher, E.; Steurer, M.; Stoiber, H. Reduction of complement factor H binding to CLL cells improves the induction of rituximab-mediated complement-dependent cytotoxicity. *Leukemia* **2013**, *27*, 2200–2208.
54. Horl, S.; Banki, Z.; Huber, G.; Ejaz, A.; Mullauer, B.; Willenbacher, E.; Steurer, M.; Stoiber, H. Complement factor H-derived short consensus repeat 18-20 enhanced complement-dependent cytotoxicity of Ofatumumab on chronic lymphocytic leukemia cells. *Haematologica* **2013**, doi:10.3324/haematol.2013.089615.
55. Kennedy, A.D.; Solga, M.D.; Schuman, T.A.; Chi, A.W.; Lindorfer, M.A.; Sutherland, W.M.; Foley, P.L.; Taylor, R.P. An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by Rituximab. *Blood* **2003**, *101*, 1071–1079.
56. Kennedy, A.D.; Beum, P.V.; Solga, M.D.; DiLillo, D.J.; Lindorfer, M.A.; Hess, C.E.; Densmore, J.J.; Williams, M.E.; Taylor, R.P. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J. Immunol.* **2004**, *172*, 3280–3288.
57. Teeling, J.L.; French, R.R.; Cragg, M.S.; van den Brakel, J.; Pluyter, M.; Huang, H.; Chan, C.; Parren, P.W.; Hack, C.E.; Dechant, M.; *et al.* Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin's lymphomas. *Blood* **2004**, *104*, 1793–1800.
58. Beum, P.V.; Peek, E.M.; Lindorfer, M.A.; Beurskens, F.J.; Engelberts, P.J.; Parren, W.H.I.; van de Winkel, J.G.J.; Taylor, R.P. Loss of CD20 and bound CD20 antibody from opsonized B cells occurs more rapidly because of trogocytosis mediated by Fc receptor-expressing effector cells than direct internalization by the B cells. *J. Immunol.* **2011**, *187*, 3438–3447.
59. Bhat, R.; Watzl, C. Serial killing of tumor cells by human natural killer cells—Enhancement by therapeutic antibodies. *PLoS One* **2007**, *2*, e326.

60. Berdeja, J.G.; Hess, A.; Lucas, D.M.; O'Donnell, P.; Ambinder, R.F.; Diehl, L.F.; Carter-Brookins, D.; Newton, S.; Flinn, I.W. Systemic interleukin-2 and adoptive transfer of lymphokine-activated killer cells improves antibody-dependent cellular cytotoxicity in patients with relapsed B-cell lymphoma treated with rituximab. *Clin. Cancer Res.* **2007**, *13*, 2392–2399.
61. Ge, X.; Wu, L.H.W.; Fernandes, S.; Wang, C.; Li, X.; Brown, J.R.; Zin, X. rILYd4, a human CD59 inhibitor, enhances complement-dependent cytotoxicity of ofatumumab against rituximab-resistant B-cell lymphoma cells and chronic lymphocytic leukemia. *Clin. Cancer Res.* **2011**, *17*, 6702–6711.
62. Teeling, J.L.; Mackus, W.J.M.; Wiegman, L.J.J.M.; van den Brakel, J.H.N.; Bees, S.A.; French, R.R.; van Meerten, T.; Ebeling, S.; Vink, T.; Slootstra, J.W.; *et al.* The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. *J. Immunol.* **2006**, *177*, 362–371.
63. Baig, N.A.; Taylor, R.P.; Lindorfer, M.A.; Church, A.K.; LaPlant, B.R.; Pavey, E.S.; Nowakowski, G.S.; Zent, C.S. Complement dependent cytotoxicity (CDC) in chronic lymphocytic leukemia (CLL): Ofatumumab enhances alemtuzumab CDC and reveals cells resistant to activated complement. *Leuk. Lymph.* **2012**, *53*, 2218–2227.
64. Wierda, W.; Kipps, T.; Durig, J.; Griskevicius, L.; Stilgenbauer, S.; Mayer, J.S.L.; Hess, G.; Griniute, R.; Hernandez-Ilizaliturri, F.J.; Padmanabhan, S.; *et al.* Chemoimmunotherapy with O-FC in previously untreated patients with chronic lymphocytic leukemia. *Blood* **2011**, *117*, 6450–6458.
65. Beum, P.V.; Kennedy, A.D.; Williams, M.E.; Lindorfer, M.A.; Taylor, R.P. The shaving reaction: Rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes. *J. Immunol.* **2006**, *176*, 2600–2609.
66. Estrov, Z.; Talpaz, M.; Ku, S.; Harris, D.; Van, Q.; Beran, M.; Hirsch-Ginsberg, C.; Huh, Y.; Yee, G.; Kurzrock, R. Z-138: A new mature B-cell acute lymphoblastic leukemia cell line from a patient with transformed chronic lymphocytic leukemia. *Leuk. Res.* **1998**, *22*, 341–353.
67. Lindorfer, M.A.; Jinivizian, H.B.; Foley, P.L.; Kennedy, A.D.; Solga, M.D.; Taylor, R.P. The B cell complement receptor 2 transfer reaction. *J. Immunol.* **2003**, *170*, 3671–3678.