Complement factor H-derived short consensus repeat 18-20 enhanced complement-dependent cytotoxicity of ofatumumab on chronic lymphocytic leukemia cells

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ABSTRACT

The antitumor activity of monoclonal antibodies in the treatment of chronic lymphocytic leukemia is mediated mainly by antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Unfortunately, the efficacy of complement-dependent cytotoxicity is strongly restricted due to the expression and acquisition of regulators of complement activation by lymphocytic leukemia cells. Whereas the role of membrane regulators of complement activation, such as CD55 and CD59, has been investigated in detail in chronic lymphocytic leukemia, the involvement of soluble regulators of complement activation, such as complement factor H, has not yet been reported. Propidium iodide staining was performed to investigate the efficacy of ofatumumab and factor H-derived short-consensus-repeat 18-20 in the induction of complement-dependent cytotoxicity on primary chronic lymphocytic leukemia cells from 20 patients. Deposition of complement C3 fragments was monitored by western blot analysis. Expression of CD20, CD55 or CD59 was determined by FACS analysis. Replacement of factor H with short consensus repeat 18-20 significantly increased the susceptibility of primary chronic lymphocytic leukemia cells to ofatumumab-induced complement-dependent cytotoxicity. More importantly, addition of short-consensus-repeat 18-20 was able to overcome complement- resistance occurring during treatment with ofatumumab alone. Use of short consensus repeat 18-20 is likely to prolong the turnover time of active C3b fragments generated on the target cells following ofatumumab-induced complement activation, thereby improving specific killing of chronic lymphocytic leukemia cells by complement-dependent cytotoxicity. The relative contribution of factor H to the protection of chronic lymphocytic leukemia cells against complement-dependent cytotoxicity was comparable to that of CD55. Our data suggest that, by abrogating factor H function, short consensus repeat 18-20 may provide a novel approach that improves the complement-dependent efficacy of therapeutic monoclonal antibodies.

Introduction

Monoclonal antibodies have considerably improved the treatment of chronic lymphocytic leukemia (CLL). To date, the best studied and most widely used therapeutic antibodies for CLL treatment are rituximab and alemtuzumab.[1] The current standard for first-line treatment of CLL is chemoimmunotherapy using rituximab in combination with purine analogs and/or alkylators; however, this therapeutic regimen may fail, in particular in patients bearing unfavorable genetic risk factors such as del(17p), del(11q) or *TP53* mutations.[2] The CD52 antibody alemtuzumab represents a treatment approach for patients with poor biological prognostic markers, but its use may be limited by its greater infusion-related, hematologic and immune toxicity.[1,2] Thus, considerable effort is being aimed at the development of new therapeutic monoclonal antibodies for first-line treatment and treatment of relapsed CLL. Ofatumumab is a fully humanized IgG_1 monoclonal antibody that binds to the CD20 antigen on the surface of B lymphocytes.[3] Phase I/II trials showed that ofatumumab as a single agent is well tolerated with an overall response rate of approximately 50% in patients with relapsed/refractory CLL, including those refractory to fludarabine and alemtuzumab.[4] In October 2009, ofatumumab was, therefore, approved by the Food and Drug Administration for the treatment of fludarabine and alemtuzumab double-refractory CLL.

The antitumor activity of ofatumumab is due to complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC).[3] The modes of action of ofatumumab were studied in depth and compared to those of rituximab.[3,5] When CLL cell lines or primary CLL cells in whole blood were treated with ofatumumab or rituximab, ofatumumab achieved notably higher lysis rates due to CDC induction.[3,5] Further studies demonstrated that ofatumumab dissociates from its target at a slower rate than does rituximab. Ofatumumab binds a segment of CD20 that is located closer to the N terminus of the molecule than is the epitope targeted by rituximab. Thus, this novel, membrane-proximal epitope together with the slow-off rate of ofatumumab[6,7] may account for the enhanced CDC potency of ofatumumab and an increased induction of macrophage-dependent phagocyto-

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sis.[3,5-8] These results demonstrate that ofatumumab has a great cytotoxic potential to kill B cells through ADCC and CDC and provides a promising therapeutic option for CLL treatment.

Although quite effective, the complement-mediated effector mechanisms induced by ofatumumab are restricted due to the expression and acquisition of regulators of complement activation (RCA) on target cells. Several membrane-bound and fluid-phase RCA have evolved to prevent potentially harmful effects of the complement system to host cells.[9] In particular, tumor cells often overexpress and bind RCA to protect themselves against complement-mediated effector mechanisms.[10] In the context of non-Hodgkin's lymphoma and CLL, the membrane-bound RCA (mRCA) CD55 and CD59 have been studied in depth and were identified as important players in protecting these malignant cells against CDC.[11-18] In addition to the mRCA mentioned above, fluid-phase RCA, especially factor H (fH) might potentially be involved in the resistance of CLL cells to antibody-induced CDC. This factor has already been demonstrated to be important in protecting different solid tumors (breast cancer, prostate cancer, lung cancer, etc.) and tumor cell lines (H2 glioblastoma cells) against CDC.[19-21] fH is a 155-kDa single polypeptide chain glycoprotein that is present in plasma at a concentration of 0.235-0.81 mg/mL.[22,23] Its mode of action is at the level of complement convertases, whereby fH accelerates the decay of alternative pathway (AP) C3 and C5 convertase. Additionally, fH serves as a cofactor for the factor I (fI)-mediated proteolytic cleavage of active C3b into inactive C3b.[24] fH is composed of 20 short consensus repeat (SCR) domains, each composed of approximately 60 amino acid residues with 3-8 amino acid spacers between the individual SCR.[25] Distinct functional regions have been identified within the full-length molecule. The complement regulatory activity is located within N terminal SCR1-4.[26] There is controversy regarding the C3b and glycosaminoglycan binding sites of fH,[25] although there is broad agreement that the C terminal portion of fH is crucial for the initial contact with its ligands and binding to cell surfaces.[26,27]

In this study, we generated human recombinant hSCR18-20 representing the C terminal binding domain of human fH. We demonstrate that blocking fH binding by means of recombinant hSCR18-20 significantly increases the susceptibility of primary CLL cells to ofatumumab-induced CDC. Moreover, our data provide evidence that among other RCA, fH is significantly involved in the protection of CLL cells against CDC.

Methods

Expression of human recombinant factor H-derived proteins

Specific DNA fragments encoding hSCR18-20 and hSCR16-17 were amplified from cDNA of full-length fH and expressed using the EasySelect™ Pichia Expression Kit.

Recombinant proteins were purified by affinity chromatography using a 6×*His* Ni-NTA column. Molecular weights and purity of the recombinant SCR fragments were verified by western blot analysis and functionality was assessed by performing heparin affinity chromatography (Figure 1). Detailed information is supplied in the *Online Supplementary Methods*.

Primary chronic lymphocytic leukemia cells

This study was approved by the Ethics Committee of Innsbruck Medical University. Heparinized peripheral blood from therapy-naive CLL patients was obtained, after receiving informed consent, at the Department of Hematology and Oncology, Innsbruck Medical University. In total, 20 patients were enrolled in this study. Following isolation of peripheral blood mononuclear cells (PBMC) by Ficoll gradient centrifugation, cells were cultured overnight before experiments were carried out. The average B-cell fraction of PBMC from all patients was 91% (range, 73% - 99%, *data not shown*). Further details are provided in the *Online Supplementary Methods*.

Immunofluorescence

To measure CD20, CD55 or CD59 expression, PBMC from patients were incubated with anti-CD20 monoclonal antibody, mouse anti-human CD55 or mouse anti-human CD59, respectively, and analyzed on a FACS Canto II cytometer (see *Online Supplementary Methods*).

Complement-dependent cytotoxicity assay

PBMC were incubated with various concentrations of ofatumumab blocking mouse anti-human CD55 (HD1A) or blocking monoclonal mouse anti-human CD59 antibody (MEM43), alone or in various combinations. The optimal concentration of hSCR18-20 (1200 mg/mL) was determined by titration of the SCR in lysis assays (*Online Supplementary Figure S1*). Normal human serum (NHS) (25%) as a source of active complement or heat-inactivated NHS (hiNHS) was added and samples were incubated for 1 h at 37°C. Prior to analysis dead cells were stained with propidium iodide (PI). Analysis was performed on a FACS Canto II cytometer measuring the PI-negative viable cell counts in the samples for 60 s at the same flow rate. Viable cell counts in control samples, i.e. patients' cells treated with hiNHS alone, were defined as 100% survival. The survival rates were calculated according to this control (percent of survival = 100% survival × count of viable cells in treated sample / count of viable cells in hiNHS control sample). In some assays cells were stained in addition for CD19 or CD3 (*Online Supplementary Methods*).

Western blot analysis to determine C3 fragments

To analyze how hSCR18-20 acts on the processing of C3b fragments, which is negatively regulated by fH, CDC assays were performed with CLL cells and C3 fragments were analyzed by western blotting (*Online Supplementary Methods*).

Statistical analysis

GraphPad Prism software was used for the statistic analyses. The unpaired *t* test was conducted to determine the difference between CD20, CD55 and CD59 expression levels. The effect of hSCR18-20 in comparison to ofatumumab alone was determined by *t* test for unpaired data. One-way ANOVA was performed to evaluate the single and synergistic impact of SCR18-20 and/or blocking antibodies.

Results

Recombinant hSCR18-20 improves the complement-dependent cytotoxicity of ofatumumab on primary chronic lymphocytic leukemia cells

We performed CDC assays with freshly isolated PBMC from 20 CLL patients. The purity of B-cell fractions was determined by flow cytometric analysis for all patients'

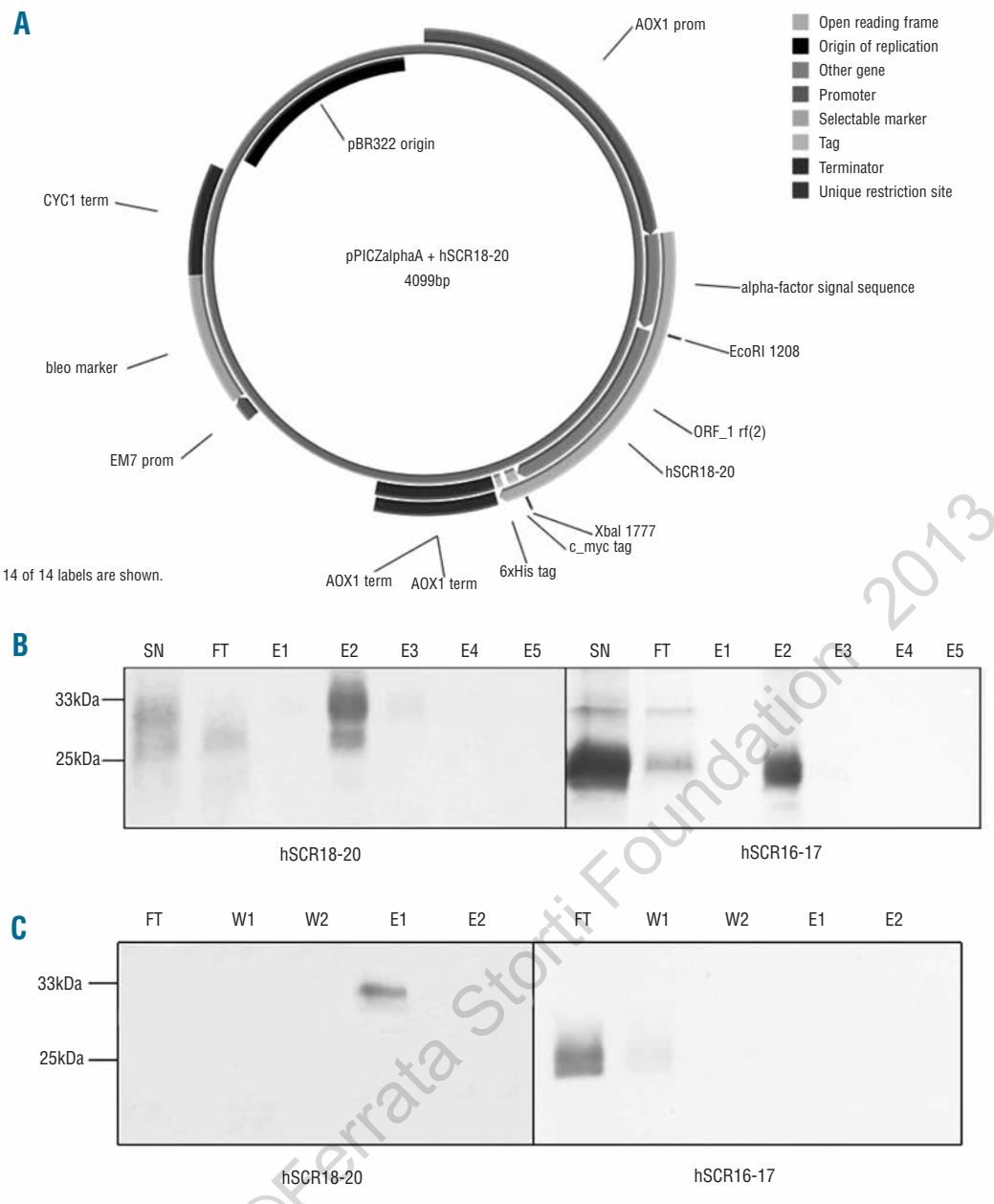

Figure 1. Production and heparin-binding activity of fH-derived proteins. (A) DNA fragments encoding hSCR18-20 and hSCR16-17 were cloned via EcoRI and XbaI restriction sites into pPICZα expression vector as indicated in the vector maps. (B) Culture supernatants were subjected to 6×*His* Ni-NTA affinity chromatography. Purified fractions were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis and western blot. The specific bands of hSCR18-20 and hSCR16-17 in the culture supernatants (SN) appear in elution fraction 2 (E2) indicating the enrichment of the recombinant proteins mediated by the *His*-tag. (C) Heparin affinity chromatography was performed with hSCR18-20 and control domain hSCR16-17. Western blot analysis of flow-through (FT), wash (W1, W2) and elution (E1, E2) fractions was carried out. The distinct band of hSCR18-20 in E1 shows the heparin-binding activity of this domain, whereas hSCR16-17 was detected mainly in the FT indicating that the control SCR did not bind to heparin.

samples and ranged from 73% to 99%, with a mean of 91% (*data not shown*). First, we assessed the CDC of CLL cells in CDC assays at increasing concentrations of ofatumumab ranging from 1 to 100 μg/mL. A pool of NHS served as a source of active complement. The surviving population was determined by flow cytometric analysis measuring the counts of PI-negative viable cells in the samples. We observed a dose-dependent response to ofatumumab (Figure 2A, open circles), resulting in complement-mediated lysis ranging from 12.4±3.9% at the lowest concentration of ofatumumab to 45.4±5.4% at the highest concentration. More importantly, addition of hSCR18-20 (1200 μg/mL) significantly enhanced ofatumumab-induced CDC (Figure 2A, closed circles). This enhancing effect of hSCR18-20 on ofatumumab-induced CDC ranged from 11.7% to 30.3% depending on the antibody concentration and boosted the ofatumumab-mediated destruction of tumor cells to 73% at the highest ofatumumab concentration used. CDC induced by ofatumumab, whether in the presence or absence of hSCR18-20, was dependent on active complement since with heat-inactivated complement (hiNHS) neither ofatumumab alone (Figure 2A, open boxes, dashed line) nor ofatumumab in the presence of hSCR18-20 (Figure 2A, closed boxes, dashed line) exerted cytotoxic effects on CLL cells.

Regarding samples from individual patients, we observed a varying sensitivity to ofatumumab-induced CDC. At an ofatumumab concentration of 100 μg/mL 30% of the study patients showed no or very poor ofatumumab-induced CDC with a cut-off value of less than 25% lysis. These patients were considered CDC non-responders (Figure 2B, open circles). At this ofatumumab concentration, the majority of patients (70%) were identified as CDC-responders (Figure 2C, open circles). Nevertheless, the enhancing effect of ofatumumab-induced CDC produced by hSCR18-20 was demonstrated

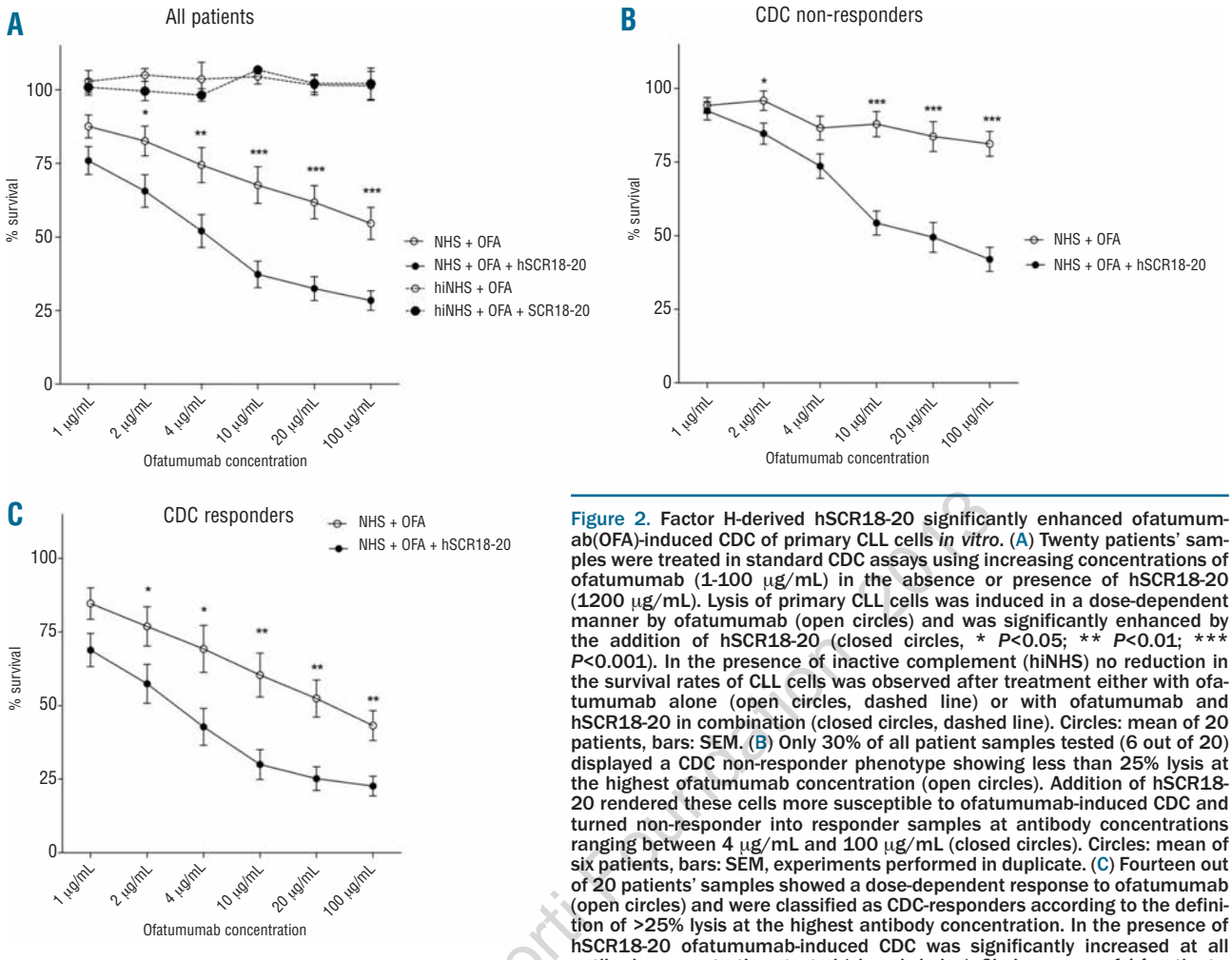

Figure 2. Factor H-derived hSCR18-20 significantly enhanced ofatumumab(OFA)-induced CDC of primary CLL cells *in vitro*. (A) Twenty patients' samples were treated in standard CDC assays using increasing concentrations of ofatumumab (1-100 µg/mL) in the absence or presence of hSCR18-20 (1200 µg/mL). Lysis of primary CLL cells was induced in a dose-dependent manner by ofatumumab (open circles) and was significantly enhanced by the addition of hSCR18-20 (closed circles, * $P<0.05$; ** $P<0.01$; *** $P<0.001$). In the presence of inactive complement (hiNHS) no reduction in the survival rates of CLL cells was observed after treatment either with ofatumumab alone (open circles, dashed line) or with ofatumumab and hSCR18-20 in combination (closed circles, dashed line). Circles: mean of 20 patients, bars: SEM. (B) Only 30% of all patient samples tested (6 out of 20) displayed a CDC non-responder phenotype showing less than 25% lysis at the highest ofatumumab concentration (open circles). Addition of hSCR18-20 rendered these cells more susceptible to ofatumumab-induced CDC and turned non-responder into responder samples at antibody concentrations ranging between 4 µg/mL and 100 µg/mL (closed circles). Circles: mean of six patients, bars: SEM, experiments performed in duplicate. (C) Fourteen out of 20 patients' samples showed a dose-dependent response to ofatumumab (open circles) and were classified as CDC-responders according to the definition of >25% lysis at the highest antibody concentration. In the presence of hSCR18-20 ofatumumab-induced CDC was significantly increased at all antibody concentrations tested (closed circles). Circles: mean of 14 patients, bars: SEM.

in both CDC non-responders (Figure 2B, closed circles) and CDC-responders (Figure 2C, closed circles). The addition of SCR in the absence of the antibody did not induce CDC. Of note, no correlation with established prognostic markers such as clinical stage, cytogenetics, *IGHV* mutational status and expression of ZAP-70 or CD38 was detectable (*data not shown*).

Improvement of ofatumumab-mediated complement-dependent cytotoxicity by hSCR18-20 is specific to B cells

Next, the B-cell specificity of ofatumumab and hSCR18-20 in heterogeneous cell mixtures was investigated. Since B-cell populations were very prominent in the PBMC of CLL patients, PBMC of healthy donors were mixed with those of CLL patients to obtain a balanced ratio of $CD19^+$ B-cell and $CD3^+$ T-cell populations in the cell suspension. The $CD3^+$ and $CD19^+$ cell fractions of the heterogeneous sample were estimated by flow cytometric analysis (Figure 3A, dot blot). CDC assays were performed in the presence of ofatumumab and/or hSCR18-20, and the survival rates were determined in the individual populations by flow cytometric analysis. Neither incubation with ofatumumab alone, nor with ofatumumab in combination with hSCR18-20 was able to reduce the viable cell counts in the $CD3^+$ T-cell population (Figure 3A). In contrast, PI-negative cell counts in the $CD19^+$ B-cell fraction were markedly diminished after treatment with ofatumumab or ofatumumab and hSCR18-20 (Figure 3A). Figure 3B illustrates that significant lysis was induced in the $CD19^+$ B-cell population after treatment with ofatumumab, which was further enhanced in the presence of hSCR18-20 (gray bars), whereas no significant influence on the survival rates of $CD3^+$ T cells was observed after treatment (black bars). Of note, neither treatment with NHS alone nor with NHS and hSCR18-20 affected the survival rates of $CD3^+$ T-cell and $CD19^+$ B-cell populations (Figure 3), indicating that complement activation by ofatumumab is necessary for hSCR18-20 to be effective.

Enhancement of ofatumumab-mediated complement-dependent cytotoxicity by hSCR18-20 is related to the presence of active C3b fragments on the cell surface

We hypothesized that enhancement of ofatumumab-mediated CDC by hSCR18-20 is due to reduced fH binding by hSCR18-20, which might influence the fI-mediated processing/inactivation of C3b fragments on the cell surface. To analyze this, CDC assays were performed with CLL cells using low amounts of ofatumumab in the presence or absence of hSCR18-20, followed by a western blot

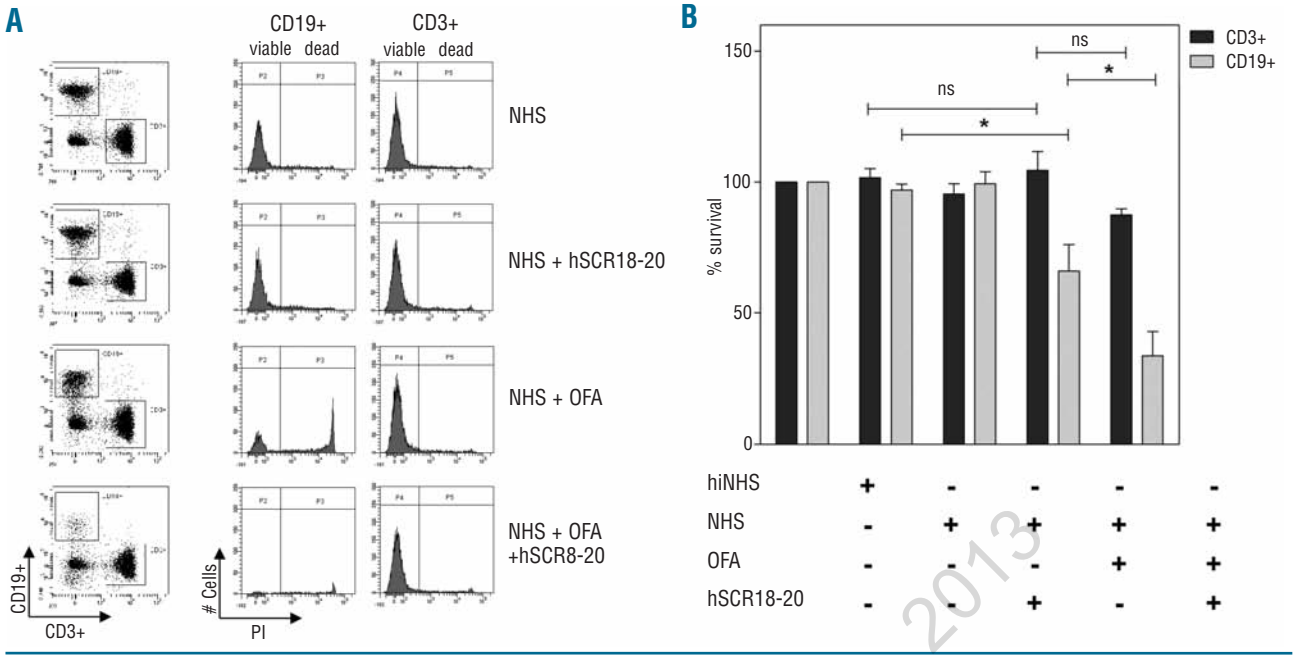

Figure 3. Complement-mediated effects of ofatumumab (OFA) and hSCR18-20 were specific to B cells. CDC assays were performed in a heterogeneous cell mixture of CLL patient PBMC and PBMC from healthy donors. Cells were stained with CD3 and CD19 antibodies and the survival rates were quantified for the individual populations. (A) Incubation of the cells in NHS only or in NHS and hSCR18-20 showed no effect on the PI-negative viable cell populations. Treatment with OFA (20 μg/mL) clearly reduced the viable CD19⁺ B-cell population (x axis) but not the CD3⁺ T-cell population. After addition of hSCR18-20 (1200 μg/mL) the viable B-cell fraction almost vanished, whereas the T-cell population was not affected. Results show one representative experiment. (B) Treatment with OFA or with OFA and hSCR18-20 significantly reduced the survival rates of the B-cell fraction (gray bars, 66% and 34%) in *in vitro* CDC assays, whereas no significant decrease in the survival rates of the T-cell population (black bars) was observed. The survival rates were calculated according to the hiNHS control, which defined 100% survival. Error bars: SEM, number of patients = 6.

analysis to characterize C3 fragments deposited on the cell surface at different time points. As a control, cells were incubated with hiNHS or NHS as a source of active complement (Figure 4, lanes 1, 2) in the absence of ofatumumab to visualize C3 (120 kDa α chain, 75 kDa β chain). After 1 min, inactive C3b (68 kDa) (Figure 4) and C3dg/C3d fragments (*not shown*) appeared. However, after 5 min, active C3b fragment (110 kDa α' chain) was observed in the presence of SCR18-20, demonstrating that cleavage of the C3 α chain (120 kDa) into C3a (10 kDa; *not shown*) and the 110 kDa α' chain had occurred (Figure 4, lane 6). In contrast, almost no active C3b-fragments were detectable in the absence of SCR18-20, suggesting a rapid inactivation of C3b into inactive C3b by fI-mediated cleavage. After 60 min incubation, C3 α' chain was still detectable when SCR18-20 was present (Figure 4, lane 10).

Expression levels of CD20, CD55 and CD59 in the complement-dependent cytotoxicity-responder and non-responder groups

CD20, CD55 and CD59 expression levels were analyzed in all the patients' samples using flow cytometric analysis since there is controversy concerning the susceptibility of malignant B cells to antibody-induced CDC.[11,12,14,28-30] As expected, the CD20 mean fluorescence intensity (MFI) was distributed heterogeneously among the 20 patients' samples and ranged from 4657 to 57575 (Figure 5A). A comparative analysis of CD20 expression levels in CDC non-responder and CDC-responder groups showed a considerably increased CD20 MFI in the responder group (16096 *versus* 6427) (Figure 5B). However, the difference did not reach statistical significance (t test for unpaired data). A low degree of heterogeneity was also observed for the expression levels of CD55 and CD59 (*data not shown*), but no difference was observed in the MFI of mRCA in the CDC non-responder and CDC-responder groups (*data not shown*). Thus, the MFI of CD20, but not CD55 or CD59, seems to determine the response to ofatumumab-induced CDC.

The relative contribution of factor H, CD55 and CD59 to protecting primary chronic lymphocytic leukemia cells against ofatumumab-mediated complement-dependent cytotoxicity

To date, only mRCA, in particular CD55 and CD59, have been discussed as diminishing the efficacy of therapeutic antibodies.[12,14] As fH has not been considered in this context, we compared the contribution of fH to protecting CLL cells from CDC to that of CD55 and CD59. The involvement of CD55 and CD59 was investigated using monoclonal antibodies (HD1A and MEM43) that functionally block these molecules. Thus, CDC assays were performed under standard conditions in the presence of ofatumumab (20 μg/mL) and blocking antibodies (10 μg/mL) or hSCR18-20 (1200 μg/mL). In an average of 20 patients ofatumumab-induced lysis was 35.5% (Figure 6A). The combination of ofatumumab and blocking anti-human CD55 (HD1A) resulted in 69.4% CDC, which was comparable to CDC induced by ofatumumab in the presence of hSCR18-20 (66.6%) (Figure 6A). In contrast, lysis obtained by ofatumumab and blocking anti-CD59 (MEM43) was less pronounced (54.7%) (Figure 6A).

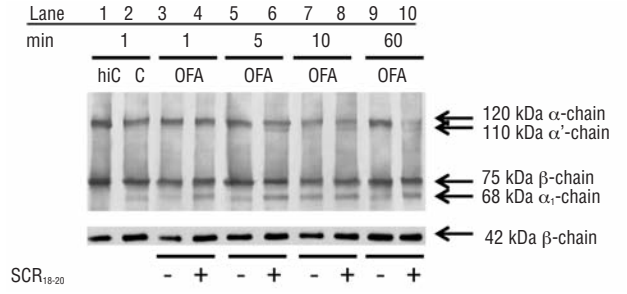

Figure 4. Western blot analysis of C3 fragments deposited on ofatumumab (OFA)-treated CLL cells in the presence or absence of hSCR18-20. CLL samples were incubated with NHS as a source of complement and with small amounts of OFA (2 μg/mL) to avoid complete lysis of the cells (lanes 3-10). SCR18-10 was added as indicated (samples 4, 6, 8, 10). C3 deposition was stopped at different time points and samples were subjected to western blot analysis. As a control, cells were incubated with hiNHS or NHS in the absence of OFA (lanes 1, 2), which allowed the detection of C3 α and β chains at 120 kDa and 75 kDa, respectively. Although the OFA concentration was suboptimal, the addition of hSCR18-20 resulted in the generation of active C3b, as indicated by the visible 110 kDa α' chain (lanes 6, 8). In the absence of the SCR, no such activation product was detectable (lanes 3, 5, 7). After 60 min, more or less all C3 α chain has been consumed, as indicated by disappearance of the 120 kDa band (lane 10). As a loading control, samples were stained for β-actin.

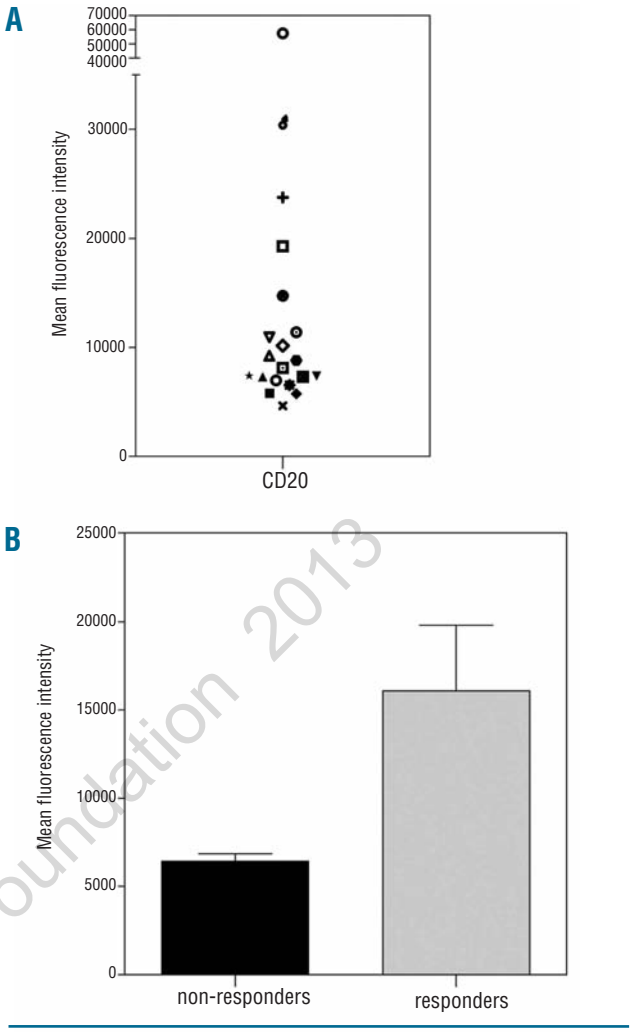

Figure 5. A trend toward higher CD20 expression was observed in CDC-responder patients. (A) A highly variable expression of CD20 was observed in 20 patients' samples. CD20 MFI ranged from 4657 to 57575. (B) CD20 expression was considerably increased in the CDC-responder group (gray bar, 16096) as compared to the CDC non-responder group (black bar, 6427). However, statistical analysis (t test for unpaired data) did not show a statistically significant difference. Bars: SEM.

Although each of the three blocking agents significantly improved CDC induced by ofatumumab, blocking fH as well as CD55 was more effective. Importantly, the fH-derived non-binding control domain hSCR16-17 did not enhance ofatumumab-induced lysis (Figure 6A), indicating that the beneficial effect of hSCR18-20 was due to specific displacement of fH from the cell surface. Of note, treatment of cells with either hSCR18-20, HD1A or MEM43 in the absence of ofatumumab did not affect survival rates (Figure 6B). We additionally investigated whether simultaneous blocking of different complement regulators would synergistically enhance ofatumumab-mediated CDC. As shown in Figure 6A, synergistic effects were obtained when cells were treated with hSCR18-20 and HD1A; the effect of the combined treatment was significantly greater than that of treatment with the individual agents. The combination of MEM43 with either hSCR18-20 alone or with hSCR18-20 and HD1A resulted in further improvement as compared to the corresponding samples.

Discussion

Recent findings indicate that the C terminal fH domain is involved in the interaction between fH and cell surfaces and renders erythrocytes more susceptible to lysis by complement.[31] In this study we investigated whether this finding can also be extended to other cell types and tested whether ofatumumab-mediated CDC is more pronounced on CLL tumor cells in the presence of fH-derived recombinant hSCR18-20. We confirmed that ofatumumab is highly effective in killing CLL cells in a complement-dependent manner.[5,32,33] In the presence of NHS as a source of active complement about 45% of the cells were lysed at the highest antibody concentration used. Ofatumumab-induced CDC was significantly boosted by the addition of hSCR18-20, resulting in the destruction of almost 75% of CLL cells. More importantly, treatment with ofatumumab in combination with hSCR18-20 was able to overcome the CDC resistance observed in distinct patients' samples when treated with ofatumumab alone. Ofatumumab-induced killing of the tumor cells was highly cell type-specific, even in the presence of hSCR18-20. The non-significant drop in T-cell counts may be due to the recently reported expression of CD20 in a small subpopulation of the $CD3^+$ T-cell pool.[34]

The previously reported increased lysis of erythrocytes in the presence of the C terminal fH domain was observed when the classical pathway was blocked by Mg-EGTA, suggesting the involvement of the alternative complement pathway.[31] In our approach it is most likely that the initial complement activation occurs via the classical pathway, as in the absence of ofatumumab no cell lysis and no presence of the active C3 α' chain was observed. In the presence of hSCR18-20 an activated C3 α' chain was

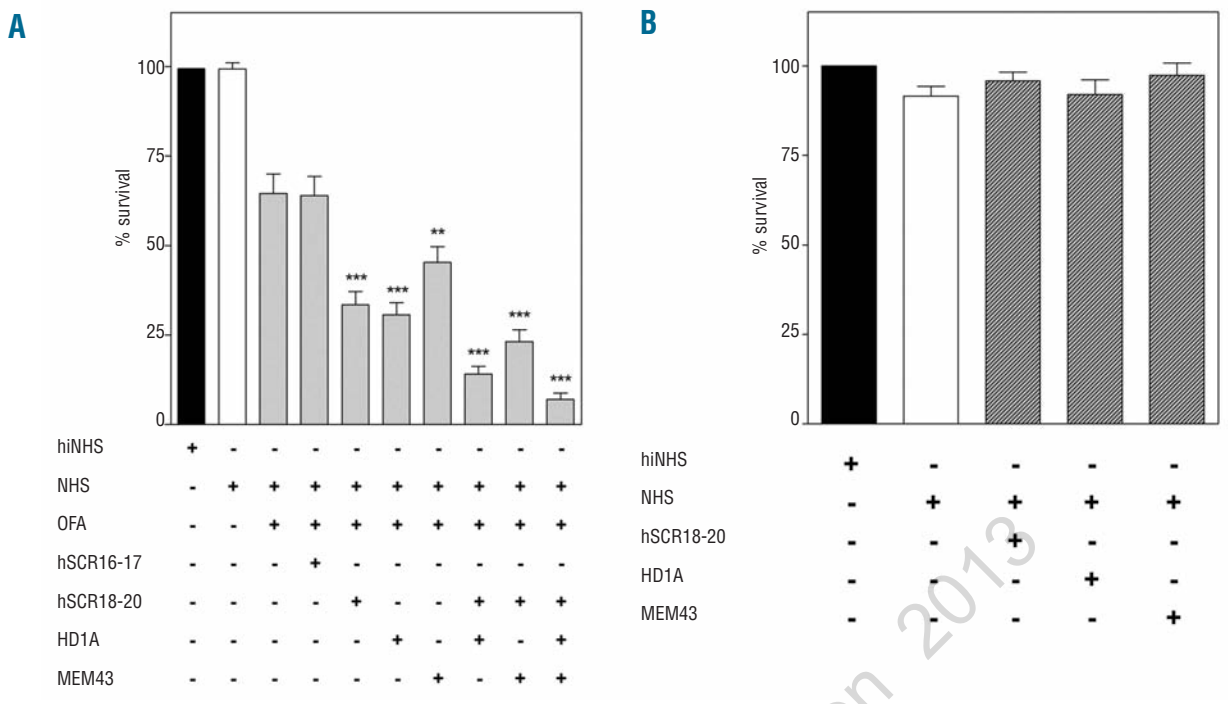

Figure 6. The effects of blocking fH, CD55 or CD59 on ofatumumab (OFA)-induced CDC of primary CLL cells. (A) CLL cells were treated in standard CDC assays using OFA (20 μg/mL), hSCR16-17 (1200 μg/mL), hSCR18-20 (1200 μg/mL), blocking HD1A (10 μg/mL) or blocking MEM43 (10 μg/mL) either alone or in various combinations depending on the experimental design. The lysis induced by OFA (35.5%) was significantly (*P<0.05; **P<0.01; ***P<0.001) enhanced by additional treatment with hSCR18-20 (66.6%), HD1A (69.4%) or MEM43 (54.7%). The fH-derived control fragment hSCR16-17 did not affect survival rates (36.1%). Simultaneous blocking of fH and CD55 by hSCR18-20 and HD1A significantly improved the effects obtained with the individual agents. Additional blocking of CD59 by means of MEM43 showed minor effects. Survival rates were calculated with reference to the hiNHS control, which defined 100% survival. Bars: SEM, n = 20. (B) No induction of lysis was observed after treatment of primary CLL cells with hSCR18-20 (1200 μg/mL), HD1A (10 μg/mL) or MEM43 (10 μg/mL) under standard conditions in the absence of OFA. Survival rates were calculated according to the hiNHS control. Bars: SEM, n = 4.

detectable in the western blot during the entire investigation period, which may indicate that active C3b fragments have a prolonged turnover time on the cell surface. After 60 min all C3 was consumed, even at the small amounts of ofatumumab used for this assay. A boosting strategy that aims to prolong the presence of active C3b fragments on the cell surface may be of importance in certain anatomical sites, such as cerebrospinal fluid, which contain insufficient complement for efficient lysis. Although not primarily relevant for CLL, such a strategy may be useful in the prevention and/or treatment of other CD20+ lymphoproliferative diseases, in particular aggressive non-Hodgkin's lymphoma. Addition of SCR may also improve the clinical response to CLL cells resistant to CDC[32] or optimize clearance of malignant cells even at high CLL tumor burden.[35]

While fH has been described as contributing to the protection of several solid tumors and tumor cell lines,[19-21,36-39] its involvement in the resistance of CLL cells to CDC has not yet been reported. By contrast, the role of CD55 and CD59 in the context of CLL has been investigated in more detail.[9,11-18,40] Both mRCA seem to contribute to protecting CLL cells against CDC, as blocking CD55 and CD59 by antibodies or a bacterial inhibitor renders the tumor cells more susceptible to complement attacks.[9] As reported in the literature, the high variability in the expression of these mRCA did not indicate a correlation with CDC11 and was not indicative of a CDC-responder or non-responder phenotype. Although in our study CD20 expression tended to be stronger in patients' samples displaying a CDC-responder phenotype, the difference in CD20 MFI between CDC-responder and non-responder groups was not statistically significant. The lesser dependence on CD20 levels may reflect the potent efficacy of ofatumumab at activating the complement system through the classical pathway of complement as compared to other therapeutic anti-CD20 monoclonal antibodies[5] and may allow the induction of efficient CDC, even when CD20 and bound CD20 monoclonal antibodies are partially internalized,[41] thus reducing expression on the cell surface. The binding of ofatumumab to a CD20 epitope close to the cell surface[3,6-8] and the segregation of anti-CD20 monoclonal antibodies into lipid rafts may be among the factors that make ofatumumab less dependent than rituximab on CD20 expression.[42,43]

In its present form, the application of SCR to improve ofatumumab is not feasible. The SCR do not only compete with fH, present at high concentrations in human serum, but also with fH-related proteins (CFHL-1 and CFHR1-5), all of which contain heparin-binding motifs.[44] However, physical coupling of SCR18-20 to ofatumumab may provide a solution to this problem. As tumor cell lysis is induced only when the monoclonal antibody has bound to its target and activated the complement system, inhibition of fH binding by SCR18-20 is necessary only locally at the tumor cell surface. By using an anti-CD20 antibody such as ofatumumab as a shuttle, the relatively low affinity binding of the SCR (and of fH)[22] is enhanced and its

effect directed specifically to B cells eventually resulting in improved CLL cell killing. Moreover, by a strategy of local fH inhibition, putative systemic side effects of an anti-fH strategy may also be reduced.

We do not expect that the increased complement activation and thus enhanced C3b deposition will decrease the overall efficacy of ofatumumab by impairing natural killer cell-mediated ADCC[45] as recently published data indicate that monoclonal antibody-dependent amplification of C3 deposition on tumor cells enhanced both macrophage-dependent, Fc-mediated ADCC and CDC *in vitro* and *in vivo*.[46] Thus, the lack of natural killer cell activation can be compensated for by increased phagocytosis and complement-mediated lysis.[5]

Taken together, fH-derived hSCR18-20 significantly boosted the CDC of ofatumumab-treated CLL cells. Not only patients' samples that were susceptible to complement lysis, but also cells that were refractory to ofatumumab-induced complement-mediated killing, became ofatumumab-sensitive when hSCR18-20 was added. Thus, combining ofatumumab with hSCR18-20 may significantly increase the efficacy of monoclonal antibody therapy and represent a novel strategy for improving treatment options in CLL.

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