

## rILYd4, a Human CD59 Inhibitor, Enhances Complement-Dependent Cytotoxicity of Ofatumumab against Rituximab-Resistant B-cell Lymphoma Cells and Chronic Lymphocytic Leukemia

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### Abstract

**Purpose:** Ofatumumab is an anti-CD20 antibody recently approved for treatment of fludarabine and alemtuzumab refractory chronic lymphocytic leukemia (CLL); it mediates much stronger complement-dependent cytotoxicity (CDC) than rituximab. Human CD59, a key membrane complement regulator that inhibits CDC, is highly expressed in B-cell malignancies and its upregulation is an important determinant of the sensitivity of B-cell malignancies to rituximab treatment. Previously, we have shown that the potent CD59 inhibitor rILYd4 sensitizes rituximab-resistant lymphoma cells to rituximab-mediated CDC. Here, we further investigated whether rILYd4 can sensitize B-cell malignancies to ofatumumab-mediated CDC and whether either ofatumumab-mediated CDC or rILYd4-enhanced ofatumumab-mediated CDC correlates with CD20 or CD59 expression, known biomarkers involved in rituximab activity.

**Experimental Design:** Rituximab-resistant cell lines and primary CLL cells were used to investigate the antitumor efficacy of the combination of rILYd4 with ofatumumab or rituximab. Propidium iodide staining or alamarBlue assay were used to evaluate the CDC effect. The levels of CD20 and CD59 on the cell membrane were analyzed by flow cytometry.

**Results:** rILYd4 enhanced CDC effects mediated by ofatumumab or rituximab on rituximab-resistant lymphoma cells and primary CLL cells *in vitro*. The sensitivity to CDC effects mediated by ofatumumab positively correlated with the ratio of CD20/CD59 and negatively correlated with CD59 levels on CLL cells. The degree to which rILYd4 enhanced CDC correlated positively with the CD59 levels on CLL cells.

**Conclusions:** These data suggest that rILYd4 may enhance the anticancer activity of ofatumumab and rituximab in B-cell malignancies that have relapsed after prior antibody-based therapies. *Clin Cancer Res*; 17(21); 6702–11. ©2011 AACR.

### Introduction

In the past 10 years, rituximab, a chimeric anti-CD20 antibody, has led to significant progress in treating B-cell

malignancies (1–5). However, rituximab efficacy remains variable and often modest when used as a single agent. Half of the patients with non-Hodgkin Lymphoma (NHL) are unresponsive to rituximab (4, 6). Some responsive NHL patients develop resistance to further treatment (4, 7). When used as a single agent, rituximab in chronic lymphocytic leukemia (CLL) is less efficacious than in indolent NHL and in the relapsed setting has little activity (3, 8–14). Although the addition of rituximab to regimens such as fludarabine with or without cyclophosphamide has been shown to improve the overall and complete response rates and prolong survival in patients with CLL, the disease remains incurable (5).

Recently, a new human IgG1 anti-CD20 monoclonal antibody, ofatumumab, has been developed. In October 2009, the U.S. Food and Drug Administration granted accelerated approval to ofatumumab for the treatment of patients with CLL refractory to fludarabine and alemtuzumab (15). The most recent clinical trial showed that single-agent ofatumumab was well tolerated with an overall

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-11-0647

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### Translational Relevance

The efficacy of rituximab and ofatumumab in cancer therapy depends, in part, on the induction of complement-dependent cytotoxicity (CDC). Human CD59 is a key complement regulator that inhibits induction of CDC. CD59 is highly expressed in B-cell malignancies, such as non-Hodgkin lymphoma and chronic lymphocytic leukemia (CLL), and upregulation of CD59 expression is an important determinant of the sensitivity of those cancer cells to rituximab treatment. Here, we show that rILYd4, a potent and nontoxic human CD59 inhibitor, enhances CDC effects mediated by ofatumumab or rituximab on rituximab-resistant B-cell malignancy cell lines and primary CLL cells. The sensitivity to CDC effects mediated by ofatumumab or rituximab with or without rILYd4 negatively correlated with the level of CD59. These data suggest that rILYd4 may be a new approach to enhance the anticancer activity of ofatumumab and rituximab in B-cell malignancies that have relapsed after prior antibody-based therapies.

response rate of 11% in heavily pretreated patients with relapsed or progressive NHL, nearly all of whom had received prior rituximab therapy (16). The overall response rate with single-agent ofatumumab was 51% in CLL patients refractory to fludarabine and alemtuzumab and 44% in the CLL patients refractory to fludarabine with bulky (>5 cm) lymphadenopathy. Although ofatumumab has a high response rate in the treatment of CLL, the progression-free survival remains quite short (17). NHL and CLL patients inevitably relapse and become increasingly refractory to further treatment (2, 16, 17). A current strategy to enhance the efficacy of ofatumumab and rituximab in the treatment of B-cell malignancies is to combine with other agents such as chlorambucil, cyclophosphamide, doxorubicin, vincristine, ifosfamide, carboplatin, cisplatin, and others (11).

Ofatumumab targets a membrane-proximal epitope encompassing both the large and the small loops of the CD20 molecule. Rituximab targets a different region involving only the large loop (18, 19). The mechanisms by which ofatumumab kills B-cell cancers are suspected to involve complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and direct cell death (1, 11, 20–22). Although rituximab and ofatumumab have comparable binding affinities to CD20, ofatumumab induces much stronger CDC than rituximab (18, 19). This may be due to the lower off-rate from CD20 of ofatumumab than that of rituximab (18, 19). In addition, ofatumumab induces ADCC and direct cell death at levels comparable with rituximab (18, 19). *In vitro*, ofatumumab requires approximately 10-fold fewer cell surface CD20 molecules than rituximab to induce detectable CDC (9, 19, 23, 24). A comparison study in a xenograft mouse tumor model showed that ofatumumab is more effective in controlling lymphoma growth than rituximab (25). These

results indicate that the ability of ofatumumab to induce CDC may play a critical role in ofatumumab-mediated cancer therapy.

When a therapeutic antibody activates the classical complement pathway, it triggers the formation of complement membrane attack complex (MAC) on cancer cells leading to the killing of cells through CDC (4). CD59, a critical membrane complement regulator, inhibits MAC formation by binding to complement proteins 8 and 9 (C8 and C9). CD59 is universally expressed on normal cells and also expressed on many kinds of cancer cells including NHL and CLL (4). Extensive evidence indicates that CD59 is highly effective at protecting NHL and CLL cells from antibody-mediated CDC (10, 26–38). Upregulation of CD59 is an important determinant of sensitivity to antibody (rituximab) treatment in CLL (10, 26–36, 39). Therefore, we developed a novel, potent, and specific human CD59 inhibitor, a recombinant 114 amino acid peptide consisting of domain 4 of intermedilysin (rILYd4), the cytolytic toxin secreted by *Streptococcus intermedius* (39–41). rILYd4 sensitized rituximab-resistant lymphoma cells and primary CLL cells from 6 patients to rituximab treatment through enhanced CDC effect, indicating that rILYd4 may be a therapeutic candidate for the treatment of antibody-resistant NHL (39). Here, by qualitative and quantitative flow cytometry, we further investigated whether rILYd4 sensitizes rituximab-resistant cells and primary CLL cells from 26 patients to CDC induced by ofatumumab. We correlated either ofatumumab-mediated CDC or rILYd4-enhanced ofatumumab-mediated CDC with known biomarkers involved in rituximab activity, that is, CD20 and CD59 expression.

### Materials and Methods

Additional information is available in Supplementary Materials and Methods.

#### Original and resistant B-cell malignancy cell lines, primary CLL cells, and cell culture

The human B-cell lymphoma cell lines ARH-77, RL, Daudi, and Raji were purchased from and authenticated by the American Type Culture Collection and passaged less than 50 times. Original and resistant B-cell malignancy cell lines were cultured in RPMI-1640 medium (Invitrogen), and rituximab-resistant Ramos, Daudi, and Raji cell lines were generated with previously published method (33, 39). Those resistant cell lines that survived complement attack induced by rituximab at concentrations of 51.2, 20, and 20  $\mu\text{g}/\text{mL}$  in the presence of 10% human serum (Valley Biomedical) as a source of complement were named as RamosR51.2, DaudiR20, and RajiR20, respectively.

The CLL patients had been previously enrolled on Dana-Farber Harvard Cancer Center (DF/HCC) protocol 99-224, a tissue banking study that links samples to clinical information. The protocol was approved by the DF/HCC Institutional Review Board, and all patients signed written informed consent. The blood from 26 CLL patients (Supplementary Table S1) was then separated by density

centrifugation through a Ficoll gradient, and peripheral blood mononuclear cells were frozen for subsequent use. The CLL cells were cultured as described in our previous publication (39).

#### Flow cytometric analysis of CD20 and CD59 levels

The cells at a density of  $5 \times 10^6$  cells per mL were harvested and washed twice with PBS. The cells suspended in 3% bovine serum albumin (BSA)/PBS were incubated with 1:100 diluted primary mouse monoclonal antibodies against CD20 or CD59 at room temperature for 30 minutes, washed in 3% BSA/PBS, and then incubated with the secondary antibody [goat anti-mouse IgG-conjugated fluorescein isothiocyanate (FITC)] at room temperature for another 30 minutes. The cells were washed in PBS before analysis. Flow cytometry was carried out with a FACScan (Becton Dickinson), and mean fluorescence intensities were converted to molecules of equivalent soluble fluorochrome (MESF) by calibrated beads (Spherotech). We divided the MESF of CD20 by the MESF of CD59 to calculate the ratio of CD20/CD59.

#### CDC assays

Cell viability was determined by either propidium iodide (PI) staining or alamarBlue assay as described (32, 39). Briefly,  $10^5$  cells were treated with rituximab or ofatumumab with or without rILYd4 in the presence of normal human serum (NHS) as a source of complement for 2 hours at 37°C. Because ARH-77 and RL were more resistant to rituximab-mediated CDC effects (18, 39, 42) than Daudi and Raji (42), 20% or 5% of NHS was used as a source of complement to carry out the experiments with ARH-77 and RL cell lines or Daudi and Raji cell lines, respectively. We used 25% final NHS for CLL cells because of the resistance of CLL to rituximab. After washing with 1% BSA/PBS, the cells (in 100  $\mu$ L) were incubated with 10  $\mu$ L PI (50  $\mu$ g/mL) at room temperature for 5 minutes and immediately analyzed on the FACScan. The PI-negative population was regarded as live cells. Percentage of cell death was calculated using the following formula: (%) =  $100 \times [1 - (\text{live cells in treated sample} / \text{live cells in untreated control})]$ . The percentage of rILYd4-enhanced effect (%) on rituximab or ofatumumab-mediated CDC was calculated as: (%) =  $[(\% \text{ of dead cells in rituximab or ofatumumab with rILYd4 sample} / \% \text{ of dead cells in rituximab or ofatumumab alone sample}) - 1] \times 100\%$ . For the alamarBlue assay, we followed the protocol as we described previously in the work of Hu and colleagues (39).

#### Determination of C1q binding, C3b(i), and C9 deposition

After cells were challenged with human complement in the CDC assay, followed by washing with 1% BSA/PBS, they were stained with FITC-1H8 [anti-C3b(i) antibody], polyclonal rabbit anti-human C1q antibody, or polyclonal goat anti-human C9 antibody followed by FITC-labeled secondary antibody, respectively. After washing with PBS, the cells were analyzed with flow cytometry, and mean fluorescence intensities were converted to MESF by calibrated beads (9).

#### Statistical analysis

Linear regression was used to evaluate the correlation between the degree of CDC induction and CD20 or CD59 expression and the ratio of the 2 with SPSS 11.5 or Graph-Pad Prism 4.0 software. The differences in the means of paired samples in the CDC assay on primary CLL cells were evaluated by the Wilcoxon signed-rank test. Most results were expressed as the mean  $\pm$  SD of data obtained from 3 to 4 separate experiments. The statistical significance of the differences between the group means was determined by one-way ANOVA to compare variance. A value of  $P < 0.05$  was considered significant.

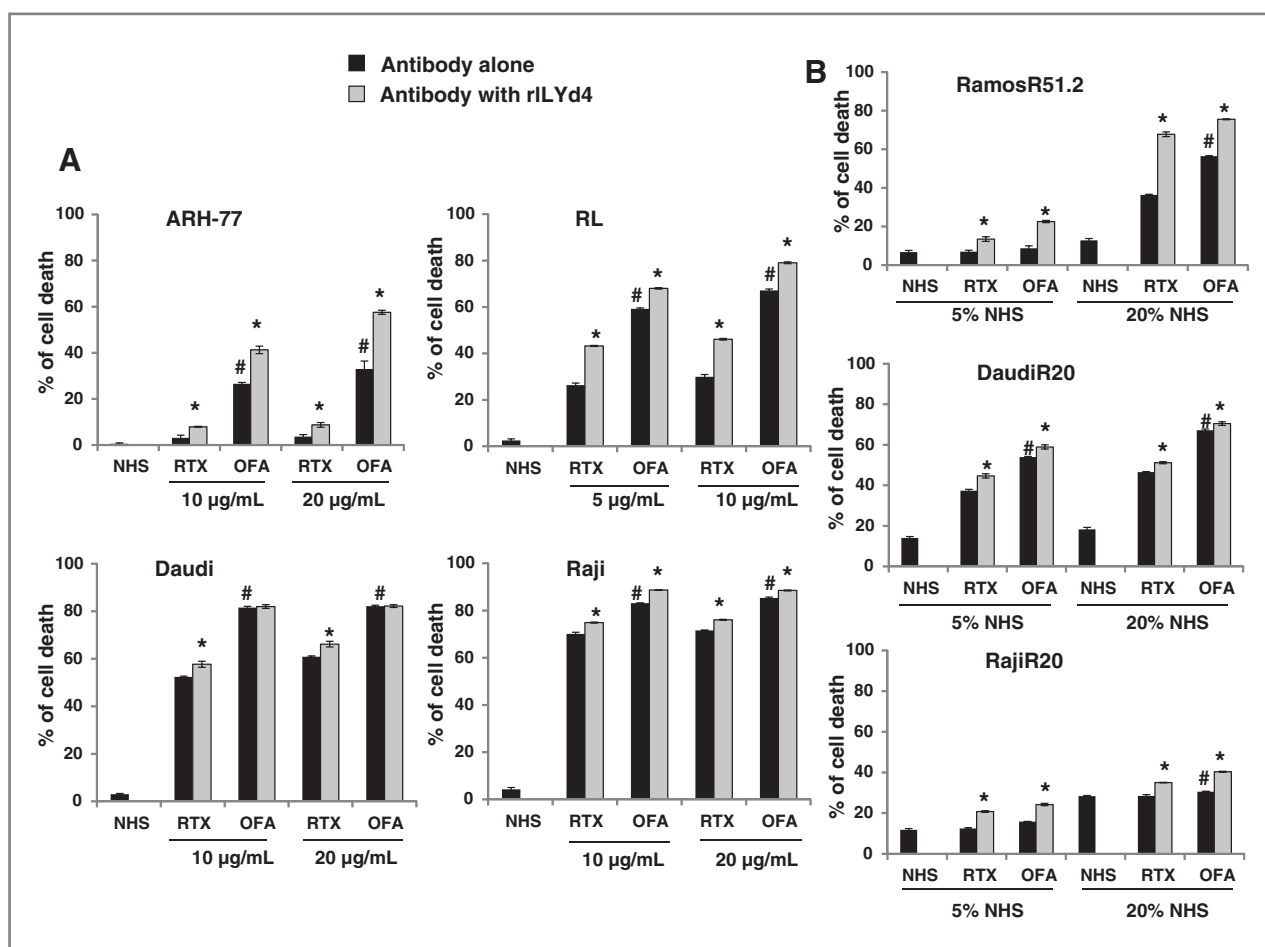
#### Results

##### rILYd4 enhances ofatumumab-mediated CDC on B-cell malignancy cell lines and sensitizes the rituximab-resistant cell lines to ofatumumab

To test whether rILYd4 enhances ofatumumab-mediated CDC, we used ARH-77, RL, Daudi, and Raji cell lines that expressed CD20 and CD59 at different levels (Supplementary Fig. S1). We also compared the CDC effect of ofatumumab with that of rituximab. We used alamarBlue assay to identify the optimal concentrations of rituximab and ofatumumab (20  $\mu$ g/mL for ARH-77 and 10  $\mu$ g/mL for RL, Daudi, and Raji cells) for each of the cell lines (Supplementary Fig. S2) and to determine the appropriate rILYd4 concentration (Supplementary Fig. S3). To carry out CDC experiments, we selected the final rILYd4 concentration to be 1,074 nmol/L that was the minimal concentration required for mediating maximal cell lysis for all cells tested. Importantly, heat-inactivated human serum (IHS) did not mediate any lysis (Supplementary Fig. S4), confirming the nature of complement-dependent cell death observed in the CDC assay with NHS. In addition, rILYd4 alone did not mediate any lysis (Supplementary Fig. S4), further confirming our previous finding that rILYd4 alone has no direct lytic effect on CD59-expressing cells (40). rILY3, a nonfunctional isotype of rILYd4, also did not mediate any more CDC than vehicle buffer (Supplementary Fig. S4), confirming the specific effect of rILYd4.

PI staining, a well established flow cytometric method for quantitative analysis of cell viability (43, 44), was used to assess cell death. Addition of 1,074 nm rILYd4 to 5% or 20% NHS for Daudi and Raji or ARH-77 and RL, respectively, led to statistically significantly higher CDC at 2 different concentrations of rituximab or ofatumumab (Fig. 1A). Ofatumumab mediated significantly higher CDC effects than rituximab in the presence or absence of rILYd4 in all 4 original cell lines, respectively (Fig. 1A). In addition, similar results were observed with 50% NHS, a more relevant physiologic condition for ARH-77 (Supplementary Fig. S5). Furthermore, the results from PI staining were comparable with those from alamarBlue assay (data not shown).

To further investigate whether rILYd4 also sensitizes rituximab-resistant B-cell lymphoma cell lines to ofatumumab-mediated CDC, we used our previously established



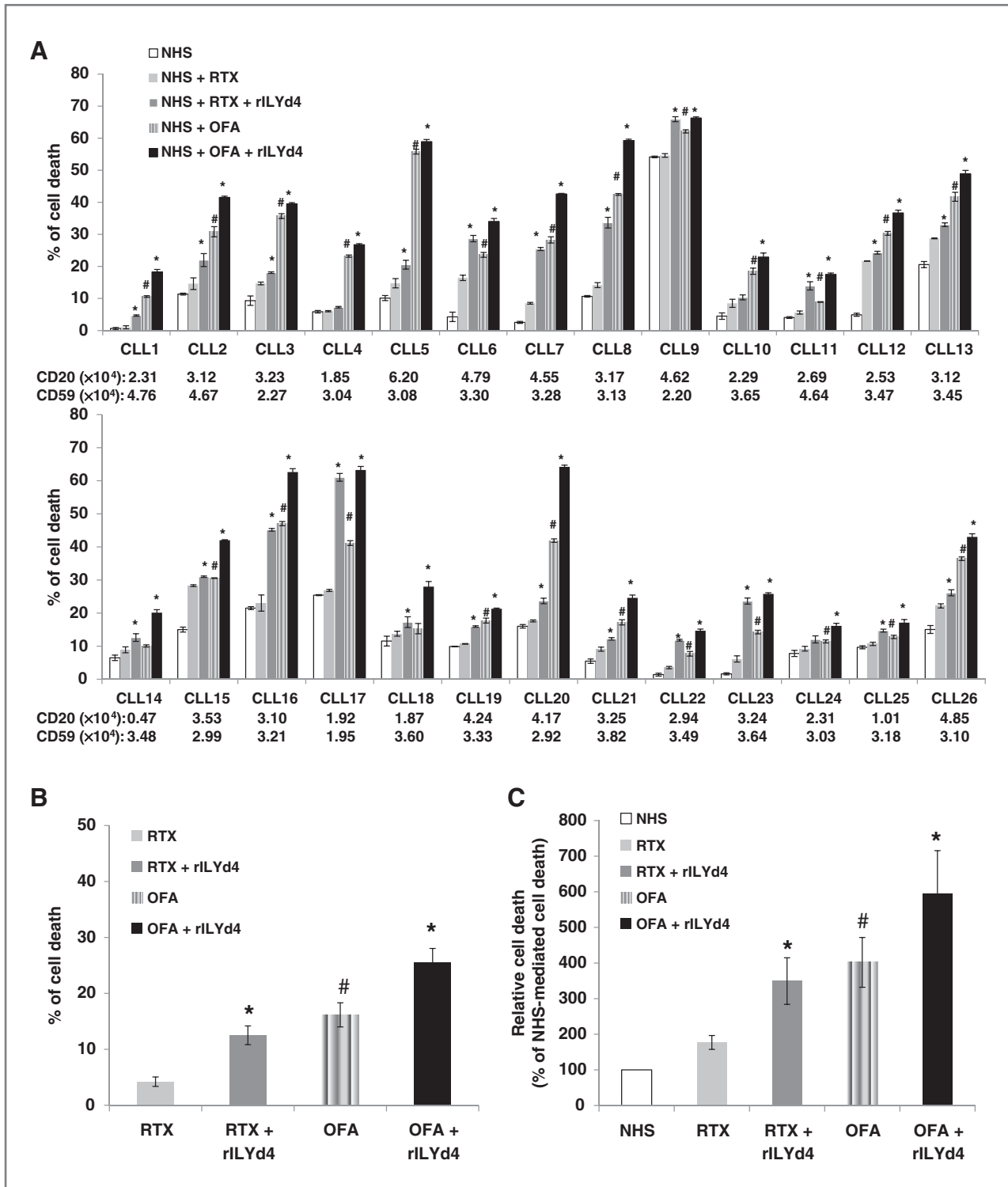
**Figure 1.** rILYd4 effect on B-cell malignancy cell lines and rituximab (RTX)-resistant cell lines. A, ARH-77, RL, Daudi, and Raji were treated with different concentrations of rituximab or ofatumumab (OFA) with 20% NHS (for ARH-77 and RL) or 5% NHS (for Daudi and Raji) in the absence or presence of 1,074 nmol/L rILYd4. B, RamosR51.2, DaudiR20, and RajiR20 were treated with 10 µg/mL rituximab or ofatumumab together with 5% or 20% NHS in the absence or presence of 1,074 nmol/L rILYd4. A and B, cells were incubated at 37°C for 2 hours. Cell viability was assessed by flow cytometry. Results are mean ± SD of 3 different experiments. \*,  $P < 0.01$  versus no rILYd4 treatment; #,  $P < 0.01$  versus rituximab.

rituximab-resistant cell lines, DaudiR20, RajiR20, and RamosR51.2 (39). These resistant cells also express higher levels of CD59 than the original cell lines (Supplementary Fig. S6A). The presence of rILYd4 resulted in higher CDC mediated by both ofatumumab and rituximab than the absence of rILYd4, with either 5% or 20% NHS (Fig. 1B). Ofatumumab also induced higher CDC activity than rituximab (Fig. 1B). Furthermore, we also observed comparable results with 50% NHS for RamosR51.2 cells (Supplementary Fig. S5). The absence of CDC in any experimental condition with NHS as a source of complement indicates that the CDC effects observed in Fig. 1B were complement dependent (Supplementary Fig. S6B).

Taken together, these results show the following: (i) rILYd4 enhances the CDC effects of ofatumumab; (ii) rILYd4 sensitizes rituximab-resistant cell lines to both ofatumumab- and rituximab-mediated CDC; and (iii) ofatumumab has more potent CDC activity than rituximab.

#### rILYd4 sensitizes primary CLL cells to ofatumumab- and rituximab-mediated CDC *ex vivo*

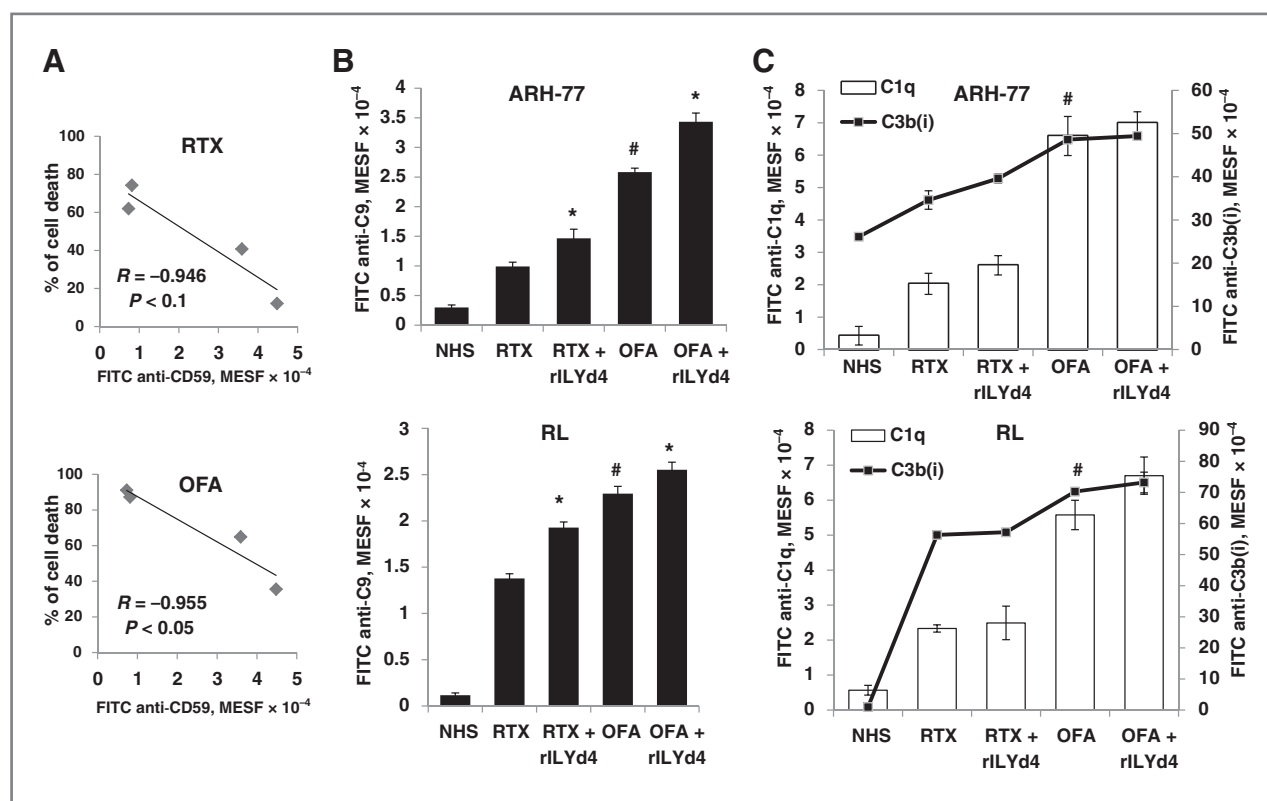
Primary CLL cells are much more resistant to antibody-mediated CDC than NHL cells because of both the lower expression of the target CD20 and higher expression of CD59 (3, 8–10). Here, we used primary CLL cells from 26 patients (Supplementary Table S1) to test the effect of rILYd4 on clinical cancer cells. rILYd4 together with ofatumumab or rituximab significantly increased the CDC effect on CLL cells from 22 of 26 patients compared with ofatumumab or rituximab alone (Fig. 2A). The pooled data of the CDC effects obtained from the 26 CLL patients also showed that rILYd4 treatment mediated statistically significant higher ofatumumab- or rituximab-induced CDC than vehicle treatment (Fig. 2B and C). In the presence or absence of rILYd4, 50% NHS, a more relevant physiologic condition, achieved stronger CDC effects mediated by rituximab or ofatumumab than 25% NHS in 4 patients with CLL samples (Supplementary Fig. S7). Of note, the cells from patient



**Figure 2.** rLYd4 enhances ofatumumab or rituximab-mediated CDC effects on primary CLL. **A**, the CDC effect on CLL cells of 26 different patients. Primary CLL cells from 26 different patients (CLL1–CLL26) were individually subjected to 10  $\mu$ M rituximab/ofatumumab-induced CDC with or without 1,074 nmol/L rLYd4 in the presence of 25% NHS. Results are mean  $\pm$  SD of 3 different experiments. Expression levels of CD20 and CD59 on CLL cells were presented by MESF. **B** and **C**, comparison of the pooled CDC data showing the net increase in cell death (**B**) or the relative increase in cell death compared with NHS-induced cell death in 26 patients (**C**). Net increase in cell death equals the cell death treated with antibody or antibody plus rLYd4 subtracting treated by NHS alone individually. The data are presented as mean  $\pm$  SEM from the 26 patients (CLL1–CLL26). A–C, \*,  $P < 0.01$  versus no rLYd4 treatment; #,  $P < 0.01$  versus rituximab.

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**Figure 3.** The CDC effect mediated by ofatumumab correlates negatively with CD59 level, and rILYd4 enhances C9 deposition on the cell surface of B-cell malignancy cells. A, negative correlation between ofatumumab-mediated CDC and CD59. B, rILYd4 increases C9 deposition. ARH-77 and RL cells were treated with 10  $\mu$ g/mL rituximab or ofatumumab in the presence of 20% NHS with or without 1,074 nmol/L rILYd4. C, C1q binding and C3b(i) deposition were not influenced by rILYd4. ARH-77 and RL cells were treated with 10  $\mu$ g/mL rituximab or ofatumumab in the presence of 20% NHS with or without 1,074 nmol/L rILYd4. The levels of C1q binding and C3b(i) and C9 deposition were represented by MESF. B and C, results are mean  $\pm$  SD of 3 different experiments. \*,  $P < 0.01$  versus no rILYd4 treatment; #,  $P < 0.01$  versus rituximab.

CLL9 are very sensitive to NHS alone. Because inactivated human serum did not lyse the cells, we reason that this phenomenon may result from an increase in complement activation on the patient cells mediated by NHS itself through unknown factors. The levels of CD20 and CD59 on the surface of the CLL cells from each patient were determined by flow cytometric analysis (Fig. 2A). The fact that cell killing was due to CDC was shown in 8 CLLs that showed no cell killing when IHS was used as a source of complement (Supplementary Fig. S8). Only 8 samples were tested because of the limiting amount of primary CLL cells. Ofatumumab mediated greater CDC than rituximab in CLL cells (Fig. 2), consistent with our observations with NHL cell lines. These results indicate that rILYd4 enhances the CDC effect of ofatumumab and rituximab on CLL cells and highlights the potential for therapeutic value of rILYd4 in the treatment of CLL.

#### Ofatumumab-mediated CDC effects negatively correlate with CD59 levels on the cell surface of B-cell malignancy cell lines

To investigate the underlying mechanism determining the sensitivity to ofatumumab-mediated CDC, we correlated the CDC-induced killing of 4 original B-cell malignancy cell lines

with levels of CD20 or CD59. We used the ratio of CD20/CD59 (divided the MESF of CD20 by the MESF of CD59) to examine whether both CD20 and CD59 determine the CDC effect. The CDC effects mediated by either ofatumumab or rituximab tended to correlate positively with the level of CD20 among these 4 cell lines, although these effects did not reach statistical significance (Supplementary Fig. S9A). In contrast, CDC effects mediated by ofatumumab correlated negatively with the level of CD59 on the surface of the 4 B-cell malignancy cell lines (Fig. 3A). Furthermore, CDC effects mediated by antibody positively correlate with the ratio of CD20/CD59, though not reaching statistical significance (Supplementary Fig. S9B). Taken together, these results confirm previous findings that CD20 and CD59 are important molecules in determining the sensitivity of lymphoma cells to rituximab-mediated CDC.

To define the underlying mechanism by which rILYd4 enhances CDC, we quantified deposition of C9 and C3b(i) and C1q binding on the antibody-treated lymphoma cells (ARH-77 and RL) in the presence or absence of rILYd4. We documented that rILYd4 treatment mediated significantly higher levels of C9 deposition but not C1q binding or C3b(i) deposition on the cells than antibody alone (Fig. 3B and C). These results further document that rILYd4 specifically

increases MAC formation through inhibiting CD59 and does not influence the level of C1q binding or C3b(i) deposition induced by rituximab or ofatumumab. We also found that ofatumumab mediated more C1q binding and more C3b(i) deposition than rituximab on the lymphoma cells (Fig. 3C), a result consistent with findings previously reported by Pawluczko and colleagues (9).

#### Ofatumumab-mediated CDC correlates negatively with CD59 levels and positively with the ratio of CD20/CD59 on the surface of CLL cells

Previous studies by others indicate that rituximab-mediated CDC correlates positively with the level of CD20 and negatively with the level of CD59 on the surface of CLL cells (32, 43). Ofatumumab mediates stronger CDC effects on CLL than rituximab, dependent on the level of CD20 but not complement regulators (45). We document here that the CDC effect induced by ofatumumab correlates positively with the level of CD20 on the surface of CLL cells (Fig. 4A). When combined with rILYd4, the extent of cell death induced by ofatumumab also correlated positively with the level of CD20 (Fig. 4A). Rituximab-mediated CDC effects with or without rILYd4 tended to correlate with the CD20 level but did not reach statistical significance (Fig. 4A). The sensitivity of CLL to ofatumumab- or rituximab-mediated CDC correlated negatively with the level of CD59 on the CLL cells (Fig. 4B). Moreover, ofatumumab-

rituximab-mediated CDC on CLL cells with or without rILYd4 correlated positively with the ratio of CD20/CD59 (Fig. 5A). These correlations were more significant in general in ofatumumab than rituximab groups (Fig. 5A). These results indicate that the levels of CD20 and CD59 on the target cell surface are associated with CDC induced by ofatumumab or rituximab and with rILYd4-enhanced CDC induced by ofatumumab or rituximab.

We further analyzed the correlation between CD59 levels and the percentage of rILYd4-enhanced effect for either therapeutic antibody on CLL cells. We showed that the percentage of rILYd4-enhanced effect positively correlates with CD59 levels on the CLL cell surface (Fig. 5B). This result directly indicates that rILYd4 enhanced the CDC effects mediated by ofatumumab or rituximab through the inhibition of CD59 activity in these primary CLL cells.

#### Discussion

We have previously reported that rILYd4 enhances rituximab-mediated CDC against Ramos cells and primary CLL cells and sensitizes rituximab-resistant Ramos cells to rituximab treatment *in vitro* and *in vivo* (39). These findings are consistent with the results shown here using 2 other rituximab-resistant cell lines (Raji and Daudi) and primary CLL cells. We also show that the CDC effect mediated by rituximab with or without rILYd4 correlated positively with the

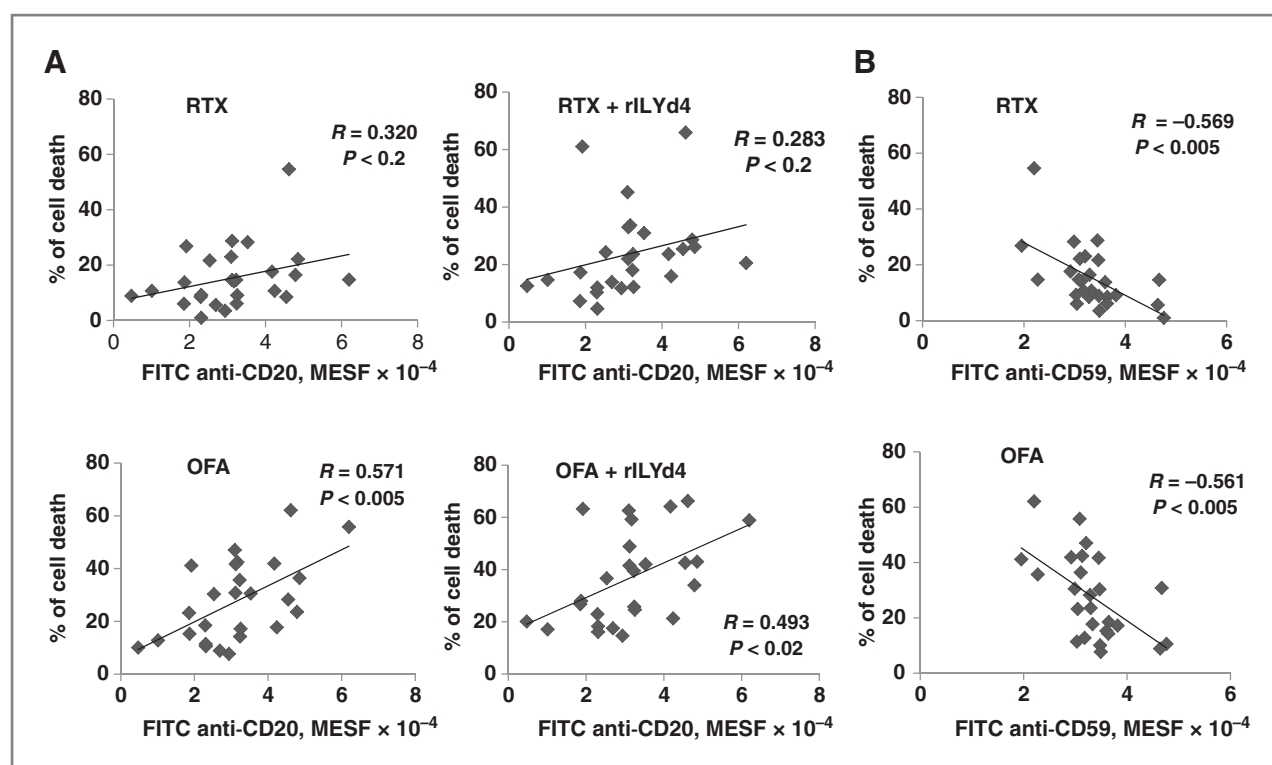
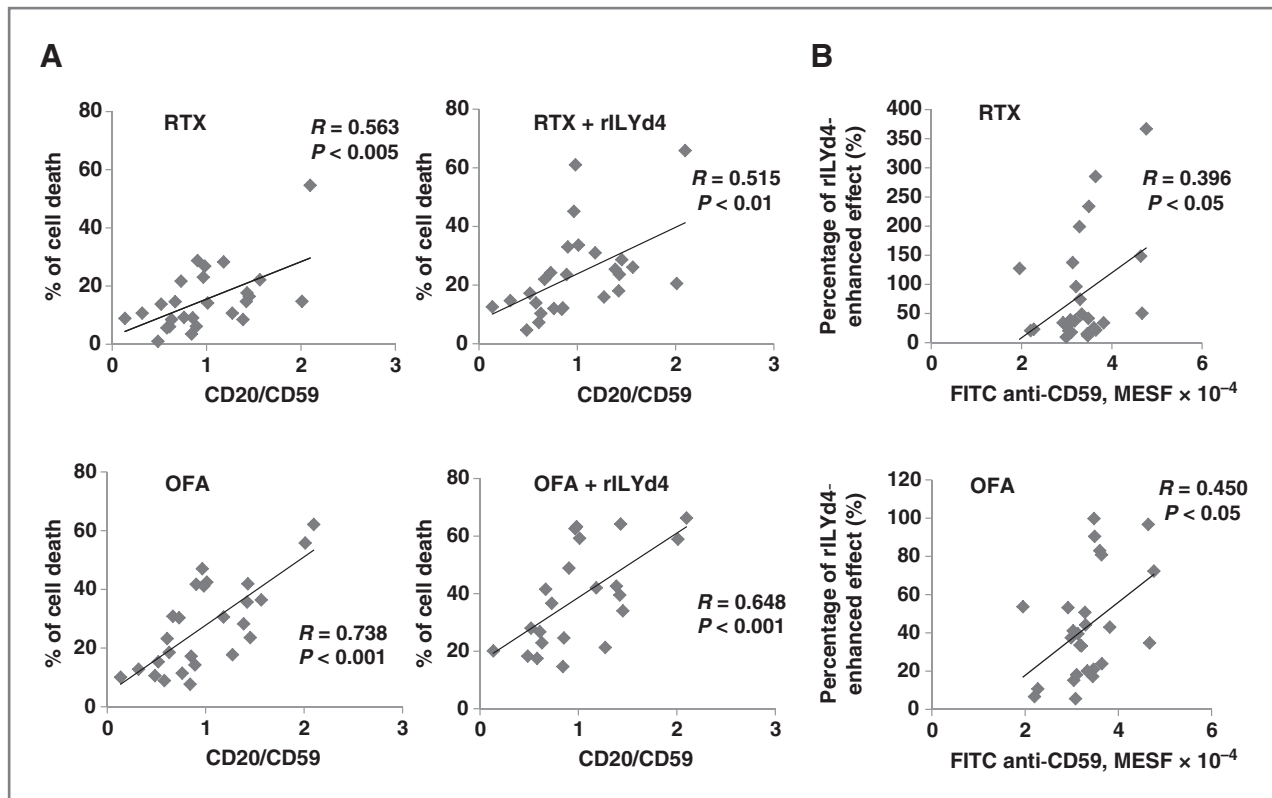


Figure 4. Correlations between the level of CD20 or CD59 on the surface of CLL cells and the CDC effect mediated by anti-CD20 antibodies with or without rILYd4. A, the extent of cell death induced by ofatumumab correlated significantly with the MESF of CD20 on the CLL cell surface. When combined with rILYd4, the extent of cell death induced by ofatumumab positively correlated with the level of CD20. B, sensitivity of CLL cells to CDC induced by ofatumumab or rituximab treatment correlated negatively with the MESF of CD59 on CLL cells.



**Figure 5.** Correlation of ofatumumab-mediated CDC with the ratio of CD20/CD59, and correlation of the percentage of rILYd4-enhanced effect with CD59 level, on the surface of CLL cells. A, the sensitivity of CLL cells to rituximab or ofatumumab mediated CDC with or without rILYd4 correlates significantly with the ratio of CD20/CD59. B, the percentage of rILYd4-enhanced effect correlates positively with the level of CD59 on the surface of CLL cells.

CD20 level on the surface of the cells and negatively with the CD59 level on the surface of the cells. These results are comparable with the findings previously reported by Golay and colleagues (32, 39, 43).

It has been extensively shown that ofatumumab-mediated CDC effects on both lymphoma cells and primary CLL cells are largely dependent on the level of CD20 on the target cell surface (1, 5, 16, 20). Although CD59 has been widely recognized to reduce the sensitivity of B-cell malignancies to rituximab, whether it has a similar role in ofatumumab treatment has not been extensively studied. An American Society of Hematology (ASH) meeting abstract reported that ofatumumab-mediated CDC effects on lymphoma cell lines and primary cells derived from diffuse large B-cell lymphoma patients correlated negatively with CD59 but not with CD46 or CD55 (45). Here, we showed the following: (i) CDC mediated by ofatumumab with or without rILYd4 on the CLL cells positively correlated with CD20 levels and CD20/CD59 ratio and (ii) ofatumumab-mediated CDC effect on the original lymphoma cell lines correlated positively and negatively with CD20 and CD59 levels, respectively. These results together with those obtained using rituximab with or without rILYd4 shed light on the importance of the inhibition of CD59 for the treatment of B-cell malignancies with rituximab or ofatumumab. Furthermore, we also document that rILYd4 is able

to enhance ofatumumab-mediated CDC. This result highlights the potential therapeutic uses of rILYd4 or rILYd4 derivatives in the treatment of B-cell malignancies, providing justification for further development and evaluation.

It is notable that the rILYd4-enhanced CDC effect on Daudi and Raji is much less than that on ARH-77 and RL lymphoma cell lines as well as CLL primary cells (Fig. 1A). This is attributable to a much lower level of CD59 on Daudi and Raji cell lines than that on ARH-77 and RL lymphoma cell lines and CLL cells. This explanation is further supported by the fact that the percentage of rILYd4-enhanced effect on CLL cells positively correlates with the level of CD59 on the CLL cells (Fig. 5B). Furthermore, 3 rituximab-resistant cell lines show variable sensitivity to rILYd4 treatment although they express a high level of CD59 on their surfaces. This observation indicates that in addition to CD59 and CD20, other resistance mechanisms may also contribute to the development of resistance to CDC effect; these alternative mechanisms warrant further investigation.

It is widely accepted that ofatumumab has much more potent CDC than rituximab (18, 19). Consistently, we found that ofatumumab mediates much stronger CDC effects than rituximab in all cells. The more potent CDC effect mediated by ofatumumab compared with rituximab is attributed to increased C1q binding and C3b(i) deposition on the target cells triggered. Previously, Pawluczko



et al (9) and Beum et al (23) showed that ofatumumab triggers more C1q binding and more C3b(i) deposition on these Daudi and Raji cells, leading to more MAC attack. These results were further confirmed by us with 2 other lymphoma cell lines expressing high levels of CD59. The application of rILYd4 to ofatumumab- or rituximab-mediated CDC specifically increases C9 deposition but not C1q binding and C3b(i) deposition on the targeted cells. This result directly highlights the specificity of rILYd4 activity and the inhibition of anti-MAC activity of CD59.

The clinical response to rituximab-containing chemotherapy varies between patients. Previously, using a live cell-imaging technique to evaluate the CDC activity of rituximab (46), Mishima and colleagues documented that CDC susceptibility of lymphoma cells freshly obtained from patients was strongly associated with response to rituximab-containing chemotherapy (46). Here, we also observed variation in response to the CDC effects mediated by ofatumumab or rituximab with or without rILYd4. For example, the rILYd4 enhancement effect mediated by both antibodies on some CLL cells such as CLL2, CLL6, CLL7, CLL8, CLL16, or CLL17 in Fig. 2A was significantly higher than that of other CLL cells such as CLL4 or CLL10. rILYd4 was able to enhance the effects of rituximab in some of the CLL cells (CLL6, CLL16, or CLL17) to the level close to or even higher than that mediated by ofatumumab alone. These observations suggest that different combinatorial strategies may be needed for different patients based on their responses to antibody-mediated CDC and their levels of CD20 and CD59. However, the therapeutic implications of these different responses among CLL patients remain to be determined in the future.

In summary, these results reported here indicate that rILYd4 may be able to function as an adjuvant for ther-

apeutic antibodies in the treatment of cancer, including in particular ofatumumab and rituximab in the treatment of B-cell malignancies. However, it is important to note that the impact of rILYd4 on ofatumumab therapy reported here is modest. Therefore, whether rILYd4 would be able to overcome resistance to ofatumumab-based cancer therapy in patients will require further investigation. Clinical testing of rILYd4 will depend on further preclinical development that reduces its immunogenicity and improve its half life.

## Disclosure of Potential Conflicts of Interest

J.R. Brown receives research funding from GSK. No potential conflicts of interest were disclosed by the other authors.

## Acknowledgments

The authors thank Dr. Ronald P. Taylor for kindly providing C3b/iC3b/C3dg monoclonal antibodies, FITC-1H8, and GSK/Genmab for kindly providing Ofatumumab.

## Grant Support

This work was supported by NIHRO1AI061174 (to X.B. Qin) and NIH R21CA141324 (to X.B. Qin), Harvard Technology Development Accelerator Fund (to X.B. Qin), and China Scholarship Council (2009628090; to X. Ge). J.R. Brown is supported by NIH K23CA115682 as well as by an ASH Scholar award and the Leukemia and Lymphoma Society Scholar in Clinical Research Award.

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Received March 10, 2011; revised August 15, 2011; accepted September 8, 2011; published OnlineFirst September 14, 2011.

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