Rituximab was the first commercially available monoclonal antibody for the treatment of lymphoma. It is now a treatment of choice for a variety of lymphoproliferative disorders including low- and high-grade non–Hodgkin’s lymphomas (NHL; refs 1–5). Various in vitro and in vivo experiments have shown that elimination of CD20+ cells by rituximab involves complement-dependent cytotoxicity (6–13), the recruitment of effector cells leading to antibody-dependent cellular cytotoxicity (14) and direct apoptotic signaling (15).

Follicular lymphoma (FL) is the most common subtype of indolent lymphoma. Rituximab is now widely used either alone or in combination with multiagent chemotherapy for the treatment of FL, either at diagnosis (16, 17), at relapse (18–20), or for maintenance therapy (2, 21, 22). However, despite its well-established clinical efficacy, a subpopulation of patients does not initially respond to rituximab and most patients will relapse after therapy. The mechanisms of action and resistance to rituximab are not fully understood.

Abstract

Purpose: Follicular lymphoma (FL) is the most common subtype of indolent lymphomas. Rituximab is widely used alone or in combination therapy for the treatment of FL. Despite its well-established clinical efficacy, a subpopulation of patients does not respond to rituximab and most patients will relapse after therapy. The mechanisms of action and resistance to rituximab are not fully understood.

Experimental Design: To study these mechanisms we developed an in vivo model of FL resistant to rituximab. This model was developed using the human RL line, isolated from a patient with FL, grown as xenotransplants in severe combined immunodeficient mice, exposed weekly to rituximab in vivo, followed by serial reimplantation and reexposure to rituximab, until a resistant phenotype was obtained.

Results: RL-derived tumors unexposed to rituximab were grown as controls and compared with the resistant tumors. Although the expression of CD46 and CD55 antigens were not differently expressed in the resistant cells, the complement inhibitor CD59 was overexpressed in a subpopulation and CD20 was found to be expressed at a lower level in a minority of cells. Bcl-XL and YY1 were also found more highly expressed in rituximab-resistant cells.

Conclusion: This model provides insight on potential in vivo resistance mechanisms to rituximab and could help contribute to the development of novel therapies in rituximab-refractory diseases.

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Sophie Dupire contributed to acquisition and interpretation of results.

Stéphanie Brunet-Manquat contributed to acquisition and interpretation of results.

Adriana Plesa contributed to acquisition and interpretation of results.

Lina Reslan contributed to acquisition and interpretation of results.

C.Dumontet contributed to the design and submission to animals ethical committee of the study, acquisition, and interpretation of results and corrected the manuscript.

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Cancer Therapy: Preclinical

In vivo Model of Follicular Lymphoma Resistant to Rituximab

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Rituximab was the first commercially available monoclonal antibody for the treatment of lymphoma. It is now a treatment of choice for a variety of lymphoproliferative disorders including low- and high-grade non–Hodgkin’s lymphomas (NHL; refs 1–5). Various in vitro and in vivo experiments have shown that elimination of CD20+ cells by rituximab involves complement-dependent cytotoxicity (6–13), the recruitment of effector cells leading to antibody-dependent cellular cytotoxicity (14) and direct apoptotic signaling (15).

Follicular lymphoma (FL) is the most common subtype of indolent lymphoma. Rituximab is now widely used either alone or in combination with multiagent chemotherapy for
Translational Relevance
Rituximab is now a standard of care in the treatment of follicular lymphoma patients. But de novo or acquired resistance to this anti-CD20 monoclonal antibody is common. Dalle et al. established an in vivo model of follicular lymphoma resistant to rituximab. This model should prove to be quite useful to identify novel mechanisms of resistance to rituximab in vivo and in the development of novel monoclonal antibodies or therapeutic strategies, including drug combinations designed to overcome resistance to rituximab.

expression of YY1 and sensitization of tumor cells to Fas and Trail-induced apoptosis (29).

There remains a large discrepancy between the results obtained in vitro using FL-derived cells (low level of cytotoxicity) and results observed in the clinical setting (high level of efficacy). Most lymphoma lines do not exhibit high sensitivity to rituximab in vitro, and most of the preclinical data on rituximab has been produced using Burkitt lines. Recently, Jazirehi et al. (30) developed a model of Burkitt lymphoma clones resistant to rituximab, whereas Czuczman et al. developed (28) follicular and Burkitt resistant cells in vitro. These clones were shown to have altered cellular signaling dynamics and to exhibit different phenotypic properties when compared with parental cells. The resistant clones were shown to have a reduced CD20 surface expression, failed to respond to rituximab-mediated inhibition of cell growth and apoptosis after cross-linking. However in vivo models of resistance to rituximab are needed to follow the progressive modifications of tumor cells induced by rituximab and their mechanisms of adaptation in the host.

We thus chose to develop such a model of FL resistant to rituximab in vivo. We then studied the characteristics of this model by focusing on the mechanisms of resistance to rituximab previously described in vitro. We also explored opportunities to overcome resistance to rituximab in this model using combination therapy.

Materials and Methods

Cell lines and culture. The RL cell line (derived from a human FL) was maintained in culture medium consisting of RPMI (Life Technologies), 10% FCS (Integro), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). All cells were cultured at 37°C in a 5% CO2 atmosphere (31).

Animals. Female CB17 severe combined immunodeficient mice purchased from Charles River Laboratories were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for the laboratory animal care and use. The animals were kept in conventional housing. Access to food and water was not restricted. All mice used in this experimental study were 5- to 6-week old at the time of tumor implantation. This study was approved by the local animal ethical committee.

Lymphoma xenograft experiments. Lymphoma cells (1 × 106; RL) were serially passed in mice exposed or unexposed to rituximab. Cells were injected s.c. on day 1. To determine sensitivity of RL cells to rituximab in vivo, mice were first divided into three experimental groups of 3 to 5 mice per group: control mice receiving no treatment, an “early treatment” group with rituximab started on day 2, and a “late treatment” group with rituximab started on day 15 after injection of the lymphoma cells. Rituximab 10 mg/kg (clinical formulation; Roche) was injected i.p. weekly. Mice were weighed and the tumor size measured twice a week with an electronic caliper. The tumor volume was estimated from two dimensional tumor measurements by the formula: tumor volume (mm3) = length (mm) × width2/2. Animals were euthanized either when tumor volume exceeded 2 mL to avoid animal discomfort or if conditions suggested the potential for animal suffering. The tumors were then gently flushed in culture medium to recover the tumor cells in the suspension. The cells were then centrifuged and counted, using trypan blue exclusion staining.

To establish the resistant model, tumor cells obtained from mice having received rituximab were reinjected the same day to a new group of mice that were then treated with the “early treatment” rituximab regimen described above.

In experiments evaluating the role of complement-dependent cytotoxicity in rituximab inhibition of tumor growth, complement inhibition was obtained by weekly i.p. injection of cobra venom factor (2 μg per mouse; Quidel Corporation).

Immunohistochemistry of tumors growing in severe combined immunodeficient mice. S.c. tumors obtained from tumor-bearing severe combined immunodeficient mice were analyzed by pathologic examination. Unstained paraffin-embedded tissue sections were used for detection of CD20 antigen by immunohistochemistry. Sections (4- to 6-μm thick) were deparaffinized by incubation at 60°C for 1 h followed by immersion in xylene. Slides were then treated with serial dilutions of alcohol (100%, 90%, and 70% ethanol/distilled water) and rehydrated in PBS. No antigen retrieval was necessary. All samples were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase. Protein blocking was performed using horse serum for 20 min, followed by a 30-min incubation with mouse antihuman CD20 clone L26 labeling an intracytoplasmic epitope of CD20 (DAKO) at 1:50 dilution.

Flow cytometry analysis. Cell surface antigen expression of RL cells was performed on a FACSCalibur flow cytometer (Becton Dickinson). Analysis of the data were performed with the Cell Quest software program (Becton Dickinson). Mouse fluorochrome-conjugated isotype control antibodies, phycocyanin 5- coupled anti-CD19, phycoerythrin-coupled APC anti-CD20, FITC-coupled anti-CD59, and phycoerythrin-coupled anti-CD55 were purchased from Immunotech. FITC-coupled anti-CD46 was purchased from Becton Dickinson. Mean Fluorescence intensity was determined by subtracting the signal of isotype-matched antibody staining from the staining observed with the specific primary antibody.

Western blot protein analysis. Protein expression was determined by Western blot analysis in rituximab-naive and rituximab-resistant tumors as previously described. Briefly, cell lysates were resolved by 12% SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Hybond-ECL; Amersharm Corp.). The blots were then incubated with the appropriate dilution of primary antibody, followed by incubation with peroxidase-conjugated secondary antibody. For this analysis, 10 × 104 cells were pelleted and proteins fractionated by SDS-PAGE (12-15% gradient gels) and transferred to a polyvinylidene difluoride membrane using an electrophoretic apparatus (Bio-Rad). The loading of equal amounts of protein was verified by Ponceau staining of the polyvinylidene difluoride membranes. The membrane was blocked with 5% nonfat, dry milk for 1 h and subsequently incubated with the primary antibody at a dilution of 1:1,000 for 1 h at room temperature. Antibody directed against Bcl2 was purchased from Dako (clone 124), YY1 from Active Motif and Bcl-xi (clone S18) from Santa Cruz, BAX from Santa Cruz (clone SC 493), BAK from Santa Cruz (clone SC 7873), BAD from Serotec (clone APH7475), BIM from Santa Cruz (clone SC 8263), CD59 from Serotec (Clone mem-43), CD55 from Abcam (MEM-118), and CD20 from Abcam (clone L26). Unbound antibody was removed by washing with PBS (pH 7.2) containing 0.1% Tween 20 and 5% nonfat, dry milk. The membrane was then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase and visualized by Chemiluminescence detection (ECL; Amersharm Corp.).
with the secondary antibody [anti mouse peroxidase-conjugated antibody (Sigma) at a dilution of 1:6,000] for 1 h at room temperature. After extensive washing with PBS, proteins were detected after addition of the staining substrates enhanced chemiluminescence (Amersham). Proteins were detected by chemiluminescence using Kodak film (Eastman Kodak Company). The Western blot analyses were performed for each animal from the different groups of animals.

Cytotoxicity assays. Tumor cells were obtained from tumors in severe combined immunodeficient mice and cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (32). The IC50 was defined as the drug concentration resulting in 50% loss of cell viability relative to untreated cells. Assays were performed in triplicate in at least three separate experiments for doxorubicin, cytarabine, and cisplatin.

Statistical analysis. The data were expressed using Student’s t test. Analyses were performed with Statistica 6.0 (StatSoft, Inc.).

Results

Induction of in vivo resistance to rituximab. Unimpeded growth of RL tumors under these experimental conditions showed palpable tumors after 17 to 18 days during the first passages, with a tendency to earlier growth during after passages. Spontaneous tumor growth required euthanasia of animals around day 30. During the first passages, mice receiving early treatment (starting on day 2) were highly sensitive to rituximab therapy when compared with the control group (Fig. 1). Early treated mice and control groups were respectively euthanized at day 35 and 46 after injection with a statistical different tumor growth at day 35 ($P = 0.004$). Conversely, in mice receiving delayed treatment (from day 15 onward), we did not observe a significant difference in tumor growth between treated and untreated groups ($P = 0.958$; Fig. 1). After four cycles of consecutive reimplantation and early treatment, the reimplanted cells progressively became resistant to rituximab. The growth of the resistant tumors in mice exposed to early rituximab therapy was comparable with that of the rituximab-naive control group, whereas the cells serially implanted in mice but never exposed to rituximab remained highly sensitive (Fig. 2A and B).

CD20 antigen expression in resistant lymphoma cells. Immunohistochemistry showed that the lymphoma cells were strongly CD20 positive both in the treated and nontreated groups. Flow cytometry analysis did not show a global difference in CD20 expression levels between parental and resistant RL cells (mean fluorescence intensities of 215 and 252, respectively). But when comparing the resistant group and the control group, a small fraction of resistant cells had become CD20 negative (4%). This suggests that iterative treatment with rituximab did not result in a global decrease of surface CD20 expression in this model, but a subset of treated cells lost this expression during the acquisition of resistance (Fig. 3A, B, C, and D). This subset of CD20 cells was not different in the expression of CD55, CD59, and CD46. When CD20 content was evaluated by immunoblots performed on lysates of control and resistant tumors, no difference was observed (data not shown). These results suggest that most of the cells exposed to repeated injection of rituximab did not lose the CD20 expression. Nevertheless, a small counterpart of treated cells did. These results was not distinguishable on the Western blot analysis that has been made with the entire population.

Role of complement in resistant lymphoma cells. Complement-dependent cytotoxicity seemed to play a role in the efficacy of rituximab in our model. When the rituximab was given in combination with cobra venom factor, we observed a significant loss of antitumor activity ($P = 0.027$; Fig. 4). To determine the possible role of complement inhibitors in the antitumor effect of rituximab, we analyzed the surface expression of CD46, CD55, and CD59. Two-color flow cytometric staining using CD19 gating and monoclonal antibodies against CD46, CD55, and CD59 was performed on cells obtained from tumors. Taking in account median fluorescence
intensity, we did not observe altered expression of CD46 in resistant cells compared with parental cells. Conversely analysis of CD59 in resistant cells showed a subpopulation over-expressing this complement inhibitor (Fig. 3B). This population also showed a down-regulation of the CD55 expression and was globally CD20+. We subsequently attempted but failed to sort this population of interest by flow cytometry. In western Blot analysis, the protein contents of CD55 and CD59 were not different between naïve and resistant cells (data not shown).

Overexpression of Bcl-X<sub>L</sub> in resistant lymphoma cells. Previous findings have established Bcl-X<sub>L</sub> as being involved in resistance to rituximab in vitro. We therefore evaluated Bcl-X<sub>L</sub> and Bcl-2 levels in the sensitive and resistant tumors. Immunoblotting showed that resistant cells expressed higher Bcl-X<sub>L</sub> protein levels compared with wild-type cells (Fig. 5). Conversely, there was no significant difference concerning the level of expression of Bcl-2. The proapoptotic factors of the Bcl-2 family BAX and BAK were also tested on Western blot analysis. Although there was no difference in BAX protein expression, BAK was found to be down-regulated in resistant cells in the same way we did not observe differences in protein expression for the BH3-only Bcl-2 family proteins BIM and BAD (data not shown).

Expression of YY1 in naïve cells and resistant cells. YY1 has been suggested to be involved in the sensitivity of NHL cells to apoptosis. It has already been shown that expression of YY1 is decreased in lymphoma cells after exposure to rituximab, resulting in a sensitization to chemotherapeutic agents. In our experiments, the protein content of YY1 was indeed found to be strongly decreased in naïve cells after exposure to rituximab (data not shown). Conversely, expression of YY1 was increased

Fig. 3. A, expression of CD20 in sensitive and resistant cells by immunohistochemistry and Facs Analysis. B, expression of complement inhibitors in sensitive and resistant cells. CD55 and CD59 were analyzed by flow cytometry on fresh tumor samples. C, expression of CD20 on CD59 higher subpopulation cells. D, expression of CD55 and CD59 on CD20- cells.

Fig. 4. Inhibitory effect of cobra venom factor on the antitumor activity of rituximab in vivo. Arrow, days of treatment by rituximab and/or cobra venom factor, respectively.
in resistant cells after in vivo treatment in comparison with the control group (Fig. 6).

**Sensitivity to chemotherapy in vitro.** Enhancing the cytotoxic effects of anticancer agents is an established property of rituximab. However, these results obtained in vitro using Burkitt's lymphoma cell lines are not reproducible on FL lines or other models of NHL. When naïve RL cells were exposed in vitro to rituximab, the cytotoxic effect was very low (>95% of cells stay alive) even with the adjunction of human serum as source of complement or accessory cells to enhance antibody-dependent cellular cytotoxicity. On the other hand, these cells were initially highly sensitive to doxorubicin (IC50 12.3 nmol/L). After having acquired resistance to rituximab in vivo, these resistant cells also proved to be significantly less sensitive to doxorubicin in vitro (IC50 153.3 nmol/L). These cells were also significantly less sensitive to cytarabine, cisplatin, vincristine, and maphosphamide, but these later differences were not significant (Table 1). Thus, in vivo resistance to rituximab seemed to be associated with in vitro resistance to a variety of chemotherapeutic agents.

**Discussion**

This is the first report of a human FL model resistant to rituximab in vivo. Comparison with the sensitive parental cells has allowed the identification of a subpopulation with increased expression of the complement inhibitor CD59, whereas the expression of CD20 was also reduced in a distinct subpopulation of resistant cells. Rituximab-resistant RL cells were found to express higher levels of Bcl-xL and YY1 protein than their sensitive counterparts.

It has been shown that FL cells can be effectively destroyed by single agent rituximab in patients, whereas B-cell chronic lymphocytic leukemia cells show a poor response. A major difference between these two diseases is the higher level of CD20 expression in FL cells in comparison with that of chronic lymphocytic leukemia cells (8, 9, 33). Several studies have addressed the question of whether CD20 expressions level could be used to predict response to rituximab (7, 8, 10, 13, 34). The results remain controversial. In vitro analysis of FL cells found no correlation between CD20 expression and sensitivity to rituximab-induced complement-dependent cytotoxicity (7, 10, 12). In contrast, a strong correlation was reported in two other studies involving patients with a variety of B-cell malignancies (9). Interestingly, in a Burkitt model resistant to rituximab in vitro, Jazirehi et al. (30) have shown that the resistant clones displayed a 50% reduction in CD20 expression. This was recently confirmed in another in vitro model by Czucman and al (28). In our in vitro model the surface expression of CD20 was not globally diminished. Nevertheless a subpopulation representing 4% of the resistant cells became CD20- during the consecutive passages. This subpopulation has emerged only in the treated group and not in the control group. It is therefore possible that longer exposure could increase the percentage of CD20- cells. Further work is needed to elucidate this point.

In our model of xenograft tumor, the complement system seems to play a critical role because its inhibition by cobra venom factor provokes a clear loss of efficacy of rituximab. Complement lysis is regulated by complement inhibitors such as CD46, CD55, and CD59. The initial expression of these inhibitors does not seem to be a predictive factor of response to rituximab therapy (8). Conversely, the blockade of these inhibitors can increase the in vitro response of rituximab through complement-dependent cytotoxicity. Increased expression of CD55 and CD59 was associated with a resistant phenotype in Burkitt's lymphoma cells derived in vitro by repeated exposure to rituximab. It has also been shown that the increased expression of the complement regulator CD59 was associated with resistance to rituximab-mediated complement lysis of multiple myeloma and NHL cell lines. Treon et al. (35) reported that complement inhibitors, particularly CD59 is present on various B-cell tumors and is associated with resistance to rituximab in patients (8). Our results tend to confirm that increased expression of CD59 is associated with the acquisition of a resistant phenotype. Conversely we did not observe modifications in CD46 expression in our resistant model. Surprisingly, the CD55 expression was down-regulated in our cell subpopulation overexpressing CD59.

Several signaling pathways have been described to be involved in rituximab-induced apoptosis. Cross-linking of
Table 1. In vitro sensitivity of sensitive and resistant cells to anticancer compounds

<table>
<thead>
<tr>
<th></th>
<th>Mean IC50 (naive cells)</th>
<th>Mean IC50 (resistant cells)</th>
<th>Resistance ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>12.3 nmol/L</td>
<td>153.3 nmol/L</td>
<td>12.46</td>
<td>0.049</td>
</tr>
<tr>
<td>Vincristine</td>
<td>16.25 pmol/L</td>
<td>24 pmol/L</td>
<td>1.48</td>
<td>(P = 0.08)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.75 μmol/L</td>
<td>12 μmol/L</td>
<td>3.2</td>
<td>(P = 0.12)</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>6.3 nmol/L</td>
<td>9.3 nmol/L</td>
<td>1.47</td>
<td>(P = 0.043)</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>0.86 ng/mL</td>
<td>1.26 ng/mL</td>
<td>1.46</td>
<td>(P = 0.07)</td>
</tr>
<tr>
<td>Maphosphamide</td>
<td>8 μmol/L</td>
<td>16 μmol/L</td>
<td>2</td>
<td>(P = 0.08)</td>
</tr>
</tbody>
</table>

NOTE: In vitro sensitivity was evaluated using an MTT cytotoxicity assay. Rituximab-resistant cells also displayed a significant resistance to doxorubicin and cytarabine with a trend toward reduced sensitivity to vincristine, gemcitabine, maphosphamide, and cisplatin. In vivo sensitivity to chemotherapeutic compounds of cells obtained from rituximab-naïve and rituximab-resistant tumors. The resistance ratio is the ratio of the mean IC50 in resistant cells and in naive cells.

rituximab, for example via FcR-positive cells, leads to the activation of an intracellular signaling cascade, which results in B-cell death by apoptosis (15, 36). Cross-linking of anti-CD20 antibodies on B cells leads to translocation of CD20 to lipid rafts, followed by activation of complement activation (37, 38). In addition, CD20 cross-linking results in a rapid up-regulation of members of the src family of tyrosine kinases, increased intracellular Ca2+ concentrations, up-regulation of the proapoptotic protein Bax, increased in RNA levels of c-myc and Berg, activation of mitogen-activated protein kinase family members p44 and 42, and increased activator protein DNA binding activity (39). Other antiapoptotic signaling pathways, including the extracellular signal-regulated kinase 1/2 and the nuclear factor-κB pathway, have been reported to be inhibited by rituximab, resulting in the sensitization of various B-cell lines to chemotherapy (40–42) and Fas receptor-mediated apoptosis induction (43). Interestingly, the protein expression of the transcription regulator YY1 was modified in our model. Although a treatment with rituximab resulted in a decreased expression of YY1 in antibody naive cells, we observed an overexpression of this protein in antibody resistant cells. This rituximab-induced overexpression of YY1 could be implicated in the resistance to combined therapy including rituximab and chemotherapeutic agents. Although it is generally believed that the mitochondrial pathway, activated via caspase-9, is the main apoptotic cascade induced by rituximab, other routes, activated via caspase-7 and caspase-8, have been reported as well (44, 45).

Several recent reports have shown that rituximab can induce a chemosensitization of NHL cell lines via the down-regulation of Bcl-XL (30, 40, 41, 46). p38 mitogen-activated protein kinase and signal transducers and activators of transcription 3 protein activity have been reported to be inhibited as a result of CD20 cross-linking by rituximab, subsequently down-regulating the antiapoptotic proteins Bcl-XL, Bcl-2, and inducing apoptosis protease activating factor 1 (46–48). In keeping with these observations, our data show increased Bcl-XL content in the resistant cells compared with the sensitive parental cells and confirm the recent results obtained in vitro by Jazirehi et al. (30), showing that the phenotype of resistant cells to rituximab may be associated with a higher expression of Bcl-XL. The onset of a rituximab-resistant phenotype has recently been shown to be associated with a down-regulation of the proapoptotic Bcl2 family proteins BAX and BAK responsible for associated resistance to chemotherapy (49). Conversely, we have not observed altered Bcl-2 or BAX content in our resistant line, whereas BAK levels were reduced. Multidrug-resistance phenotype (MDR) associated with the overexpression of transmembrane efflux pumps such as P-glycoprotein (MDR1) was not observed in the RI. cells, either rituximab-naïve or resistant (data not shown).

In conclusion, we have established and begun the characterization of a model of human FL resistant to rituximab in vivo. Two phenotypically different subpopulations emerged from these repeated exposures: a CD59+ and a CD20- population. The YY1 expression was also found to be increased in resistant cells. This model has a certain number of limitations, including its fast growth rate due to the fact that it is a transformed FL and is thus not representative of indolent disease, or its lack of immune human effector cells. In spite of these limitations, this model and others currently under development should prove to be quite useful to identify novel mechanisms of resistance to rituximab in vivo and in the development of novel monoclonal antibodies or therapeutic strategies, including drug combinations designed to overcome resistance to rituximab.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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