Lenalidomide down-regulates the CD20 antigen and antagonizes direct and antibody-dependent cellular cytotoxicity of rituximab on primary chronic lymphocytic leukemia cells

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Lenalidomide, an immunomodulatory agent that enhances antibody-dependent cellular cytotoxicity (ADCC), is currently being investigated as a therapy for chronic lymphocytic leukemia (CLL). The anti-CD20 antibody rituximab is active in CLL and represents a rational agent to combine with lenalidomide. We therefore examined whether lenalidomide combined with rituximab enhances direct apoptosis and ADCC in CLL cells. In contrast to previous reports using CD20-positive lymphoma cell lines, lenalidomide down-regulated CD20 surface antigen expression in CLL patient cells via enhanced internalization, without influencing transcription. The CD20 surface antigen internalization enhanced delivery of an oligonucleotide incorporated into anti-CD20 immunoliposomes. In addition, CD20 surface antigen down-modulation by lenalidomide in CLL was accompanied by diminished rituximab-mediated apoptosis and ADCC. These observations suggest a need for alternative sequencing strategies to avoid antagonism between lenalidomide and rituximab therapy in CLL. In addition, they suggest that lenalidomide therapy might be useful to enhance targeted delivery of RNAi-based therapies using CD20 immunoliposomes in B-cell malignancies.

Methods

Cell isolation

Blood was obtained from patients with CLL as described by National Cancer Institute (NCI) Working Group criteria. All patients provided informed consent under an Ohio State University Institutional Review Board–approved protocol in accordance with the Declaration of Helsinki. The clinical features of each of these patients are summarized in Table 1. CLL B cells were isolated by Ficoll centrifugation using Rosette-Sep reagent (StemCell Technologies, Vancouver, BC) according to the manufacturer’s instructions. CLL cells were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated human serum (Valley Biomedical, Winchester, VA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and ...
Table 1. Clinical characteristics of patients whose samples were used for in vitro studies

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100 U/mL penicillin/100 μg/mL streptomycin (Sigma-Aldrich, St Louis, MO) at 37°C in 5% CO₂. For ADCC experiments, CD56⁺ and CD19⁺ cells were negatively purified from whole blood obtained from either healthy volunteers or CLL patients by magnetic-activated cell sorting according to the manufacturer’s recommendations (MiniMACS; Miltenyi Biotec, Auburn, CA). The purity of enriched populations was always greater than 95% of the total yield as detected by CD19 and CD3 staining.

### Lenalidomide extraction and purification

The lenalidomide used for the in vitro CLL experiments was extracted from commercial capsules donated by several patients who had stopped treatment. Powdered material was stirred in a mixture of 250 mL ethyl acetate with 10 mL triethylamine for 3 hours and then filtered. This process was repeated with the residual powder 2 additional times. The collected organic solvent was dried to yield a white solid, which was used for biochemical testing. The purity of capsule-extracted lenalidomide was evaluated with nuclear magnetic resonance (NMR) and liquid chromatography/mass spectrometry in The Ohio State University Pharmacanalytical Shared Resource as published. The NMR spectra contained only lenalidomide and derivations thereof. Powdered material was stirred in a mixture of 250 mL ethyl acetate and 10 mL triethylamine for 3 hours and then filtered. This process was repeated with the residual powder 2 additional times. The collected organic solvent was dried to yield a white solid, which was used for biochemical testing.

### Immunophenotyping studies

Immunophenotyping was performed to determine the percentage and mean fluorescence intensity of CD20, CD19, CD5, CD23, CD38, CD3, and CD52 on CLL cells relative to a specific isotype control. Cells (10⁶) were washed with PBS, stained with fluorochrome-labeled antibodies at 4°C, then rinsed in PBS and analyzed by flow cytometry (EPICS- XL; Beckman Coulter, Fullerton, CA). A total of 10,000 events were collected and analyzed using Windows Multiple Document Interface for Flow Cytometry (WinMDI). CD20, CD19, CD3, CD5, CD23, and CD38 antibodies were obtained from BD Biosciences (San Jose, CA), and CD52 antibody was obtained from Serotec (Raleigh, NC).

### Intracellular CD20 staining

CLL cells were incubated with a molar excess of rituximab to saturate the binding of extracellular CD20. Cells were washed twice with PBS and then fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer’s recommendations. Cells were then incubated with CD20-phycocerythrin (PE) antibody, washed twice in PBS, and analyzed by flow cytometry. To ensure the specificity of the staining procedure, binding of IgG1-k isotype antibody was also measured.

### Immunoblotting

Immunoblot assays were performed with the multiple antigen detection immunoblotting method as described previously. Whole-cell lysates were prepared and stored in –80°C. Protein concentration was quantified by the bicinchoninic acid method ( Pierce Chemical, Rockford, IL). Protein samples (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) and transferred to 0.2-μm nitrocellulose membranes ( Whatman Schleicher & Schuell, Keene, NH) and incubated with goat anti-human CD20 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit antiactin (Cell Signaling Technology, Danvers, MA) antibodies. Horseradish peroxidase–conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA) was used as secondary antibody. Immune complexes were detected using chemiluminescent substrate (SuperSignal; Pierce Chemical).

### Analysis of cytotoxicity in presence of cross-linking

Cells (10⁶ cells/mL) were treated with commercially purchased alemtuzumab (anti-CD52; Ilex Pharmaceuticals, San Antonio, TX), rituximab (anti-CD20; Genentech, San Francisco, CA), or trastuzumab (anti-HER2; Genentech) at a concentration of 10 μg/mL. Goat antihuman IgG cross-linker (Fc specific; Jackson ImmunoResearch Laboratories, West Grove, PA) was added to the cell suspension 5 minutes after adding the primary antibodies at a concentration of 50 μg/mL. In addition, samples with no treatment or crosslinker alone were collected at matched time points. Apoptosis of cells at 24 and 48 hours after treatment was measured by annexin V–fluorescein isothiocyanate/propidium iodide (PI) flow cytometry analysis according to the supplier’s instructions (BD Biosciences). Cytotoxicity results were expressed as percentage of total positive cells over untreated control [% positive cells = (% annexin + and/or PI¹ cells of treatment group) – (% annexin + and/or PI¹ cells of untreated control)].
Analysis of ADCC

ADCC activity was determined by standard 4-hour $^{51}$Cr-release assay. $^{51}$Cr-labeled target cells (10$^5$ CLL cells/mL) were incubated with media alone or in the presence of various antibodies (10 $\mu$g/mL) at 37°C for 30 minutes. Unbound antibody was washed off and the cells plated at 10$^4$ cells/well. Effector cells (natural killer [NK] cells from healthy donors or CLL patients) were then added to the plates at indicated effector-to-target (E:T) ratios. After a 4-hour incubation, supernatants were removed and measured using a gamma counter. The percentage of specific cell lysis was determined by the equation: % lysis = 100 × (ER − SR)/(MR − SR), where ER, SR, and MR represent experimental, spontaneous, and maximum release, respectively. Data were normalized to the untreated control.

Semiquantitative reverse-transcribed polymerase chain reaction

RNA was extracted with TRizol reagent (Invitrogen) according to the manufacturer’s directions and converted to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). Real-time polymerase chain reaction was performed using custom-designed primers for CD20 (ID: Hs00544819_m1; Applied Biosystems, Foster City, CA) and an ABI Prism 7700 sequence detection system (Applied Biosystems). The expression of CD20 relative to an internal control gene was calculated by plotting the Ct (cycle number), and the average relative expression for each group was determined using the comparative method (2$^{-\Delta\Delta Ct}$).

Preparation of antibody-coated immunoliposomes

3β-[N-(N,N¢-Dimethylaminooethane)-carbamoyl]-cholesterol (DC-Chol) and DSPE-PEG-maleimide (DSPE-PEG-Mal) were purchased from Avanti Polar Lipids (Alabaster, AL). Methoxy-polyethylene glycol (molecular weight = 2000 Da)–distearyl phosphatidylthanolamine (DSPE-PAG) and egg phosphatidylcholine (Egg PC) were obtained from Lipoid (Newark, NJ). 2-Iminodiethanolamine (Traut reagent), 5.5'-dithiobis-(2-nitrobenzoic acid) (Eillon reagent), and other chemicals were purchased from Sigma-Aldrich. A carboxyfluorescein (FAM) terminus modified oligoethoxynucleotide (ODN) (5’-6 FAM- TAC CGC GTG CGA CCC TCT) was custom-synthesized by Alpha DNA (Montreal, QC). An ethanol dilution method was modified to prepare the FAM-ODN–loaded immunoliposomes. Briefly, protamine sulfate in citric acid (20 mM, pH 4) was mixed with lipids (DC-Chol/Egg PC/PEG-DSP-E [molar ratio] = 28.0:7.0:2.0) at a mass ratio of lipids:protamine = 12.5:0.3, followed by addition of ODN in citric acid (20 mM, pH 4) at ODN/lipids/protamine (weight ratio) = 1:12.5:0.3. The complexes were then dialyzed against citric acid (20 mM, pH 4) for 1 hour and then further dialyzed against N,N¢-2-ethanesulfonic acid–buffered saline (145 mM NaCl, 20 mM N,N¢-2-ethanesulfonic acid, pH 7.4) overnight, using a Dispo-Dialyzer (Spectrum Labs, Rancho Dominguez, CA) with a molecular weight cutoff of 10 000 Da. Liposome size distribution was analyzed by dynamic light scattering on a NICOMP Particle Sizer Model 370 (Particle Sizing Systems, Santa Barbara, CA). Volume-weighted analysis showed an average particle size of approximately 50 nm.

A postinsertion method was adopted to incorporate antibody ligands into preformed liposomes containing FAM-ODN. In this method, rituximab, trastuzumab (anti-HER2), or alemtuzumab (anti-CD52) was incubated with 20× Traut reagent (2 hours at room temperature) to yield sulfhydryl-modified antibodies. The anti-CD20–sulfhydril was then incubated with micelles of Mal-PEG-DSPE at a molar ratio of 1:10 and then incubated with FAM-ODN loaded liposomes for 1 hour at 37°C. Targeted liposomes with antibody-PEG-DSPE-to-lipid ratios of 1:1000 (0.1 mol/dL) were thus prepared.

Rituximab immunoliposome uptake studies

CLL cells were incubated at 10$^6$ cells/mL for 1 hour at 37°C with rituximab-, trastuzumab-, or alemtuzumab-coated immunoliposome-FAM-ODN (CODN = 1.0 $\mu$M) and washed twice in PBS. Cells were kept in culture in RPMI 10% fetal bovine serum for an additional 1, 4, and 24 hours, respectively, in the presence of either 0.5 $\mu$M lenalidomide or vehicle control. Cells were then washed once with cold 0.5 M glycine buffer (pH 2.8) containing 0.15 M NaCl followed by cold PBS wash to remove extracellular immunoliposome particles resulting from acid wash has been shown to induce detachment of antibody from antigen.26,27 We further verified that this was the case by flow cytometry (data not shown). Cells were visualized on a Nikon fluorescence microscope (magnification ×200).

Laser-scanning confocal microscopy

Binding and internalization of anti-CD20 in CLL cells were examined by laser scanning confocal microscopy. CLL cells were incubated with lenalidomide for 48 hours at 37°C and then collected and washed twice with PBS followed by fixation with 2% paraformaldehyde. Cells were then attached on a slide glass using Cytospin (Thermo Electron, Waltham, MA) and permeabilized with cold acetone for 5 minutes. Before cell membrane and nucleus staining, cells were preincubated with 1% bovine serum albumin in PBS for 1 hour at room temperature to reduce nonspecific binding. Cells were incubated with 50 $\mu$g/mL concanavalin A (Invitrogen) for 5 minutes at room temperature to stain cell membrane and with 1 $\mu$g/mL DRAQ5TM (Biostatus, Leicestershire, United Kingdom) for 5 minutes at room temperature to stain nucleus. Finally, slides were rinsed twice with PBS and covered with glass for confocal microscopy observation. Green fluorescence of fluorescein isothiocyanate–CD20, red fluorescence of concanavalin A, and blue fluorescence of DRAQ5 were analyzed, and merged images were produced using Zeiss 510 META Laser Scanning Confocal Imaging Systems and LSM Image software (Carl Zeiss, Thornwood, NY).

Statistical analyses

All analyses were performed by the Center for Biostatistics, The Ohio State University. Mixed effects models were fitted to the cytotoxicity data. Random effects associated with the interactions were always included in the models to ensure that error was not underestimated. SPSS software, version 13.0 (SPSS, Chicago, IL) was used for the statistical analysis. Nonparametric paired Wilcoxon signed ranks tests were performed to examine both the proportion and mean fluorescence intensity change of cells expressing specific antigens after treatment with lenalidomide or media for 48 hours. A paired t test was used to evaluate whether there was a change in CD20 expression level between cells treated with lenalidomide and untreated control.

Results

Lenalidomide causes down-regulation of the CD20 antigen in primary CLL cells

Given that previous studies in lymphoma cell lines showed modest apoptosis and cell-cycle arrest with lenalidomide, we assessed the ability of lenalidomide to promote apoptosis in CLL patient cells. CLL cells incubated with 0.5 $\mu$M lenalidomide for 48 or 72 hours exhibited no apoptosis in vitro (data not shown) in agreement with previous reports.28 However, as shown in Figure 1A, at least 40% reductions in the proportion (left panel, P < .001) and relative mean fluorescence intensity (right panel, P = .001) of CD20–expressing cells were observed in samples treated with lenalidomide compared with vehicle-treated controls (n = 18). Table 2 reports CD20 relative mean fluorescence intensity and percentage values for all the samples analyzed. No differences were observed between vehicle (PBS)–treated and untreated cells (data not shown); therefore, vehicle treatment was chosen as experimental control for all the in vitro studies. Lenalidomide treatment induced also increased levels of CD23 and CD38 activation markers on CLL cells (P = .001 and P < .05, respectively; Figure 1D) whereas no changes in CD19, CD5, and CD52 (data not shown) expression
was observed. A representative case is shown in Figure 1B. The effect of a wider dose range of lenalidomide (0.05-3 μM) on the CLL cells was tested. The levels of CD20 and CD52 antigens were evaluated at 24, 48, and 72 hours (Figure 1C) for each dose. The maximum effect on CD20 down-regulation was achieved at 72 hours with 0.5 μM lenalidomide. Therefore, this concentration was chosen to perform all the in vitro studies described in this report.

We next sought to determine whether the mechanism by which lenalidomide down-regulates CD20 antigen was via decreased transcription. To study this, CD20 mRNA expression level was assessed by real-time reverse-transcribed polymerase chain reaction in lenalidomide- and vehicle-treated CLL cells at 24 and 48 hours. Figure 1E demonstrates that lenalidomide treatment resulted in a very modest increase in CD20 mRNA transcription at 24 and 48 hours compared with vehicle control. Previous reports have suggested that, in the setting...
of B-cell activation, CD20 can be internalized.\textsuperscript{29} We therefore tested whether the lenalidomide-mediated reduction in CD20 expression observed by flow cytometry was the result of CD20 internalization. Reduction of CD20 antigen surface expression was observed in 6 of 8 CLL patient samples after lenalidomide treatment, and this was associated with increased intracellular fluorescence compared with vehicle-treated controls ($P = .006$; Figure 2A). This was further confirmed by confocal microscopy analysis (Figure 2B). These results suggest that CD20 is actively internalized in CLL cells after lenalidomide treatment. Consistent with this hypothesis, immunoblot analysis of CLL cells treated with lenalidomide for 48 and 72 hours showed no variation in the total level of CD20 protein relative to vehicle control (Figure 2C).

**Lenalidomide-mediated internalization of CD20 enhances intracellular delivery of ODN in rituximab immunoliposomes.**

Given the internalization of the CD20 antigen after lenalidomide treatment, we hypothesized that this may enhance CD20-liposome-mediated intracellular drug delivery. To test this hypothesis, we used a FAM-ODN formulated into rituximab-coated liposome-mediated intracellular drug delivery. To test this hypothesis, we formulated a FAM-ODN into rituximab-coated liposomes to deliver ODN into CLL cells. To determine whether this treatment may enhance CD20-mediated ODN delivery, we hypothesized that this may enhance CD20-liposome-mediated intracellular drug delivery. To test this hypothesis, we used a FAM-ODN formulated into rituximab-coated liposomes. CLL cells were incubated at 10$^6$ cells/mL for 1 hour at 37°C with rituximab-coated immunoliposome-FAM-ODN ($C_{ODN} = 1.0 \mu M$) and washed twice in PBS. Cells were kept in culture in RPMI 10% FBS for an additional 1, 4, or 24 hours in the presence of either 0.5 $\mu M$ lenalidomide or vehicle control. Extracellular bound rituximab immunoliposomes were then washed. Cells were visualized on a Nikon fluorescence microscope (magnification, $\times 200$). Lenalidomide treatment enhanced internalization of fluorochrome-labeled ODN in CLL samples as early as 1 hour after treatment with a maximum effect seen at 24 hours ($N = 3$; Figure 3A). In contrast, no enhancement of internalization of fluorochrome-labeled ODN was noted in vehicle-treated cells. This phenomenon requires binding of the CD20 antigen as demonstrated by the lack of fluorochrome-labeled ODN delivery using trastuzumab-coated immunoliposome (Figure 3B). Furthermore, no differences were observed in the internalization of fluorochrome-labeled ODN with alemtuzumab immunoliposome between lenalidomide- and vehicle-treated cells in agreement with the observation that lenalidomide does not modulate the levels of the CD52 antigen. These studies provide further support that lenalidomide causes antigen-specific internalization of CD20 on CLL cells that leads to enhanced delivery of a fluorochrome-labeled ODN.

**Lenalidomide treatment antagonizes rituximab-mediated apoptosis in CLL cells**

Given the observed modulation of CD20 antigen on CLL cells by lenalidomide, we next assessed whether this treatment adversely...
Influenced cell death promoted by rituximab. We observed that lenalidomide treatment at 48 hours significantly decreased antibody-mediated apoptosis promoted by rituximab in presence of cross-linking (25% ± 11% cell death compared with 11% ± 8% in vehicle- and lenalidomide-treated CLL cells, respectively; \( P < .017, \) \( N = 10; \) Figure 4A). No significant change in alemtuzumab-mediated apoptosis was observed under these same conditions.

**Lenalidomide treatment induces CD56 expression in CD16^−CD56^+ cells and enhances NK cell–mediated ADCC**

Previous studies have demonstrated that lenalidomide treatment of normal NK cells enhances CD16 expression and ADCC-mediated by the anti-CD40 antibody SGN40.\(^{30}\) Treatment of purified NK cells with lenalidomide for 72 hours resulted in an increase in the percentage of CD16^−CD56^+ cells (31% ± 14% compared with 18% ± 10% in lenalidomide- and vehicle-treated NK cells, respectively; \( N = 12, P < .01; \) Figure 4B left panel). Interestingly, the increase in CD56^+ cells was associated with a decrease in CDC16^−CD56^− cells (20% ± 11% compared with 35% ± 13% in lenalidomide- and vehicle-treated NK cells, respectively; \( N = 12, P < .005 \)). This suggests the activation of competent CD16^+ CD56^−NK cells by lenalidomide (Figure 4B right panel).

A representative result for CD56 and CD16 surface expression of lenalidomide-treated NK cells is shown in Figure 4C. Consistent with this hypothesis, Figure 5A demonstrates that lenalidomide-treated NK cells from a healthy donor resulted in an overall enhancement of rituximab-mediated ADCC against target CLL patient cells (\( N = 4, P = .009 \)). Similar results were observed with NK cells derived from patients with CLL (\( N = 2, P = .02; \) Figure 5B).

**Lenalidomide treatment of CLL target cells antagonizes rituximab-mediated ADCC**

Given our findings that lenalidomide induced down-regulation of CD20 in CLL cells while enhancing NK-mediated ADCC function, we tested the effect of lenalidomide treatment of B cells on CD20-mediated ADCC using rituximab. Pretreatment of CLL B cells at concentrations of lenalidomide previously shown to down-regulate CD20 resulted in a decrease in rituximab-mediated ADCC by either allogeneic (\( N = 5, P < .05; \) Figure 6A) or autologous (\( N = 2, P < .05; \) Figure 6B) NK cells. As both target CLL cells and NK cells would be subject to lenalidomide effects in vivo, we tested the effect of cotreatment of target CLL B cells and NK cells on rituximab-mediated ADCC function. Peripheral blood mononuclear cells from CLL patients were incubated for 72 hours with 0.5 \( \mu \)M lenalidomide or vehicle control. After this, B- and NK-cell fractions were purified by magnetic activated cell sorting. Compared with NK-only controls, the cotreatment of autologous NK cells and primary CLL cells with lenalidomide resulted in diminished rituximab-mediated ADCC (\( N = 2, P = .02; \) Figure 6C). In contrast, no significant change in ADCC was observed between lenalidomide versus untreated cells using alemtuzumab (data not shown).

**Discussion**

Herein, we have demonstrated that the innate immune-activating agent lenalidomide promotes different biologic effects against patient-derived CLL cells compared with NHL cell lines as previously reported. Specifically, we demonstrate that lenalidomide at concentrations attainable in vivo causes down-regulation of CD20 antigen on the surface of CLL cells. This CD20 surface antigen down-modulation is not generalized to other surface antigens, as the levels of other B-cell antigens, such as CD52 and CD19, do not change. The mechanism of this lenalidomide-mediated down-regulation of surface CD20 does not involve decreased transcription but probably instead represents internalization of this molecule. We have demonstrated a potential application of this internalization, in that delivery of oligonucleotide to CLL cells via CD20 immunoliposomes is enhanced. More importantly, this finding may have particular clinical relevance as simultaneous lenalidomide treatment significantly antagonizes rituximab-mediated apoptosis and ADCC in vitro. This effect was not generalized, as the effects of alemtuzumab were not altered by lenalidomide treatment. Overall, these findings emphasize the complexity of introducing new biologic agents such as lenalidomide with other established agents such as rituximab for CLL.

The lenalidomide used in this study was not derived from the pharmaceutical company Celgene (Summit, NJ) that markets...
it but rather was acquired after several patients donated their pharmaceutical-grade medication that they had ceased taking. The lenalidomide was extracted from these tablets using the procedures outlined in “Methods” and confirmed to have very high purity by NMR and liquid chromatography/mass spectrometry analysis performed by The Ohio State University Pharmacoanalytical Shared Resource using a highly sensitive assay.22 The NMR spectra contained only lenalidomide resonance peaks and had no indication of contaminating materials. This same assay is used to measure lenalidomide plasma concentrations as part of ongoing clinical trials at The Ohio State University. Repeated experiments showed consistent biologic activity of lenalidomide as suggested by consistent and reproducible activation of CLL cells and also innate immune effector cells. Obtaining lenalidomide in this manner allowed rapid performance of the research described in this report. In addition, it facilitated performance of 2 other recently published studies where the etiology of tumor flare induced by lenalidomide31 or reversal of the T-cell defect32 by this same agent in CLL patients was reported. The careful analytical assessment performed to assure purity of extracted lenalidomide provides assurance that the reagent used is of high purity. The research performed with extracted lenalidomide also used methodology to assure protection of laboratory research workers from undue exposure to this agent as is typically applied for any biohazardous compound. Studies that use extracted reagent as performed with lenalidomide in this study should always include bioanalytical assessment to ensure purity of the compound.

NK cells from CLL patients have been reported to be defective relative to those from healthy volunteers.33-36 Our data show that autologous NK cells from CLL patients demonstrate similar behavior as normal NK cells with respect to enhanced ADCC after treatment with lenalidomide.30 As observed with direct apoptosis, ADCC of CLL target cells was also diminished by lenalidomide treatment using both allogeneic and autologous NK cells. The use of primary CLL cells as targets, as opposed to B-cell lymphoma cell lines, reduces the ability to fully determine whether this antagonism is the result of CD20 down-regulation and diminished FcγRIIIa binding versus an alteration of the CLL cell that makes it...
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Figure 5. Lenalidomide treatment of allogenic or autologous derived NK cells resulted in enhanced rituximab-mediated ADCC of untreated CLL cells. (A) ADCC was measured using lenalidomide-treated NK cells from normal volunteers and B-CLL cells at 12.5:1, 25:1, and 50:1 E/T in the presence or absence of 10 μg/mL rituximab or trastuzumab. Columns, average of triplicate wells, were representative of 3 independent experiments; bars represent SD. The overall lenalidomide versus vehicle rituximab-mediated ADCC was significantly higher for lenalidomide (P = .009). (B) ADCC was measured using lenalidomide-treated NK and B cells from CLL patients at 12.5:1 and 25:1 E/T in the presence or absence of 10 μg/mL rituximab or trastuzumab. Columns, average of triplicate wells, were representative of 2 independent experiments; bars represent SD (N = 2, P = .02).

Figure 6. Lenalidomide treatment of B-CLL cells resulted in diminished ADCC mediated by rituximab. (A) ADCC was measured using freshly isolated NK cells from normal volunteers and lenalidomide- (0.5 μM) or vehicle control-treated B-CLL cells at 12.5:1 and 25:1 E/T in the presence or absence of 10 μg/mL rituximab or trastuzumab. Columns, average of triplicate wells, were representative of 5 independent experiments; bars represent SD. The overall lenalidomide- versus vehicle rituximab-mediated ADCC was significantly lower for lenalidomide (P < .05). (B) ADCC was measured using untreated NK and lenalidomide- or vehicle-treated B cells from CLL patients at 12.5:1 and 25:1 E/T in the presence or absence of 10 μg/mL rituximab or trastuzumab. Columns, average of triplicate wells, were representative of 2 independent experiments; bars represent SD (N = 2, P < .05). (C) ADCC was measured using lenalidomide- or vehicle control-treated NK and B cells from CLL patients at 12.5:1 and 25:1 E/T in the presence or absence of 10 μg/mL rituximab or trastuzumab. Columns, average of triplicate wells, were representative of 2 independent experiments; bars represent SD (N = 2, P = .02).

more resistant to NK cell–mediated killing. In other systems with IgG1 antibodies, antigen density on the target cell influences the degree of ADCC observed.37 Further investigation of how lenalidomide diminishes rituximab-mediated ADCC is warranted. Interestingly, the effect of antibody-dependent cellular phagocytosis by human monocytes was not modulated by lenalidomide (data not shown).

Previous studies have demonstrated that activation of CLL cells results in decreased expression of cell surface CD20 expression.38 In prior studies examining the influence of CD40 antigen activation on normal B cells, CD20 surface antigen was observed to decrease. This was not accompanied by changes in transcription but was blocked by protein kinase C inhibitors. Our data described herein demonstrate that lenalidomide promotes internalization of CD20 in CLL cells possibly through a similar activating mechanism. Indeed, preliminary studies by others39 and our own group (in a full peer-reviewed manuscript) demonstrate that lenalidomide treatment of CLL cells increases costimulatory molecules, including CD86, CD40, CD80, and CD95 as is observed with B-cell activation. The CD20 antigen generally does not internalize significantly in CLL cells, which is one of the advantages of rituximab. However, treatment of CLL cells with lenalidomide in this study greatly enhanced CD20 internalization and hence the ability to deliver oligonucleotide therapy using CD20 antibody-mediated immune liposomes. Intracellular delivery of oligonucleotide-based therapeutics, including CpG oligonucleotides, siRNA, and microRNA are all relevant to CLL therapy, and our data suggest that lenalidomide treatment might significantly improve the efficacy of B-cell–specific targeted therapy with CD20 immunoliposomes.

Our data have several implications for the clinical use of lenalidomide in the treatment of CLL and other B-cell malignancies. First, they show important differences with lenalidomide between primary CLL cells and B-cell tumor lines, indicating that preclinical findings with this agent should be confirmed in primary
tumor cells before translating a concept to the clinic. Our data also suggest that concurrent administration of rituximab with lenalidomide in CLL may indeed be counterproductive because of reduction in target expression. However, alternative sequences of administration, such as administering rituximab first and then treating with lenalidomide, might be beneficial. Ideally, we would like to study this in a xenograft murine model designed to test therapeutic human antibodies against engrafted primary CLL cells. To our knowledge, lenalidomide is one of the first biologic agents that favorably alters the innate immune system introduced for the therapy of B-cell malignancies, but its use must be evaluated carefully based on its potential influence on target antigens.

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Authorship
Contribution: R.L. performed the experiments related to lenalidomide and participated in analysis of the data; Q.L. set-up analytical methods to measure lenalidomide and participated in analysis of the data; J.P.B. performed the monococyte/macrophage experiments and participated in analysis of the data; G.L. provided technical advice related to the internalization studies and participated in analysis of the data; A.R., L.L.S., and G.M. assisted with the lenalidomide experiments; W.B. and L.A. participated in obtaining reagents for this study and assisted with patient sample acquisition; D.-S.W. performed the lenalidomide extraction for this study; A.L. participated in the statistical analysis of the data; C.-S.C. performed the lenalidomide extraction for this study and provided input to experimental design; A.J.J. assisted with the lenalidomide experiments and provided input into experimental design; R.J.L. and L.J.L. provided input into design of immune-targeted liposomes and reviewed drafts of the manuscript along with the final version of the paper; S.T. provided input into design of the monococyte/macrophage experiments and reviewed drafts of the manuscript along with the final version of the paper; N.M. provided design of the experiments and reviewed drafts of the manuscript along with the final version of the paper; J.C.B. oversaw the entire research project, including obtaining funding for the project, planning the experiments, drafting the first version of the paper, and reviewing each draft version and the final paper; and all authors reviewed the final version of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests. A provisional patent was filed on using this method to deliver RNA-based therapy.

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References


Lenalidomide down-regulates the CD20 antigen and antagonizes direct and antibody-dependent cellular cytotoxicity of rituximab on primary chronic lymphocytic leukemia cells

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