Targeting CD38 Enhances the Antileukemic Activity of Ibrutinib in Chronic Lymphocytic Leukemia

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Abstract

Purpose: CD38 has emerged as a high-impact therapeutic target in multiple myeloma, with the approval of daratumumab (anti-CD38 mAb). The clinical importance of CD38 in patients with chronic lymphocytic leukemia (CLL) has been known for over 2 decades, although its relevance as a therapeutic target in CLL remains understudied.

Experimental Design: We investigated the biological effects and antitumor mechanisms engaged by daratumumab in primary CLL cells. Besides its known immune-effector mechanisms (antibody-dependent cell-mediated cytotoxicity, complement-dependent death, and antibody-dependent cellular phagocytosis), we also measured direct apoptotic effects of daratumumab alone or in combination with ibrutinib. In vivo antileukemic activity was assessed in a partially humanized xenograft model. The influence of CD38 on B-cell receptor (BCR) signaling was measured via immunoblotting of Lyn, Syk, BTK, PLCγ2, ERK1/2, and AKT.

Results: In addition to immune-effector mechanisms; daratumumab also induced direct apoptosis of primary CLL cells, which was partially dependent on FcγR cross-linking. For the first time, we demonstrated the influence of CD38 on BCR signaling where interference of CD38 down-regulated Syk, BTK, PLCγ2, ERK1/2, and AKT; effects that were further enhanced by addition of ibrutinib. In comparison to single-agent treatment, the combination of ibrutinib and daratumumab resulted in significantly enhanced anti-CLL activity in vitro and significantly decreased tumor growth and prolonged survival in the in vivo CLL xenograft model.

Conclusions: Overall, our data demonstrate the antitumor mechanisms of daratumumab in CLL; furthermore, we show how cotargeting BTK and CD38 lead to a robust anti-CLL effect, which has clinical implications.

Introduction

CD38 is a highly conserved transmembrane type II glycoprotein expressed on B lymphocytes and other hematopoietic cells (1). Physiologically, CD38 functions as an ectoenzyme and a coreceptor, the latter depending on its spatiotemporal association with other cell surface (and cell-type specific) antigens. On B lymphocytes, CD38 associates with the B-cell receptor (BCR) complex [(BCR)/CD81/CD19/CD21] and amplifies signal intensity transmitted through the complex to drive cell proliferation (2). Patients with CLL with a higher proportion of CLL cells expressing CD38 (>30%) have a shorter time to symptomatic disease and a more aggressive clinical course with inferior survival versus patients who have <30% of CD38 + CLL cells (3, 4), thus establishing CD38 expression as a marker of poor prognosis (5, 6). Despite its known association with an aggressive CLL phenotype, the role of CD38 as a therapeutic target remains unclear.

Daratumumab is a first-in-class anti-CD38 therapeutic mAb approved for the treatment of relapsed/refractory multiple myeloma (7). It comprises a fully human IgG1κ mAb, which binds the C-terminus of CD38 at an epitope composed of β-strand–containing amino acids 233–246 and 267–280 (8). A report by Matas-Cespedes and colleagues demonstrated the antileukemic effects of single-agent daratumumab in ex vivo and in vivo CLL models (9). The cytotoxicity reported was modest, with partial insight into the direct killing mechanism of daratumumab in CLL cells. We hypothesized that CD38 is a high value target in CLL and blocking of its receptorial function can be translated into a clinically beneficial therapeutic strategy through improved understanding of the mechanism(s) that link CD38 to CLL cell survival. Here, we provide evidence that CD38 engagement

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Translational Relevance

Increased CD38 expression on chronic lymphocytic leukemia (CLL) cells is linked to aggressive disease features and poor clinical outcome. Biologically, CD38 promotes CLL cell proliferation through association with multiple cell surface receptors, including the B-cell receptor (BCR). As therapeutic opportunities to disrupt CD38 function become increasingly available, we investigated the antitumor activity of daratumumab (anti-CD38 human mAb) in patient-derived CLL cells. Daratumumab was noted to promote immune-effector-mediated cytosis, as well as direct apoptosis of CLL cells. In vivo, the combination of ibrutinib and daratumumab significantly delayed tumor growth in B-cell leukemia-bearing mice and prolonged their survival. Altogether, our results suggest that combination of ibrutinib and daratumumab yields greater anti-CLL activity than either agent alone and support clinical evaluation of this regimen in patients with CLL.

Materials and Methods

Written informed consent was obtained from all patients whose samples were used in this study, approved by the Mayo Clinic Jacksonville Institutional Review Board and in accordance with the Declaration of Helsinki. Peripheral blood was collected from patients with a confirmed diagnosis of CLL who were not on active anti-CLL treatment or those off anti-CLL therapy for ≥1 month. This was followed by isolation of CD19+/CD5+ B cells (primary CLL cells). Peripheral blood mononuclear cells (PBMC) from human donors were used in antibody-dependent cell-mediated cytotoxicity (ADCC) assays, 10% human serum was used in complement-dependent death (CDC) assays, and human macrophages were used in antibody-dependent cellular phagocytosis (ADCP) assays, as described by de Weers and colleagues (8). Apoptosis, mitochondrial transmembrane permeability, and Western blotting assays were conducted as per prior methods (10–13). All cells were cultured in AIM-V media under conditions previously reported by us (10, 11). CD38 receptor density on CLL cells was quantified as mean fluorescence intensity (MFI) and cell surface antibody bound/cell (sAbc). For certain experiments, PBMCs were isolated from patients 4, 18, 19, 28, and 31 and CD19/CD5+ CLL cells were selected out using magnetic beads, followed by flow sorting with an anti-CD38 APC antibody for separation of CD19+/CD38hi and CD19+/CD38lo-purified cells. Cells were then treated with trypsin-EDTA for 10 minutes and washed twice followed by culture in AIM-V serum-free media for ≥24 hours. CD38 expression in purified cells was again reassessed using a multi-epitope FITC-conjugated anti-CD38 antibody (Cytoxons CD38 multi-epitope-FITC antibody). Our sorting strategy is presented in Supplementary Figs. S1 and S2. JVM13 (CD38+) and MEC1 (CD38−) cell lines were also used in experiments. An in vivo model of disseminated disease (14, 15) was established using luciferase-labeled JVM13 (JVM13-Luc) cells, injected via tail vein intravenously into 6- to 8-week old NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice, following a protocol approved by the Mayo Clinic Institutional Animal Care and Use Committee. Ibrutinib and kuromanin were purchased from Selleckchem. Daratumumab was acquired through the Mayo Clinic pharmacy and came predissolved/diluted. Statistical analyses were performed using R Statistical Software (version 3.2.3; R Foundation for Statistical Computing), further detailed in figure legends. Data are represented as mean ± SEM, unless otherwise stated in the figure legend.

A full description of all assays is presented in Supplementary Materials and Methods.

Results

Patient and sample characteristics

Patients with CLL (n = 36) representing all clinical and genetic subsets were included in the study (clinical characteristics in Supplementary Tables S1 and S2). Relatively consistent with prior reports, we noted 27.7% (n = 10) of patients had CD38+ disease (using standard 30% cutoff; refs. 16, 17), whereas the remaining patients (n = 26, 72.2%) had CD38− disease. Notably, 16.6% (n = 6) of patients carried a deletion in chromosome 17p positive (Del17p+).

Daratumumab induces immune-mediated cytotoxicity in CLL cells

We first investigated the ability of daratumumab to induce CLL-specific lysis through immune-effector mechanisms (ADCC, CDC, and ADCP). Mean specific lysis from ADCC was 17.59% ± 1.30% (Fig. 1A). Subset analysis in CLL cells from patients defined as having CD38+ and CD38− disease showed significantly greater ADCC (P = 0.023) in CD38+ cases (24.63% ± 3.46%) versus CD38− cases (14.11% ± 1.11%; Fig. 1B). In flow-sorted CD19+/CD38hi clones treated with daratumumab, ADCC was noted in 22.45% ± 2.36% of cells. And in CD19+/CD38lo clones, ADCC was noted in 16.47% ± 1.03% of cells (Fig. 1C). We then assessed cell death via CDC in all CLL cells (n = 30) and noted that mean specific lysis was in the order of 14.88% ± 0.92% (Fig. 1D). Marginally higher levels were observed in CLL cells from CD38+ patients (18.22% ± 2.41%) versus CD38− patients (13.58% ± 0.95%; Fig. 1E). In flow-sorted cells, CDC was noted in 19.10% ± 1.98% and 9.23% ± 1.81% of CD19+/CD38hi clones and CD19+/CD38lo clones, respectively. (Fig. 1F). Comparative analysis in JVM13 and MEC1 cells did not show significant induction of ADCC or CDC with daratumumab alone (Supplementary Fig. S3A–S3C). Cell death through ADCP has been previously reported with daratumumab in myeloma cells (18). We noted ADCP in 8.52% ± 0.44% of CLL cells (Fig. 1G); with little difference observed between CLL cells from CD38+ (9.37% ± 0.77%) versus CD38− patients (8.79% ± 0.92%). Similar findings were seen in flow-sorted CD19+/CD38hi or CD19+/CD38lo-purified cells. (Fig. 1H). Comparative analysis in the cell lines showed higher ADCP in JVM13 (18.01% ± 1.0%) versus MEC1 (3.55% ± 0.14%; Supplementary Fig. S3D). Correlation between ADCP results, CD38 MFI (r = 0.49; P = 0.006), and CD38 sAbc (r = 0.41; P = 0.021) was significant (Supplementary Fig. S4A and S4B). Similarly, correlation between CDC results and percent of
Figure 1.
Daratumumab induces CLL cell death through immune effector-mediated mechanisms. A, ADCC induced by single-agent daratumumab (Dara, 0.1 μg/mL) was determined in Calcein-AM–labeled primary CLL cells (target) from 30 patients, ex vivo, in the absence or presence of effector (peripheral blood mononuclear) cells from healthy human donors at an effector:target (E:T) ratio of 50:1 for 6 hours. Specific lysis was calculated as described in Supplementary Materials and Methods. Spontaneous release was determined using a nonspecific IgG1-b12 isotype antibody at 0.1 μg/mL. B, ADCC in primary CLL cells from CD38<sup>+</sup> (n = 8) and CD38<sup>-</sup> (n = 22) patients (Pts) was assessed as a subset analysis and showed significantly higher specific lysis in cells from CD38<sup>+</sup> patients. C, ADCC was also assessed in flow-sorted CD19<sup>+</sup>/CD38<sup>hi</sup> and CD19<sup>+</sup>/CD38<sup>lo</sup> CLL cells from five patients (patients 4, 18, 19, 28, and 31) and revealed a similar trend. D, Specific lysis from CDC was measured in CLL cells in the presence of 10% human serum from a single healthy donor for 1 hour. E, Subset analysis of specific lysis from CDC induced in CLL cells from CD38<sup>+</sup> (n = 8) versus CD38<sup>-</sup> (n = 22) patients was also determined. F, Similarly, CDC was determined in flow-purified CD19<sup>-</sup>/CD38<sup>hi</sup> and CD19<sup>-</sup>/CD38<sup>-</sup> CLL cells. G, Cell death through phagocytosis was assessed in Calcein-AM–labeled primary CLL cells (n = 30), with subset analysis in CD38<sup>-</sup> versus CD38<sup>+</sup> cases and separately in flow-purified CD19<sup>-</sup>/CD38<sup>hi</sup> and CD19<sup>-</sup>/CD38<sup>-</sup> CLL cells (H). Results are expressed as mean ± SEM. Comparative significance analyses between the groups (brackets) show P (*, P ≤ 0.05; **, P < 0.01; ***, P < 0.001).
CD38-expressing cells was also significant ($r = 0.49$; $P = 0.006$; Supplementary Fig. S4C). ADCC cell death assay results did not show any significant correlation with either CD38 MFI/sAbc or percent of CD38-expressing cells (Supplementary Table S3).

**Daratumumab can directly induce apoptosis of CLL cells**

Prior studies in multiple myeloma cells suggest that daratumumab can directly induce apoptosis (19). In CLL cells treated with daratumumab, we noted $31.33\% / 2.56\%$ annexin-V/propidium iodide (PI) positivity overall (Fig. 2A). Subset analysis of CD38$^+$ and CD38$^-$ cases showed greater annexin-V/PI positivity in CD38$^+$ patients ($36.41\% / 3.34\%$) versus CD38$^-$ patients ($23.39\% / 1.70\%$; $P = 0.002$; Fig. 2B and C); with similar effects in JVM13 ($37.58\% / 1.88\%$) versus MEC1 cells (14.82% $\pm$ 1.37%; Supplementary Fig. S5). In unsorted CLL cells from patients with either CD38$^+$ or CD38$^-$ disease, a significant correlation was observed between percent apoptosis with CD38 MFI ($r = 0.39$; $P = 0.036$), CD38 sAbc ($r = 0.53$; $P = 0.003$), and percent CD38 positivity of CLL cells ($r = 0.45$; $P = 0.012$; Supplementary Table S3). Interestingly, in flow-sorted CLL cells, a significant degree of apoptosis ($P < 0.001$) was noted in CD19$^+$/CD38$^{hi}$ cells ($70.10\% / 12.18\%$), with comparatively lower...
apoptosis in CD19+/CD38lo cells (31.57% ± 11.22%; Fig. 2D and F). A significant correlation between apoptosis results with percent CD38hi CLL cells (r = 0.45; P = 0.012), CD38 MFI (r = 0.39; P = 0.036), and CD38 sAbc (r = 0.53; P = 0.003) was observed (Supplementary Fig. S4D–S4F). Other than association between degree of CDC induction and patient age (P = 0.036), no other significant correlation was observed between daratumumab-mediated cell death and clinical characteristic of patients (Supplementary Table S4).

To test whether small-molecule–based inhibition of CD38 could induce apoptosis, we used kuromanin, a flavonoid inhibitor of CD38 enzymatic activity (20, 21), which has been previously used to interrogate CD38 biology (22). In flow-sorted CLL cells, apoptosis induced by kuromanin was significantly greater (P < 0.001) in CD19+/CD38hi versus CD19+/CD38lo cells (54.97 ± 4.99 vs. 15.60 ± 1.28, respectively; Fig. 2E and F).

In a subset of CLL patient cells, we also examined whether daratumumab-induced apoptosis was due to FC-gamma receptor (FcγR) cross-linking. CLL cells were noted to express FcγRII (CD64, 73.67% ± 3.37%), FcγRIII (CD32, 95.18% ± 1.85%), and FcγRI (CD16, 19.31% ± 3.89%; Supplementary Fig. S6). When treated with daratumumab + an FcγR blocker, the percentage of apoptotic CLL cells was significantly lower (18.7% ± 0.13%) than those treated with daratumumab alone (27.21% ± 1.67%; P = 0.007). Addition of an F(ab’)2 fragment to daratumumab-treated CLL cells triggered further apoptosis (39.43% ± 0.56%); however, a decrease was noted when FcγR blocker was added (29.91% ± 1.33% apoptosis; P = 0.0079; Fig. 2G and H). Altogether, these results indicated to us that daratumumab-mediated apoptosis in CLL cells is partially dependent on FcγR cross-linking.

**Targeting CD38 modulates proteins associated with BCR signaling**

CD38 colocalizes with CD19 and CD81 in the lipid rafts at the cell membrane and this result in amplification of BCR and collateral signaling events (23). CLL cells with high proliferative potential inherently depend on BCR signaling and consequently are also reliant on CD38 coreceptorial function (24–26). Indeed, prior studies have demonstrated an increase in ERK activity (one of the terminal effectors of BCR signaling) upon CD38 agonistic ligation (21–23, 27). Given our interest in targeting CD38, its established role in BCR signal amplification (23, 27) and the high clinical relevance of BCR-targeting agents in CLL (28), we questioned whether therapeutic interference of CD38 would modulate the BCR pathway. Basal expression of BCR-signaling components: (p) p-Lyn, p-Syk, p-BTK, p-PLCγ2, p-ERK1/2, and p-AKT in CLL cells from CD38hi versus CD38lo patients is shown in Supplementary Fig. S7A–S7F. We treated (IgM-stimulated) CLL cells from both CD38hi and CD38lo patients with daratumumab, ex vivo, and noted a significant decrease in p-Syk, p-BTK, p-ERK1/2, and p-AKT (Fig. 3A–C for CD38hi CLL patient cells and Fig. 3H–N for CD38lo CLL patient cells; compare red bars with black bars; P < 0.05). As expected, comparative analysis between CLL cells from the CD38hi versus CD38lo patients revealed the percent decrease in proximal BCR-signaling proteins (p-Lyn, p-BTK, and p-PLCγ2) elicited by daratumumab was significantly more notable in CLL cells from CD38hi patients (P < 0.05). Intriguingly, we observed the opposite for p-ERK and p-AKT, which decreased more significantly in daratumumab-treated CLL cells from CD38lo patients (relative to CLL cells from CD38hi patients; Supplementary Figs. S8A–S8F).

Ibrutinib significantly augments the cytotoxic activity of daratumumab

Previous studies have shown that inhibition of BTK can downregulate cell surface antigens on CLL cells (29). Thus, we first examined whether ibrutinib modulates CD38 expression and noted that ibrutinib did not downregulate CD38 on CLL cells (24 hours exposure; Supplementary Fig. S10). As such, we proceeded toward antitumor testing of the ID combination in primary CLL cells. Cytolysis from ADC in ibrutinib-treated CLL cells was 9.92% ± 0.88%, which significantly (P < 0.001) increased to 42.81% ± 1.12%, in ID-treated cells (Fig. 4A). This effect was more pronounced in cells from CD38hi (63.73% ± 4.43%) versus CD38lo (35.21% ± 1.61%) patients with CLL (Fig. 4B). In flow-sorted CD19+CD38hi and CD19+CD38lo CLL cells, we probed for p-BTK and p-PLCγ2. CD38hi clones appeared to have higher BTK expression versus CD38lo clones. In addition, all drugs (including the ID combination) appeared to more effectively downregulate p-BTK, as well as p-PLCγ2 in CD38hi clones (Supplementary Fig. S9H). Altogether, these results allowed us to conclude that blocking either the receptor function (daratumumab) or the enzymatic properties (kuromanin) of CD38 leads to downregulation of BCR-associated proteins in CLL cells and which can be further amplified through simultaneous inhibition of BTK with ibrutinib.
1.02% (Fig. 4G). And the difference in ADCP was significantly appreciable between ID-treated CLL cells from CD38⁺ (29.96% ± 1.41%) versus CD38⁻/C0 patients (18.42% ± 0.94%; P < 0.001; Fig. 4H). Examination in flow-sorted CD19⁺/CD38hi cells, ADCP was noted in 17.35% ± 0.90% of ID-treated clones versus 10.64% ± 0.88% in CD19⁺/CD38lo cells (P < 0.01; Fig. 4I). Of note, comparative analyses (for ADCC, CDC, and ADCP) in JVM13 and MEC1 cell lines was also performed and showed similar results as in primary CLL cells, with some expected variations (Supplementary Fig. S3). A significant correlation between ADCC, CDC, and ADCP in ID-treated CLL cells with percent CD38⁺ CLL cells, CD38 MFI and CD38 sAbc was noted (P < 0.05; Supplementary Table S3). A significant correlation between degree of immune-mediated cytolysis (ADCC, CDC, and ADCP) and CD38 expression (percent CD38⁺ cells, MFI/sAbc) was observed in ID-treated CLL cells. Interestingly, significantly higher ADCC, as well as CDC were noted in ID-treated CLL cells from Del17p⁺ patients (P = 0.021 and P = 0.036; Supplementary Table S5). These data demonstrate that cotargeting CD38 and BTK results in a significant increase in immune-directed CLL cell killing (vs. either daratumumab or ibrutinib alone) and although this effect was perceptible in both CLL cells from CD38⁻ and CD38⁺ patients, it was more pronounced in the latter.

Figure 3.
Cotargeting CD38 results in downregulation of BCR-signaling proteins, which are further augmented by ibrutinib treatment. Phosphorylated (p-) and total protein levels for Lyn, Syk, BTK, PLCγ2, ERK1/2, and AKT were probed for by western blot analysis in CLL cell lysates (–/+ BCR stimulation with anti-IgM for 1 hour), from CD38⁺ patients (patients 11, 16, 26, 33, and 34; A–G) and CD38⁻ patients (patients 8, 14, 28, 35, and 36; H–N). Primary CLL cells were treated for 2 hours with isotype IgG1-b12 Ab (control, 0.1 μg/mL), ibrutinib (Ibr, 1 μmol/L), daratumumab (Dara, 0.1 μg/mL), or the combination of ibrutinib + daratumumab (ID) before lysate preparation. G and N, Representative Western blots from a patient with CD38⁺ and CD38⁻ CLL are shown. Results shown are mean ± SD. **, P < 0.01; #, statistically significant (P < 0.05) difference compared with control or single-agent-treated cells; #d, significant (P < 0.05) compared with all single-agent or control-treated cells, except daratumumab-treated cells; #i, significant (P < 0.05) compared with all single-agent or control-treated cells, except ibrutinib-treated cells.
We also examined apoptosis and observed 36.86% ± 2.56% annexin-V/PI positivity in CLL cells treated with ibrutinib, which increased to 61.90% ± 2.41% in ID-treated cells (Fig. 5A). Subset analysis of cells from patients with CD38⁺ versus CD38⁻ CLL revealed 45.26% ± 2.11% versus 33.81% ± 3.18% apoptosis in ibrutinib-treated and 70.71% ± 3.44% versus 58.7% ± 2.77% apoptosis in ID-treated CLL clones, respectively (Fig. 5B). Scatter plots from two representative patients are shown in Fig. 5C. Using a different combination of probes (7AAD/annexin-V), we noted a similar trend (56.6% ± 4.92%; 7AAD/annexin-V positivity) in ID-treated CLL cells from seven patients (five of who had CD38⁻ disease; Supplementary Fig. S11). In flow-purified CD19⁺/CD38⁺ and CD19⁺/CD38⁻ cells, ID combination treatment resulted in apoptosis of 89.53% ± 2.16% and 49.27% ±
in cells from patients that were CD38\textsuperscript{+} agents showed significant effect (Fig. S5). We also assessed apoptosis in CLL cells exposed to ibritinib (49.53\% ± 2.39\%; Fig. 5A), compared with either kuromanin or ibritinib alone. This validates the fact that the cytotoxicity of ibritinib is enhanced with concurrent targeting of CD38 and this effect is independent of whether a mAb or an anti-CD38 small molecule is used. Apoptosis was mirrored by a loss of mitochondrial membrane permeability by 69.22\% ± 2.49\% in CLL cells (n = 30 patients), the percent change of which was marginally higher in cells from patients that were CD38\textsuperscript{−} (Dilc15 MFI 687.3 ± 111.11 vs. 2,145 ± 289.8 in controls) compared with those who were clinically categorized as CD38\textsuperscript{+} (Dilc15 MFI 1,100 ± 193.7 vs. 4,340 ± 608.2 in control cells) compared with those who were clinically categorized as CD38\textsuperscript{+} (Dilc15 MFI 1,100 ± 193.7 vs. 4,340 ± 608.2 in control cells) compared with those who were clinically categorized as CD38\textsuperscript{+}.

The combination of daratumumab and ibritinib reduces tumor burden in a mouse model of CLL

We tested the anti-CLL activity of daratumumab ± ibritinib in an \textit{in vivo} disseminated disease model system. NSG mice were injected with JVM13-Luc cells via tail vein injection and disease burden was monitored by bioluminescent signal. On day 7 postimplantation, mice were randomly divided into eight groups (6 mice/group) to receive either: (i) vehicle, (ii) effector cells only on the days that daratumumab was given, (iii) daratumumab alone, (iv) ibritinib, (v) daratumumab + effector cells, (vi) ibritinib + effector cells, (vii) ID combination, and (viii) ID combination + effector cells (Fig. 6A). On postimplantation day 28, treatment was concluded in all groups before onset of any signs/symptoms of xeno-GVHD (typically occurring at week 4), postimplantation of human effector cells; ref. 15) and final antitumor response was assessed. Compared with vehicle- or effector cell–only groups, all other groups showed significantly reduced tumor burden (P < 0.01). Tumor burden in mice treated with daratumumab + effector cells versus ibritinib + effector cells was not significantly different (P = 0.063). However, in mice treated with ID therapy (± effector cells), significantly lower disease burden was noted, approximately 3.5- and 2.8-fold lower than that observed in vehicle (P < 0.01) or effector cell alone–treated (P < 0.01) mice, respectively (Fig. 6B and C). No significant changes in weight were noted in any of the treatment groups (Fig. 6D). Mice in groups 2, 5, 6, and 8, which were administered effector cells, were sacrificed on day 30; whereas mice remaining in the other groups (that did not receive effector cells) were followed up to 107 days for survival analysis (Fig. 6E). A trend for longer survival in the daratumumab monotherapy versus vehicle-treated cohort was noted (median overall survival 89 vs. 59 days, respectively, P = 0.057). By day 107, 50\% of the mice in the ID combination–treated arm were alive and healthy.

Discussion

Targeting CD38 for therapeutic purposes has been largely examined in multiple myeloma (30). In CLL, preclinical proof-of-concept for disrupting CD38 function was first reported by Vaisitti and colleagues where inhibition of its enzymatic activity with the flavonoid kuromanin slowed CLL cell homing and adhesion \textit{in vitro} and in a murine model (22). Subsequently, Matas-Cespedes and colleagues reported on the anti-CLL activity...
of daratumumab showing its mechanism of action to be through ADCC and ADCP, with a trend for higher cytolytic activity in CLL cells from CD38² patients (9). Our results herein support these findings and show that in addition to immune-effector-mediated cell kill, daratumumab induces apoptosis in CLL cells, partially dependent on FcR-mediated cross-linking and which is actualized through destabilization of the mitochondria.

Although the enzymatic functions of CD38 and its inhibition in CLL cells have been described previously (1), the receptorial properties of CD38 and particularly their role in signal transmis-
sion through the BCR complex are less understood. Studies by Deaglio and colleagues and Malavasi and colleagues have pro-
vided significant insight on localization of CD38/BCR complexes in cell membrane lipid rafts. Indeed, CD38 ligation with an
agonistic anti-CD38 mAb (IB4) results in calcium flux and increased ERK1/2 activity (22, 27), however, antagonism of CD38 and its effects on BCR signaling have not been previously reported. We show for the first time that mAb-based engagement of CD38 (with daratumumab, which minimally inhibits CD38 enzymatic function) or small molecule–based targeting of CD38 (with kuromanin, which primarily inhibits enzymatic activity of CD38) results in significant downregulation of proximal (Syk and BTK), terminal (PLCγ2 and ERK), and collateral (AKT) proteins involved in BCR signaling. Our observation that CD38 enzymatic inhibition can to a large extent mimic CD38 receptorial block in terms of apoptosis induction and BCR signaling attenuation (mostly in CD38+CD38lo CLL cells) opens avenues of investigation for the use of highly specific small-molecule inhibitors of CD38 such as 76c, reported by Tarrago and colleagues (31). Moreover, highly specific inhibition of CD38 (receptor and NADase activity) may also be able to shift the T-cell repertoire from a pro- to anti-tumor disposition as eloquently shown by Chatterjee and colleagues using CD38 knockout mice (32).

The effects of individually targeting CD38 and BTK yielded modest downregulation of BCR components. Thus, we considered whether disrupting CD38 and BTK simultaneously could further decrease the aforementioned proteins; translating to enhanced lethality in CLL cells. As expected, cotargeting of CD38 (with daratumumab/kuromanin) and BTK (ibrutinib) significantly reduced most of the phosphorylated BCR-signaling proteins and was associated with not only increased apoptosis and mitochondrial disturbance, but also significantly greater immune-effector–mediated cell death. In the case of ADCC, this is not entirely surprising as ibrutinib shifts Th1, Th2, and CD8+ T-cell populations toward an overall anti-tumor disposition (33, 34). This immunomodulation in turn potentially synergizes with the T-cell–modulating properties of daratumumab in both the multiple myeloma (35) and CLL microenvironment (36). Although this may explain enhanced ADCC, it does not explain improved CDC or ADCP. Further studies on complement inhibitor protein expression changes, as well as the effects of BTK ± CD38 inhibition on macrophages are being conducted by us under both ex vivo and in vivo conditions. Altogether, the overall anti-tumor activity of daratumumab is significantly enhanced when partnered with ibritinib and this is reflected by increased CLL cytolysis in every underlying assay: ADCC, CDC, ADCP, and apoptosis (Supplementary Table S6).

To more accurately gauge CLL cell sensitivity toward daratumumab, kuromanin, and/or ibritinib based on CD38 expression/receptor density, we used FACS-purified CD38hi and CD38lo CD19+/CD5+ CLL cells in our workflow. Although results measuring ADCC, CDC, and ADCP showed a similar trend as seen in unsorted CD19+/CD5+ CLL cells from patients with CD38+ and CD38– CLL, the results from the apoptosis assays were more noteworthy. In CD38hi CLL clones, we detected significant apoptosis from ibritinib alone, which did not increase with the addition of daratumumab. In contrast, albeit lower overall compared with CD38lo cells, the magnitude of apoptosis induced by ID therapy was more prominent in CD38lo clones. The significance of these findings is unclear; however, overall our data suggest that CD38 receptor levels and survival dependency on BCR signaling are intricately linked and associated with response to BCR/CD38-targeting agents. These associations, however, should be cautiously interpreted as they were determined in ex vivo assays, whereas the effect of ibritinib or daratumumab in humans is remarkably enhanced through engagement and reshaping of the innate and adaptive immune environment (35, 37). In addition, as CD38 status has not been reported to be a determinant of clinical response to ibritinib, it is plausible that patients with CD38hi and CD38lo CLL alike would demonstrate equivalent response to ibritinib + daratumumab combination treatment.

As daratumumab does not bind murine CD38 (thus precluding use of transgenic Eu-TCL1 mice), we established a disseminated disease xenograft model to study the anti-CLL activity profile of daratumumab (± ibrutinib). In this short-course study, the goal was to measure time to tumor growth before onset of xenogeneic GVHD could impact the results. We noted significant activity of daratumumab relative to mice treated with effector cells only (serving as a control for all mice groups that received drug + effectors). Survival analysis was only performed on mice that received drug without effector cells. Although underpowered, this analysis suggested that the ID combination was superior in prolonging the survival of mice compared with vehicle-treated mice ($P = 0.0113$). Although the median survival of mice treated with single agent daratumumab or ibritinib was lower than mice that received combination ID treatment, the differences were not statistically significant. Further experiments that can incorporate the immune-effector activity of daratumumab in an appropriate mouse model system are needed to comprehensively evaluate the survival advantage conferred with use of daratumumab ± ibrutinib.

In summary, our data highlights that: (i) daratumumab induces cell death in primary CLL cells through various mechanisms ex vivo (ADCC, CDC, ADCP, and apoptosis); (ii) statistically significant correlations between CD38 receptor density (MFI or sAbs) with ADCC, ADCP, and apoptosis were observed; (iii) targeting CD38 with either daratumumab or kuromanin can significantly modulate BCR-associated and these effects are more prominent in CD38+ cases, and (iv) the combination of daratumumab and ibritinib induces significantly more immune-effector–mediated and direct apoptosis of CLL cells from CD38+ and CD38– patients alike. On the basis of our results, although single-agent daratumumab may be more effective in patients with CLL with CD38+ disease, the combination of daratumumab and ibritinib may be highly effective in all treatment requiring patients irrespective of CD38 expression status. Our data provide the framework for future clinical investigations entailing therapeutic strategies targeting CD38 and BTK together.

**Disclosure of Potential Conflicts of Interest**

F. Malavasi reports receiving speakers bureau honoraria from Takeda and Sanofi, and is a consultant/advisory board member for Takeda, Sanofi, and Tusk Therapeutics. S. Ailawadi reports receiving other commercial research support from Pharmacyciles, Inc., and is a consultant/advisory board member for Amgen, Celgene, Takeda, and Janssen. No potential conflicts of interest were disclosed by the other authors.

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