Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095

Cancer Therapy: Preclinical

Clinical Cancer Research

# The Human CD38 Monoclonal Antibody Daratumumab Shows Antitumor Activity and Hampers Leukemia–Microenvironment Interactions in Chronic Lymphocytic Leukemia

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## Abstract

**Purpose:** To establish a proof-of-concept for the efficacy of the anti-CD38 antibody daratumumab in the poor prognosis CD38$^+$ chronic lymphocytic leukemia (CLL) subtype.

**Experimental Design:** The mechanism of action of daratumumab was assessed in CLL primary cells and cell lines using peripheral blood mononuclear cells to analyze antibody-dependent cell cytotoxicity (ADCC), murine and human macrophages to study antibody-dependent cell phagocytosis (ADCP), or human serum to analyze complement-dependent cytotoxicity (CDC). The effect of daratumumab on CLL cell migration and adhesion to extracellular matrix was characterized. Daratumumab activity was validated in two *in vivo* models.

**Results:** Daratumumab demonstrated efficient lysis of patient-derived CLL cells and cell lines by ADCC *in vitro* and ADCP both *in vitro* and *in vivo* whereas exhibited negligible CDC in these cells. To demonstrate the therapeutic effect of daratumumab in CLL, we generated a disseminated CLL mouse model with the CD38$^+$ MEC2 cell line and CLL patient–derived xenografts (CLL-PDX). Daratumumab significantly prolonged overall survival of MEC2 mice, completely eliminated cells from the infiltrated organs, and significantly reduced disease burden in the spleen of CLL-PDX. The effect of daratumumab on patient-derived CLL cell dissemination was demonstrated *in vitro* by its effect on CXCL12-induced migration and *in vivo* by interfering with CLL cell homing to spleen in NSG mice. Daratumumab also reduced adhesion of CLL cells to VCAM-1, accompanied by downregulation of the matrix metalloproteinase MMP9.

**Conclusions:** These unique and substantial effects of daratumumab on CLL viability and dissemination support the investigation of its use in a clinical setting of CLL. *Clin Cancer Res; 23(6); 1493–505. ©2016 AACR.*

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

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**doi:** 10.1158/1078-0432.CCR-15-2095



## Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults and is characterized by progressive accumulation of nonfunctional, apoptosis-resistant mature B cells in peripheral blood, bone marrow, and lymphoid tissues (1, 2). The majority of the tumor cells in the blood are resting. However, heavy-water experiments have shown that CLL contains a small fraction of actively proliferating cells, with approximately 2% of cells newly generated each day (3). This proliferation occurs in specific structures known as proliferation centers localized in the lymph nodes and in the bone marrow. Thus, CLL is considered a disease characterized by a dynamic balance between cells circulating in the blood and cells located in permissive niches in lymphoid organs (1, 2). The former are primarily mature-looking small lymphocytes resistant to apoptosis, whereas the latter are composed by lymphocytes that undergo either proliferation or apoptosis depending on the microenvironment.

CD38 was first reported to be associated with inferior outcome by Damle and colleagues in 1999 (4) and confirmed later as a

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## Translational Relevance

Chronic lymphocytic leukemia (CLL) remains an incurable disease where high CD38 expression is associated with poor prognosis and identifies cells that are prone to proliferate. CD38 cooperates in migration, adhesion, and invasion through its molecular association with CXCR4, MMP9, and CD49d. The human anti-CD38 monoclonal antibody daratumumab has shown efficient cell killing and a good safety profile in clinical trials in multiple myeloma. Here, we demonstrate that daratumumab also exerts significant cytotoxicity against patient-derived CLL cells, via ADCC and ADCP *in vitro* and *in vivo*. Furthermore, daratumumab interferes with CD38 signaling and reduces CLL cell adhesion, migration, and homing. Moreover, daratumumab shows therapeutic activity in two mouse models. Thus, daratumumab improves overall survival in a systemic $CD38^+$ MEC2 cell line mouse model and reduces tumor burden in CLL patient–derived xenografts. These results provide scientific rationale for the clinical development of daratumumab in poor prognosis $CD38^+$ CLL.

prognostic factor independent of *IGHV* mutation status (5). Patten and colleagues demonstrated that CD38 expression in CLL is dynamic and changes as a result of contact with activated $CD4^+$ T cells in proliferation centers, being CD38 specifically expressed on cells that are primed to proliferate in the lymph node (6). As a consequence, the expression of CD38 on CLL differs among lymphoid compartments, being higher in bone marrow and lymph node than in peripheral blood (7, 8) and in the proliferating fraction of the tumor (9). The functional importance of CD38 in CLL extends beyond proliferation, as it appears to be linked to the tyrosine kinase ZAP-70 and characterizes CLL cells with high migratory potential (10). CD38 cooperates with CXCR4-induced migration (11) and sustains B-cell receptor (BCR)-mediated signaling (12). Finally, a role of CD38 in adhesion and tissue invasion was recently recognized. CD38 forms a macromolecular complex with the integrin CD49d and the matrix metalloproteinase MMP9, enhancing CD49d-mediated cell adhesion as well as MMP9 expression and activity (13–15). This is of key relevance because CD49d surface expression strongly correlates with overall survival in CLL (16). All these properties make CD38 an attractive target for antibody therapy in CLL and other $CD38^+$ hematologic malignancies such as multiple myeloma (17), non–Hodgkin lymphoma (NHL), and B- and T-acute lymphoblastic leukemia.

The human anti-CD38 antibody daratumumab has progressed to phase III clinical trials in patients with multiple myeloma. Daratumumab is a human IgG1 therapeutic monoclonal antibody (mAb) that binds to CD38. In 2015, the U.S. FDA has approved daratumumab for patients with multiple myeloma, who have received at least 3 prior lines of therapy including a proteasome inhibitor and an immunomodulatory agent, or patients double refractory to these agents. Approval was based on 2 phase II studies of daratumumab monotherapy (16 mg/kg) in heavily treated patients (18, 19). A pooled analysis of these studies revealed an overall response rate of 31%, including responses that deepened over time, and median overall survival of 19.9 months. Daratumumab induces killing of tumor cells, mainly via complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC; ref. 20), and antibody-dependent cellular phagocytosis (ADCP) by macrophages (21) in multiple myeloma and Burkitt lymphoma cell lines. In addition, daratumumab induces apoptosis upon secondary cross-linking (22). Recent studies have revealed previously unknown immunomodulatory effects of daratumumab where CD38-expressing immunosuppressive regulatory T and B cells and myeloid-derived suppressor cells are highly sensitive to daratumumab treatment (23). It has also been shown that daratumumab can modulate the enzymatic activity of CD38 and potentially may lead to a reduction in immunosuppressive adenosine levels (24, 25). This shift away from an immunosuppressive environment may lead to the generation of protective immune responses. Indeed, a concomitant increase of helper and cytotoxic T-cell absolute cell counts and production of IFNγ in response to viral peptides was observed. In addition, an increase in T-cell clonality in subjects who responded to daratumumab versus subjects who did not respond was observed, indicating an improved adaptive immune response (23).

Two additional anti-CD38 antibodies have also entered clinical trials for multiple myeloma and other $CD38^+$ hematologic malignancies, MOR202 (26) and isatuximab (SAR650984; ref. 27), that are being tested alone and in combination with standard therapy.

The aim of this study was to evaluate the cytotoxic effect of daratumumab on CLL cells via CDC, ADCC, and ADCP, as well as its effect on tumor cell–microenvironment interactions, using patient-derived CLL cells and CLL cell lines in *in vitro* and *in vivo* settings.

## Materials and Methods

### Cell lines and patient samples

Primary tumor cells from 18 patients with CLL (see clinical characteristics in Table 1), diagnosed according to the World Health Organization (WHO) classification criteria, were used. Written informed consents of the patients were granted following the guidelines of the Hospital Clínic Ethic Committee (IRB) and the Declaration of Helsinki. Mononuclear cells were isolated from peripheral blood by gradient centrifugation on Ficoll (GE Healthcare) and used fresh or cryopreserved in liquid nitrogen in RPMI-1640 containing 10% DMSO (Sigma-Aldrich) and 60% heat-inactivated FBS (Life Technologies) and maintained within the hematopathology collection of the institution (IDIBAPS-Hospital Clínic Biobank, R121001-094). The prolymphocytic leukemia (PLL) cell lines, MEC1, MEC2, and JVM13, as well as the Burkitt lymphoma Daudi cell line were obtained from DSMZ. CLL primary samples and cell lines were cultured in RPMI-1640 or IMDM supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 μg/mL penicillin/streptomycin (Life Technologies) and were maintained in a humidified atmosphere at 37°C containing 5% $CO_2$. Normocin (100 μg/mL; InvivoGen) was added to the cell line cultures to prevent mycoplasma contamination in cell lines that were routinely tested for mycoplasma infection by PCR. The identity of all cell lines was verified by using AmpFISTR identifier kit (Life Technologies).

### Therapeutic and complement regulatory proteins blocking antibodies

A human IgG1 targeting CD38 (daratumumab) was generated by immunization in a HuMAb mouse (20). The human mAb IgG1-b12, specific for the HIV-1 gp120 envelope glycoprotein

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**Table 1.** CLL patient characteristics

| Study label | Gender | Binet/Rai stage | % tumor cells[a] | IgVH status[b] | CD38[c] | CD38 sABC[d] | CD46[c] | CD55[c] | CD59[c] | CD49d[c] | %CDC[e] | %ADCC[f] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CLL1 | M | C/IV | 95 | nd | 98 | 816 | 98 | 91 | 9 | 4 | nd | 32.7 |
| CLL2 | M | A/I | 96 | UM | 63 | 897 | 889 | 88 | 96 | 6 | 6.4 | 20.8 |
| CLL3 | M | C/IV | 86 | UM | 80 | 1,875 | 90 | 88 | 79 | 68 | 8.4 | 20.8 |
| CLL4 | M | A/I | 97 | UM | 31 | 355 | 92 | 90 | 75 | 79 | nd | 44.5 |
| CLL5 | M | B/II | 97 | UM | 44 | 562 | 92 | 90 | 97 | 1 | 0.1 | 39.4 |
| CLL6 | F | C/III | 86 | M | 50 | nd | nd | nd | nd | nd | 25.1 | 20 |
| CLL7 | M | C/III | 96 | UM | 2 | nd | 98 | 99 | 90 | 0.7 | 5.6 | 0.2 |
| CLL8 | M | A/0 | 95 | M | 53 | 736 | 93 | 94 | 83 | 5 | 3.4 | 26.6 |
| CLL9 | M | A/I | 82 | nd | 73 | nd | 98 | 97 | 98 | 63 | 0.9 | 22.9 |
| CLL10 | M | B/III | 93 | M | 64 | nd | 92 | 91 | 94 | 100 | 25.6 | 47 |
| CLL 11 | M | A/0 | 97 | M | 0.5 | 294 | 95 | 94 | 98 | 0.1 | 0 | 0 |
| CLL 12 | F | A/III | 94 | UM | 95 | 2,132 | 97 | 87 | 90 | 80 | 17.4 | 31.4 |
| CLL 13 | M | A/0 | 60 | nd | 55 | nd | 99 | 100 | 99 | 98 | 10.4 | nd |
| CLL14 | M | B/II | 98 | UM | 66 | nd | 97 | 93 | 86 | nd | nd | 27.9 |
| CLL15 | F | A/I | 86 | UM | 71 | 1,531 | 96 | 98 | 88 | 83 | nd | 27.2 |
| CLL 16 | M | A/I | 53 | nd | 75 | nd | 98 | 94 | 96 | 95 | 23.2 | 38.1 |
| CLL17 | M | A/0 | 85 | UM | 83 | nd | 99 | 98 | 96 | nd | 0.3 | nd |
| CLL 18 | M | A/0 | 91 | UM | 70 | nd | 98 | 98 | 97 | 92 | 0 | 22 |

Abbreviations: F, female; M, male; nd, not determined.

[a]Percentage of tumor cells assessed by flow cytometry on the basis of $CD19^+CD5^+CD23^+$ cells.

[b]Determined by direct sequencing. M, mutated, sequence homology < 98%; UM, unmutated, sequence homology > 98%.

[c]Percentage of positive cells for CD38, CD46, CD55, CD59, and CD49d determined by flow cytometry in $CD19^+CD5^+$ population, referred to as isotype control. CD38 was considered positive when the percentage of positive cells exceeded 30%.

[d]sABC, number of surface antibodies bound per cell evaluated by QuantiBRITE CD38-PE.

[e]Percentage of CDC induction at 10 μg/mL daratumumab.

[f]Percentage of ADCC induction at 0.1 μg/mL daratumumab.

(28), was included in all experiments as an isotype control mAb. Both antibodies were provided by Genmab.

Anti-CD46 (clone TRA-2-10, Biolegend), anti-CD55 (clone 1C6, Hycult Biotech), and anti-CD59 (clone YTH 53.1, AbD Serotech) antibodies were used to block complement regulatory proteins (CRP).

### Antibody-dependent cellular cytotoxicity

Target cells were labeled with 1 μmol/L Calcein-AM (Life Technologies) for 30 minutes at 37°C. Afterward, cells were washed 3 times with PBS, plated in triplicate at $1 \times 10^4$ cells per well in 96-well round-bottom plates, and preincubated (room temperature for 15 minutes) with 10-fold serial dilutions of either isotype control (IgG1-b12) or daratumumab (range, 1 to 0.0001 μg/mL) in RPMI-1640. Daratumumab doses for *in vitro* studies were previously established (20). Culture medium was added instead of mAb to determine the spontaneous calcein release, and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, fresh human peripheral blood mononuclear cells (PBMC) were added at an effector:target (E:T) ratio of 50:1, optimized in a previous report (20), and cells were incubated for 4 hours at 37°C. The plates were centrifuged, supernatant transferred into black plates (Thermo Scientific) and fluorescence was measured in a Synergy spectrophotometer (Bio-Tek; excitation filter: 485 ± 20 nm; bandpass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$\textit{specific lysis} = 100 \times \frac{\textit{experimental release (RFU)} - \textit{spontaneous release (RFU)}}{\textit{maximal release (RFU)} - \textit{spontaneous release (RFU)}}$$

### Antibody-dependent cellular phagocytosis

Macrophages were generated from monocytes isolated from bone marrow of the hind legs of female SCID mice (C.B-17/Icr-Prkdc$^{scid}$/Crl; Janvier Labs) by flushing the femurs. The cells were cultured for 7 days in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 μg/mL penicillin/streptomycin, and 50 U/mL M-CSF (Cell Guidance), and the culture medium was renewed every 3 days. On day 7, macrophages were detached with 0.1% trypsin-EDTA and characterized by flow cytometry ($CD11b^+$, $F4/80^+$; mouse antibodies obtained from eBiosciences and Invitrogen, respectively). The macrophages were seeded at $2.5 \times 10^5$ cells per well into non–tissue cultured–treated 24-well plates and allowed to adhere overnight. Target cells (primary CLL and cell lines) were labeled with 0.01 μmol/L Calcein-AM and added to the macrophages at an E:T ratio of 1:1 in the presence of a fixed mAb concentration of 1 μg/mL. After 4 hours of incubation, the nonphagocytosed target cells were collected. The macrophages were detached with 0.1% trypsin-EDTA, added to the nonphagocytosed target cells, and stained for F4/80 expression. The amount of remaining target cells ($calcein^+$ $F4/80^-$) was determined on an Attune acoustic cytometer, and the percentage of killed target cells in the presence of daratumumab compared with isotype control was calculated using the following formula:

$$\%\ \textit{eliminated target cells} = 100 - \left[100 \times \frac{\textit{remaining target cells after DARA treatment}}{\textit{remaining target cells after isotype control treatment}}\right]$$

### *In vivo* phagocytosis assay

*In vivo* phagocytosis assay was carried out as described by Overdijk and colleagues (21). SCID beige mice (CB17.CG-PRKDC-LYST/CR, Charles River Laboratories), which lack natural killer (NK) cells, were inoculated with primary CLL cells or MEC2 cells ($2 \times 10^7$ cells per mouse) into their peritoneal cavity, following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona (Barcelona, Spain). Mice were randomly assigned into cohorts of 3 to 5 mice and received one intraperitoneal injection of 20 mg/kg of daratumumab or isotype control. Forty-eight hours later, mice were

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sacrificed and peritoneal lavage done by injecting the cavity with 5 mL of cold PBS. Total recovery of the peritoneal cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies (provided by Invitrogen and BD Pharmingen, respectively). The relative percentage of remaining CLL cells from daratumumab-treated mice was derived from the isotype control group, which was set at 100%.

$$\%\ remaining\ target\ cells = 100 \times \frac{remaining\ target\ cells\ after\ DARA\ treatment}{remaining\ target\ cells\ after\ isotype\ control\ treatment}$$

### Complement-dependent cytotoxicity

Target cells were labeled with 1 μmol/L Calcein-AM (Life Technologies) for 30 minutes at 37°C. Afterward, cells were washed 3 times with PBS, plated in triplicate at $1 \times 10^5$ cells per well in 96-well round-bottom plates, and preincubated [room temperature (29), 15 minutes] with 10-fold serial dilutions of either isotype control (IgG1-b12) or daratumumab (range, 10 to 0.01 μg/mL) in RPMI-1640. Culture medium was added instead of mAb to determine the spontaneous calcein release and 1% Triton X-100 was used to determine the maximal calcein release. Thereafter, 10% normal human AB serum was added and incubated for 45 minutes at 37°C. The plates were centrifuged, supernatants transferred into black plates (Thermo Scientific) and fluorescence measured in a Synergy spectrophotometer (Bio-Tek; excitation filter: 485 ± 20 nm; bandpass filter: 530 ± 20 nm). The percentage of cellular cytotoxicity was calculated using the following formula:

$$specific\ lysis = 100 \times \frac{experimental\ release\ (RFU) - spontaneous\ release\ (RFU)}{maximal\ release\ (RFU) - spontaneous\ release\ (RFU)}$$

### *In vivo* homing

Homing experiment was done as previously described by Vaisitti and colleagues (11). Briefly, NOD/SCIDγ null (NSG) mice (bred in-house, animal facility, University of Barcelona) were randomly assigned into cohorts of 4 mice and pretreated intraperitoneally with 10 mg/kg of daratumumab, isotype control, or anti-CXCR4 (R&D Systems). Twenty-four hours later, mice were inoculated with fresh primary CLL ($2 \times 10^7$ cells per mouse) via the tail vein following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. Mice were sacrificed 24 hours after tumor cell inoculation; peripheral blood, spleen, and bone marrow were recovered and the presence of tumor cells evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

### Systemic MEC2 xenograft mouse model

SCID mice were preconditioned with 25 mg/kg of busulfan 24 hours before inoculation via tail vein of MEC2 cells ($10^7$ cells per mouse), following a protocol approved by the Animal Testing Ethic committee of the University of Barcelona. One week later, mice were randomly assigned into cohorts of 6 to 7 mice. A saturating loading dose of 20 mg/kg daratumumab or isotype control intraperitoneally was given on day 7 and thereafter 10 mg/kg weekly for 3 weeks. Mice were sacrificed if they lost 15% to 20% of weight and/or showed signs of disease. Survival studies were extended up to day 90 when the study was terminated. The presence of tumor cells was evaluated first macroscopically and then by flow cytometry. Cells from infiltrated organs were obtained by tissue homogenization. Bone marrow cells were obtained after flushing the femoral and tibia bones with RPMI-1640 media. These samples were filtered through 70-μm nylon sieves (BD Falcon). Erythrocytes were lysed using ACK buffer (Quality Biological Inc.). The cells were labeled with huCD45/CD19/CD5 antibodies and analyzed by flow cytometry. Organ samples were snap-frozen in OCT medium (Sakura Tissue Tek) or formalin fixed and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) and CD19 (Dako) antibody and evaluated by Cell B Basic Imaging Software (Olympus).

### CLL patient–derived mouse xenograft

On day 1, NSG mice were inoculated intravenously with fresh PBMCs from CLL ($2 \times 10^7$ cells per mouse). On day 2, mice were randomly assigned to 2 groups (3–4 mice per group) and dosed intraperitoneally with 20 mg/kg of daratumumab or control isotype. Mice were sacrificed on day 5; peripheral blood, spleen, and bone marrow were recovered and the presence of tumor cells was evaluated by flow cytometry after staining with huCD45/CD19/CD5 antibodies.

### Statistical analysis

Unpaired and paired *t* tests or one-way ANOVA were used to assess statistical differences between groups by means of GraphPad Prism software 4.0. For Kaplan–Meier survival curves, SPSS19 software was used.

## Results

### Daratumumab induces ADCC

Antibody-dependent killing via ADCC by FcγR-bearing effector cells accounts for the antitumor activity of daratumumab in models of multiple myeloma and Burkitt lymphoma (20, 30). The ability of daratumumab to induce ADCC on CLL cells was assessed by calcein-AM release assay using PBMCs from healthy donors as a source of effector cells (mainly NK cells and monocytes). CLL cell lines and primary cells were treated with increasing concentrations of daratumumab or isotype control. Daratumumab induced significant cell lysis starting at doses as low as 0.01 μg/mL in $CD38^+$ CLL cell lines (Fig. 1A) and at 0.001 μg/mL in primary CLL cells (Fig. 1B), reaching its maximum killing activity at 0.1 to 1 μg/mL (mean ± SD = 31.9% ± 11.6%). In contrast, the isotype control antibody did not induce significant cell lysis, tested at the maximum concentration of 1 μg/mL (mean ± SD = 12.4% ± 11.6%; Fig. 1B). No ADCC induction was detected in $CD38^-$ CLL cases (Table 1: CLL7, CLL11, and MEC1 cell line [Table S1]). A summary of ADCC induction in $CD38^+$ versus $CD38^-$ CLL primary cases is shown in Fig. 1C. The degree of ADCC induction did not correlate with CD38 sABC for CLL cell lines and primary cells ($r^2 = 0.088$; Fig. 1D). Altogether, these data indicate that ADCC constitutes a mechanism of daratumumab activity in $CD38^+$ CLL cells, but the extent of ADCC does not strictly correlate with CD38 expression.

### Daratumumab promotes CLL cell clearance by phagocytosis *in vitro* and *in vivo*

Recent results indicate that ADCP is a potent mechanism of action for daratumumab (21). We explored ADCP of CLL both

Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095



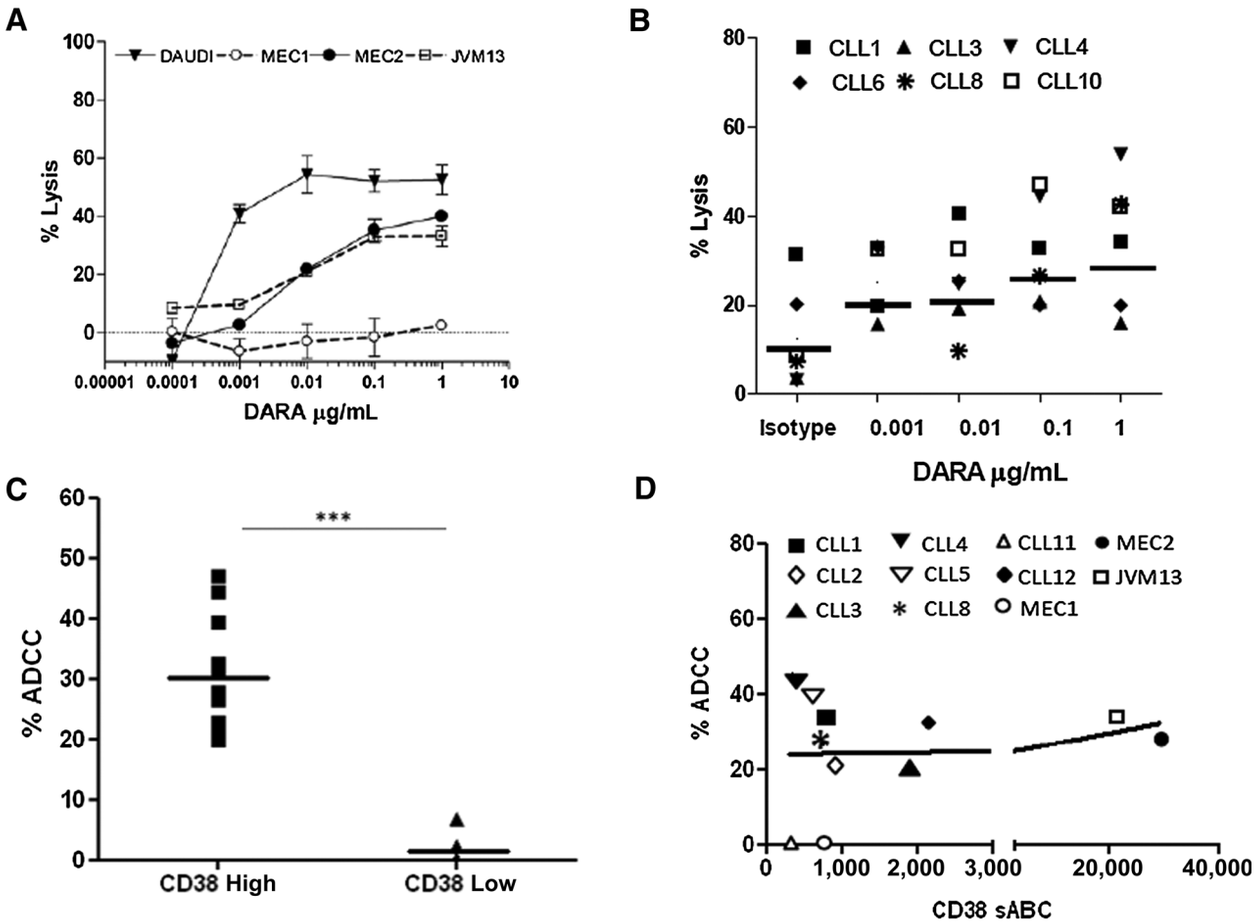


**Figure 1.**

Daratumumab (DARA) induces ADCC in the presence of external effectors. **A,** Daudi cells, CLL cell lines, both CD38$^{high}$ (MEC2 and JVM13) and CD38$^{low}$ (MEC1) were treated with increasing daratumumab doses (0.0001–1 μg/mL) in the presence of PBMCs from healthy donors at an E:T ratio of 50:1 for 4 hours. Viability was then evaluated by calcein release assay. **B,** Results in primary CLL cells with daratumumab doses (0.001–1 μg/mL). ADCC induced by isotype control at the maximal dose of 1 μg/mL is also depicted and the horizontal line represents the mean lysis. **C,** ADCC induction by daratumumab (0.1 μg/mL) in CD38 high (≥30%) versus CD38 low (<30%) CLL primary cases. **D,** Number of surface antibodies bound per cell (sABC) of CD38 was quantified in primary CLL cells and cell lines and plotted for correlation with ADCC induction by daratumumab (cell lines, 0.1 μg/mL and primary CLL, 0.01 μg/mL).

*in vitro* and *in vivo*. To assess ADCP *in vitro*, macrophages were generated from bone marrow mouse monocytes stimulated with M-CSF. Daratumumab induced ADCP in primary CLL cells (mean ± SD = 23% ± 4%; Fig. 2A). Representative flow cytometry profiles of CLL cells and macrophages after daratumumab treatment are depicted for CLL1 (Fig. 2B). As observed for ADCC, phagocytosis was specimen-dependent and not strictly related to CD38 expression levels.

We next demonstrated the occurrence of ADCP *in vivo*. SCID *beige* mice, devoid of NK cells but with active macrophages, were inoculated intraperitoneally with primary CLL cells or the MEC2 cell line as described in Materials and Methods. As shown in Fig. 2C, the percentage of remaining viable CLL cells after daratumumab treatment was significantly reduced (mean ± SD = 46% ± 5%; $P < 0.05$, unpaired $t$ test) compared with the isotype control group at 100%. Remarkably, this decrease in cell number was detectable as early as 2 hours after daratumumab administration (data not shown). Figure 2D represents flow cytometry profiles showing the number of CLL cells (huCD45$^+$/CD19$^+$/CD5$^+$) recovered from the intraperitoneal cavity after isotype control or daratumumab treatment.

Taken together, these results demonstrate that ADCP may contribute to daratumumab antitumor activity against CLL cells both in *in vitro* and *in vivo* settings.

### Daratumumab induces limited CDC of CLL cells

Daratumumab was selected from a panel of human antibodies for its broad-spectrum killing activity against hematologic cell lines. Daratumumab was particularly differentiated for its potent CDC activity (20). We evaluated daratumumab-induced CDC activity in a panel of CLL primary cells and cell lines (Table 1 and Supplementary Table S1). In the majority of primary CLL samples, CD38$^+$ PLL cell lines (MEC2 and JVM13) and in CD38$^-$ cell line (MEC1), daratumumab did not induce significant cell death in the presence of normal human serum (10%). In 5 of 18 primary CLL cells,

Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095



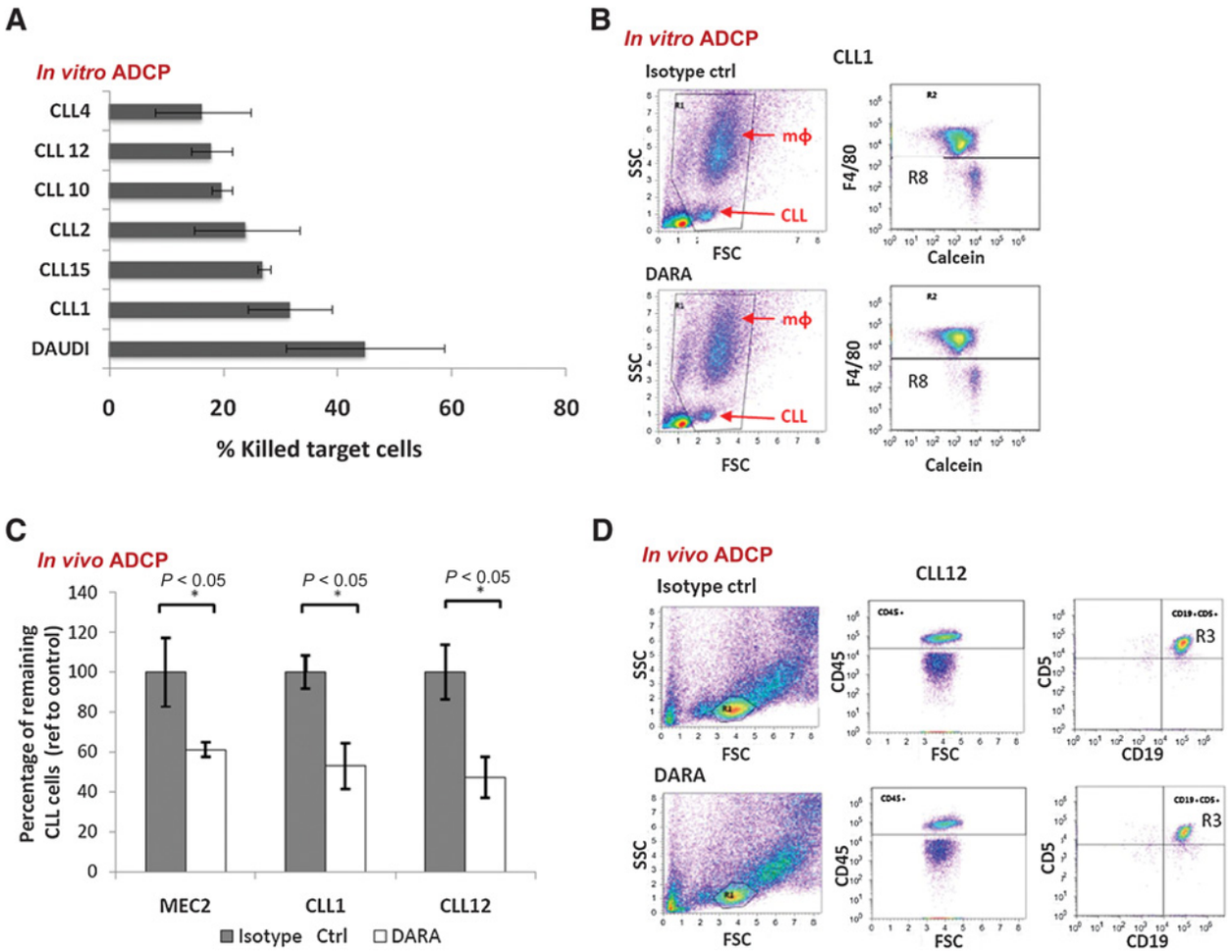


**Figure 2.**

Daratumumab (DARA) induces ADCP *in vitro* and *in vivo*. **A,** CLL cells were treated in triplicates with a fixed concentration (1 μg/mL) of daratumumab or isotype control in the presence of mouse macrophages at a E:T ratio of 1:1 for 4 hours. Percentage of killed target cells was calculated by flow cytometry. CLL cells were identified as calcein$^+$ F4/80$^-$, and the percentage of killing by daratumumab was calculated according to the formula included in Materials and Methods. Daudi cell line was used as positive control. **B,** Representative flow cytometric plots of *in vitro* ADCP. CLL cells and macrophages (mφ) are clearly seen in the forward scatter (FSC)/side scatter (SSC) density plot. The number of cells in the R8 gate was used to calculate the percentage of killed tumor cells. **C,** *In vivo* phagocytosis was evaluated in SCID *beige* mice ($n$ = 3–5 per group) that were inoculated intraperitoneally with primary CLL cells or MEC2 cell line ($2 \times 10^7$ cells per mouse) and subsequently treated intraperitoneally with one dose of 20 mg/kg of daratumumab or isotype control. The mice were sacrificed 48 hours later and cells from the peritoneum recovered and counted by flow cytometry as huCD45$^+$CD19$^+$CD5$^+$ for primary CLL cells and CD45$^+$CD19$^+$CD5$^-$ for MEC2 cells. The percentage of residual leukemia cells after daratumumab treatment is plotted where the total number of cells remaining treated with the isotype control was set to 100% ($P < 0.05$, unpaired $t$ test). **D,** Representative flow cytometric plots where the R3 gate was used to calculate the percentage of killed tumor cells. The gating strategy started with cells in FSC/SSC (R1), then gating on CD45$^+$, and finally CD19$^+$CD5$^+$.

daratumumab induced just more than 10% CDC (range, 10.4%–25.6%). This limited CDC induction was not increased in the presence of higher human serum concentrations (data not shown). To explain the poor induction of CDC, we assessed the expression of CRPs and the number of CD38 molecules per cell (CD38 sABC) on CLL cells. High expression of the CRPs [CD46, CD55, CD59 (mean ± SD = 94% ± 3%, 92% ± 4%, 92% ± 8%, respectively)] was detected by flow cytometry in all CLL cell lines and primary cases, whereas only CD46 (88%) was highly expressed in the Burkitt lymphoma cell line Daudi (Table 1 and Supplementary Table S1) which was used as a positive control for CDC. Blocking antibodies against these CRPs probed to increase CDC induction by daratumumab in Daudi, whereas no effect was observed in CLL cells. However, blockade of CRPs increased CDC induced by the anti-CD20 antibodies rituximab (RITUX) or ofatumumab (OFA) in some CLL cases (Supplementary Fig. S1).

CD38 expression in CD38$^+$ CLL primary tumor samples (mean sABC ± SD = 1,053 ± 677) was lower than in CD38$^+$ CLL cell lines (mean sABC ± SD = 25,024 ± 6,031), which was roughly 10-fold below that detected in Daudi (mean sABC ± SD = 292,131). A summary of mean fluorescence intensity ratio (MFIR) for CD38 and CRPs in CLL cells and cell lines is included in Supplementary Table S2. Previous results in multiple myeloma by Nijhof and colleagues have demonstrated that all-trans retinoic acid (ATRA) increases CD38 expression. We have analyzed this

Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095



possibility in CLL. CLL cells ($n = 6$) were pretreated with ATRA or left untreated for 48 hours. CD38 expression was analyzed subsequently on these CLL cells and challenged to CDC assay. As shown in Supplementary Fig. S2A, CD38 MFI of $CD19^+CD5^+$ cells, was significantly ($P < 0.05$) increased after ATRA treatment (average increase, 30%), in a similar proportion than that shown for multiple myeloma patient samples. A representative example is shown (Supplementary Fig. S2B). However, no CDC induction by daratumumab was observed in the cases analyzed (Supplementary Fig. S2C), indicating that this increase in CD38 expression was not sufficient to engage CDC.

In conclusion, these results indicate that daratumumab did not induce significant CDC in either CLL cell lines or primary CLL cells and is probably due to high expression of CRPs and insufficient CD38 expression.

**Daratumumab interferes with *in vitro* migration and *in vivo* homing**

Homing of CLL cells to secondary lymphoid organs is mainly coordinated by the CXCL12/CXCR4 axis (31). Using CLL primary cells and a xenograft mouse model, Vaisitti and colleagues demonstrated that CD38 synergizes with the CXCR4 signaling pathway and controls chemotaxis/homing of CLL cells through a close interaction between CD38 and CXCR4 in the membrane (11). Following this line of investigation, the effect of daratumumab on CLL cell migration was evaluated using a CXCL12 gradient. An anti-CXCR4 antibody was used as a positive control of migration blockade. In $CD38^+$ CLL cells, daratumumab inhibited CXCL12-mediated migration up to 70% (mean ± SD = 44% ± 16%; $P < 0.01$; $n = 5$), which was comparable with anti-CXCR4 treatment (Fig. 3A). These results are in agreement with that previous report using the blocking anti-CD38 antibody SUN-4B7 (11). We next examined daratumumab-mediated signaling following CXCR4–CXCL12 interaction. The immediate early effect of stimulation for migration is the activation of ERK1/2 (11). Phosphorylation of ERK1/2 in CLL tumor cells occurred shortly after CXCR4–CXCL12 ligation and peaked at 5 minutes after CXCL12 addition. Treatment with daratumumab reduced ERK activation by CXCR4–CXCL12 in CLL1 and CLL12, whereas the ERK inhibition was less pronounced in CLL3 (Fig. 3B), illustrating heterogeneity in primary tumor cells.

We then validated these *in vitro* migration results using the *in vivo* homing mouse model described previously by Vaisitti and colleagues (11). Using NSG mice, which lack NK cells and active macrophages, we analyzed the effect of daratumumab on primary CLL cell migration from peripheral blood to bone marrow and spleen. In this model, NSG mice were pretreated (day 0) with daratumumab, isotype control, or anti-CXCR4, followed by fresh CLL cell inoculation on day 1. Peripheral blood, bone marrow, and spleen cells were isolated on day 2, and CLL cells were identified as $CD45^+/CD19^+/CD5^+$. Representative flow cytometric profiles from a mouse spleen are shown in Fig. 3C. Cell enumeration showed that CLL cells rapidly move from peripheral blood and mainly migrated to the spleen and that daratumumab significantly reduced this migration (55% inhibition on average, $P < 0.05$; Fig. 3D). Migration of CLL cells to bone marrow was limited and was not affected by pretreatment of mice with daratumumab (data not shown). In conclusion, *in vivo* and *in vitro* results suggest that daratumumab hampers dissemination of CLL cells to secondary lymphoid organs.

**Daratumumab inhibits CD49d-mediated CLL cell adhesion by reducing MMP9 levels**

In addition to migration, CD38 also plays a key role in cell adhesion through physical interaction with the integrin CD49d/CD29 (α4β1 integrin; ref. 15) which is the strongest flow cytometry marker associated with poor prognosis in CLL together with *IGVH* mutational status (16) and MMP9 (13). In addition, the expression of CD38 correlates with that of CD49d. We analyzed the effect of daratumumab on CD49d/CD29-mediated adhesion of CLL cells to vascular cell adhesion molecule-1 (VCAM-1), an essential component of extracellular matrix. As shown in Fig. 4A and B, when compared with isotype control antibody, daratumumab significantly impeded the adhesion to VCAM-1 of CLL primary cells ($n = 4$) and MEC2 cell line (mean ± SD = 38% ± 11%, $P < 0.01$), with no significant differences with anti-CD49d blocking antibody used here as a positive control (mean ± SD = 49% ± 30%).

Moreover, CD38–CD49d complex also recruits MMP9 leading to the upregulation and activation of this metalloproteinase (13–15). Thus, we investigated the effect of daratumumab on MMP9 expression, by analyzing the variations in *MMP9* transcripts levels in $CD38^+CD49d^+$ CLL cells. As depicted in Fig. 4C, CLL cell adhesion to VCAM increased *MMP9* mRNA levels (mean ± SD = 2 ± 1), and daratumumab completely abrogated both constitutive ($P < 0.01$) and VCAM-induced ($P < 0.05$) *MMP9* expression. Altogether, these results demonstrate that daratumumab counteracts VCAM-1–mediated adhesion of CLL cells and induces the transcriptional downregulation of *MMP9*.

**Daratumumab prolongs overall survival in a systemic CLL mouse model and reduces tumor burden in CLL-PDX**

We successfully established a systemic MEC2 model by intravenous cell inoculation in busulfan-preconditioned SCID mice, which retain NKs and macrophages that can function as effectors for daratumumab activity. Previous attempts to establish a MEC2 mouse model failed using subcutaneous cell inoculation in nude mice (32).

One week after cell inoculation, mice were randomly assigned into 2 groups and were administered a total of 4 doses of daratumumab or isotype control following a weekly schedule (20/10/10/10 mg/kg). These doses were chosen on the basis of dose escalation studies in multiple myeloma (33). The isotype control–treated mice started to show signs of disease (mainly weight loss > 20% and rough hair) starting at day 32 post cell inoculation (Fig. 5A and B), and all mice in the control group were sacrificed by day 40. These mice showed systemic dissemination of disease in lung, kidney, ovaries, parathyroid glands, and enlarged lymph nodes (identified as $CD45^+/CD19^+$; Fig. 5C and D and Supplementary Fig. S3), resembling aggressive CLL. In several mice, bone marrow and spleen infiltration was also observed (data not shown). Of note, a similar disseminated CLL mouse model was described by Bertilaccio and colleagues using the $CD38^-$PLL cell line MEC1 in $Rag2^{-/-}\gamma^{-/-}$ mice, and the authors demonstrated its value as a tool to assess the efficacy of chemotherapeutic agents (34). In several mice, bone marrow and spleen infiltration was also observed (data not shown). In contrast, in the daratumumab-treated group, only one mouse harbored signs of illness and required euthanasia at day 41, whereas the remaining animals survived and did not develop life-threatening symptoms up to day 90, when the experiment was terminated (Fig. 5A and B).

Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095



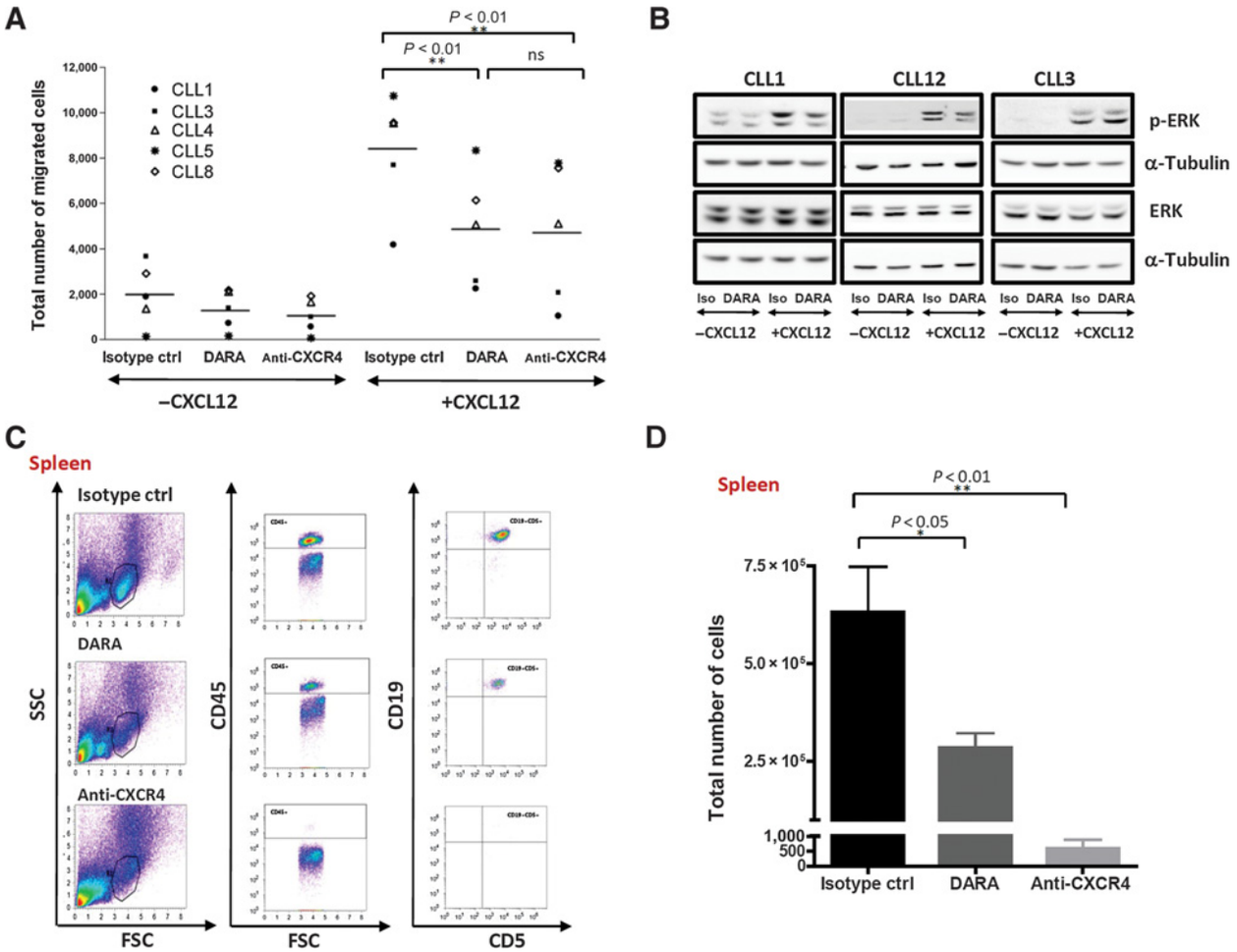


**Figure 3.**

Daratumumab interferes with CLL cell migration and *in vivo* homing. **A,** CLL cells were preincubated with the antibodies for 30 minutes at 4°C (30 μg/mL for isotype control and daratumumab and 25 μg/mL anti-CXCR4) and then assayed for migration in a CXCL12 gradient. After 4 hours, CLL cells ($CD19^+CD5^+$) in the lower chamber were counted in triplicates in a flow cytometer at fix flow rate. Total number of cells is graphed for representative patients with CLL ($n = 5$). Statistical differences between groups were assessed by paired *t* test. **B,** Western blot analysis of ERK activation after stimulation of CLL cells for 5 minutes with CXCL12 (200 ng/mL). Before stimulation, cells were serum-starved for 2 hours and pretreated for 30 minutes with the corresponding antibodies (30 μg/mL). **C** and **D,** *In vivo* homing was assessed by intravenous inoculation of fresh CLL cells via tail vein in NSG mice, previously pretreated with the corresponding antibodies (10 mg/kg, $n = 4$ mice/group). After 20 hours, cells were recovered from spleen, labeled with huCD45/CD19/CD5, and counted in a flow cytometer. Representative density plots for each treatment are shown (**C**). The gating strategy started with cells in forward scatter (FSC)/side scatter (SSC; R1), then gating on $huCD45^+$, and finally $huCD19^+CD5^+$. **D,** Total number of $huCD45^+CD19^+CD5^+$ recovered from the spleen is plotted. Statistical differences between groups were assessed by unpaired *t* test.

By this day, the antibody concentrations in the serum of daratumumab-treated animals had dropped to 1.5 μg/mL (Supplementary Fig. S4). Interestingly, in this group of mice, no MEC2 cells were found by flow cytometry or immunohistochemistry in the commonly infiltrated organs like lung and kidney, contrasting with the remarkable predominance of malignant, human $CD19^+$ cells observed in these secondary sites in control isotype–treated mice (Fig. 5C and D), suggesting that these mice may be free of disease. These data suggest a strong antitumor activity and long-term survival of daratumumab-treated mice in this model.

To develop a mouse model closer to CLL biology, we established a short time CLL-PDX model using NSG mice, needed to avoid CLL clearance by mouse NKs. To provide this mouse model with FcγR-bearing effector cells, we selected CLL cases enriched in NKs and monocytes (Fig. 5E). Fresh PBMCs from these patients were intravenously inoculated (day 1) and treated the following day (day 2) with daratumumab or control isotype. On day 5, mice were sacrificed and cells recovered from peripheral blood, bone marrow, and spleen. As described previously, cells mainly homed to the spleen, where a significant (*, $P < 0.05$) decrease of CLL cells was found in the spleen of daratumumab-treated group. No significant differences were found in bone marrow or peripheral blood resident CLL cells between the 2 groups (Supplementary Fig. S5A). Off note, when the experiment was performed with a CLL sample with reduced numbers of effectors, the antitumoral effect of daratumumab was diminished (Supplementary Fig. S5B and S5C)

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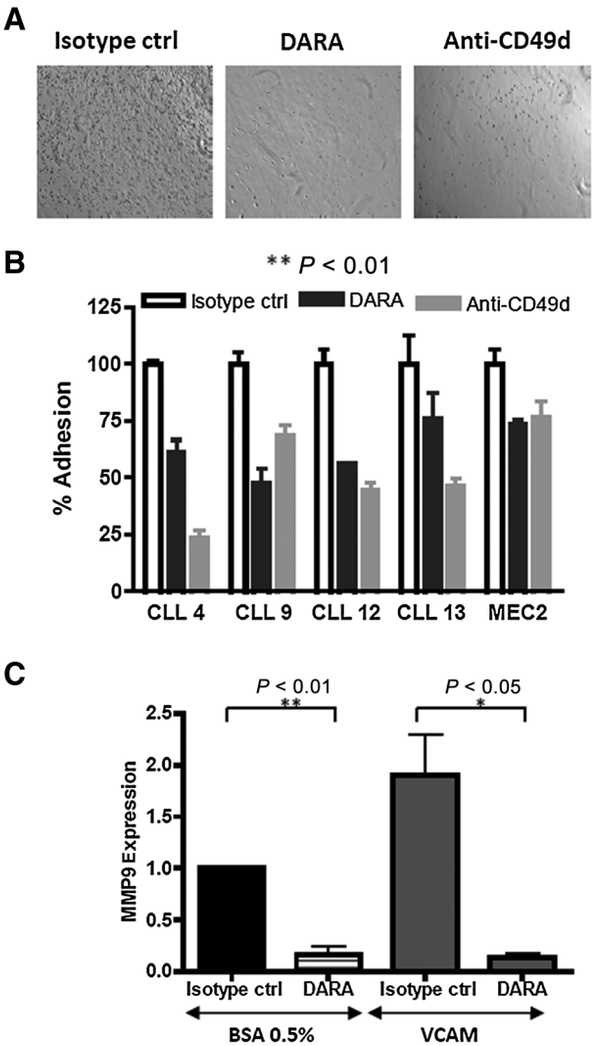


**Figure 4.**

Daratumumab hampers CLL adhesion to VCAM. Calcein-labeled primary CLL and MEC2 cells were preincubated with the corresponding antibodies (30 μg/mL) and left to adhere for 30 minutes to plates precoated with VCAM-1 or BSA (nonspecific adhesion). Anti-CD49d was used as positive control for inhibition of adhesion. Nonadhered cells were removed by extensive washing. **A,** Representative phase-contrast microscopy field images (100×) from adhesion to VCAM-1 of CLL9. **B,** Adhered cells were then lysed and supernatants analyzed in a fluorimeter. Percentage of VCAM-1 adhesion is expressed normalized to isotype control and after subtraction of BSA nonspecific adhesion ($P < 0.01$, one-way ANOVA comparing the 3 groups together). **C,** RT-PCR for *MMP9* was performed on the adhered cells to VCAM, using *mGUS* as endogenous control. $n = 3$, CLL4, CLL12, and CLL13. Expression levels for each sample are normalized to the corresponding isotype control and adhesion to BSA. $^{**}$, $P < 0.01$; $^{*}$, $P < 0.05$; unpaired $t$ test.

## Discussion

Targeted immunotherapy with mAbs has become the standard of care for successful treatment of many forms of cancer. In CLL, anti-CD20 antibodies (rituximab, ofatumumab, and obinutuzumab) have demonstrated therapeutic benefit, alone and in combination with chemotherapy (35, 36). Identification of new targets with a broader expression spectrum and potential for distinctive mechanisms could yield novel antibody therapeutics for a wider range of hematologic malignancies.

In the last years, CD38 has gained momentum as a novel therapeutic target for patients with hematologic malignancies, namely multiple myeloma (37, 38), CLL, and NHL. CD38 is an ectoenzyme belonging to the family of nucleotide-metabolizing enzymes, involved in the scavenging of extracellular nucleotides. CD38 catalyzes the synthesis of cyclic ADP-ribose and ADP-ribose from NAD, leading to an increase in cytoplasmic $Ca^{2+}$ concentration. CD38 is used as a disease marker for leukemias and myeloma and it is considered a negative prognostic marker for CLL (4, 5). Moreover, recent evidence indicates that CD38 forms a complex molecular network delivering growth and survival signals in CLL cells. CD38 cooperates with chemokines and their receptors to influence cell migratory responses (11). These characteristics make CD38 an attractive target for CLL therapy. The use of an antibody such as daratumumab that specifically blocks CD38 might provide a new approach for interfering with deleterious growth circuits and for increasing the susceptibility of leukemic cells to conventional chemotherapy.

In this study, we have analyzed the potential therapeutic activity of daratumumab in CLL. Daratumumab showed limited CDC activity in both primary CLL cells and CLL cell lines. Complement activation is strongly regulated by CRPs *in vivo* to prevent its uncontrolled amplification, including CD46, CD55, and CD59 that have been shown to mediate resistance to CDC induced by rituximab and less by ofatumumab (39). In fact, these proteins are overexpressed in a number of tumor types, and their upregulation has been postulated to contribute to mAb resistance *in vivo* (40). Our data show that CLL cell lines and primary cells display very high expression of the CRP, which may explain the limited sensitivity to daratumumab-mediated CDC *in vitro*.

Our results are the first to provide strong evidence that daratumumab induces lysis of CLL cells by FcγR-mediated ADCC and ADCP through NK cells and macrophages, respectively. This cytotoxic effect is remarkable in CLL, where the mean ADCC induction was 35% at 0.1 μg/mL, being in the same range to those published for primary multiple myeloma (19, 32). In addition, ADCC does not correlate strictly with CD38 sABC in CLL cell lines or primary cells, indicating that at least in CLL, the number of CD38 molecules on the cell surface may not be the only factor in driving Fc-mediated cytotoxicity of daratumumab. This observation suggests that other molecules within the immune synapse must control the extent of ADCC/ADCP by daratumumab. Activating receptors expressed on NK cells include FcγRIIIA, activating forms of killer cell Ig-like receptors (KIRs; KIR2DS and KIR3DS), NKG2D, and the natural cytotoxicity receptors (NCR) called NKp30, NKp44, and NKp46 that are critical for optimal ADCC activity (41). NKG2D and NCRs are the most relevant receptors that stimulate responses to tumor target cells. In addition, inhibitory receptors counteract activating receptors as a means to tolerate mature NK cells. Thus, the overall makeup of these activating and inhibitory molecules on each individual CLL tumor cell may dictate the extent of ADCC by daratumumab (42). Along these lines, a recent study in multiple myeloma has shown that blocking inhibitory KIRs with IPH2102, a human IgG4 monoclonal antibody that blocks the interaction of the 3 main inhibitory KIRs with their ligands, improves ADCC induced by daratumumab against multiple myeloma cells (43).

Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095



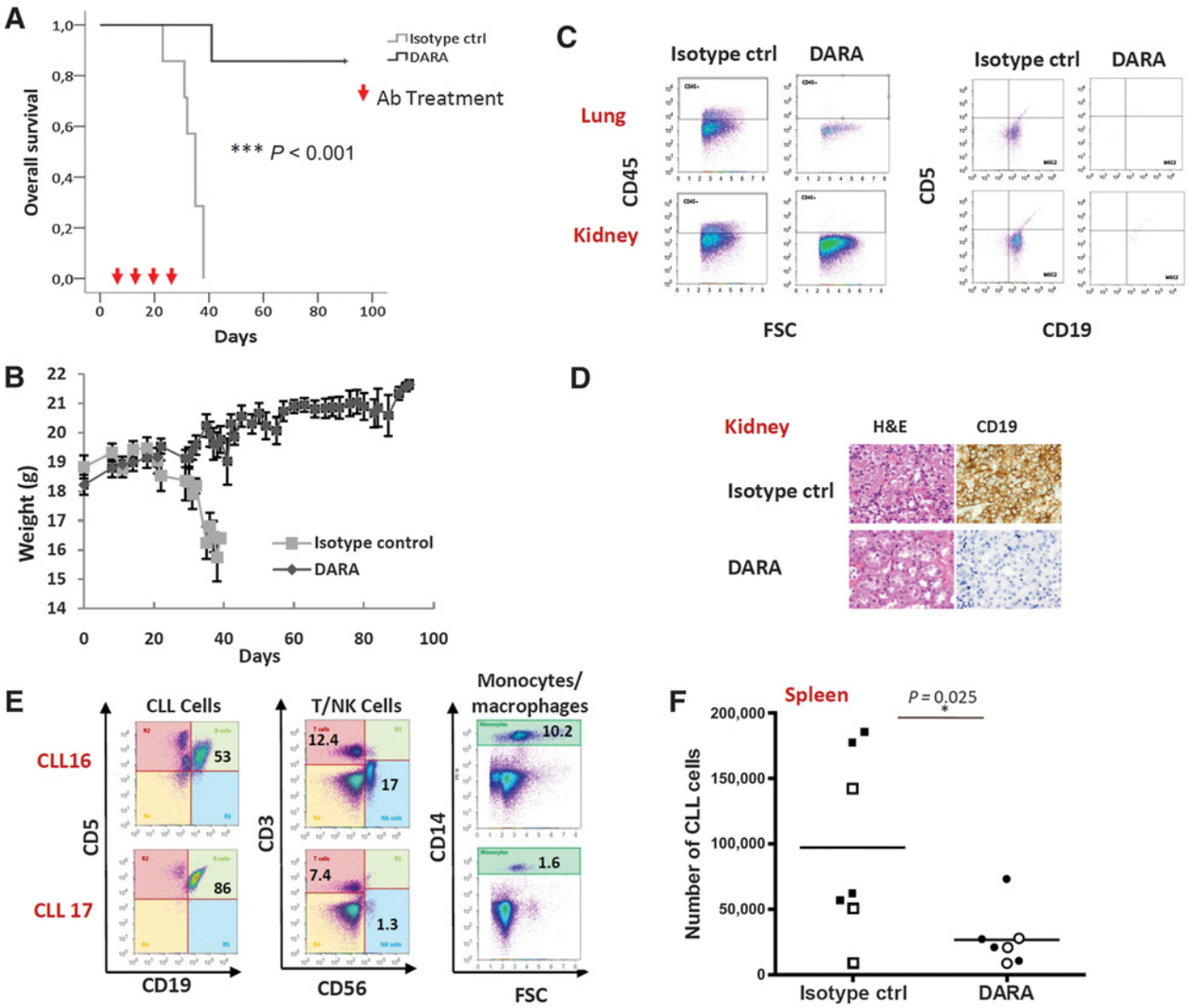


**Figure 5.**

*In vivo* efficacy of daratumumab (DARA). **A,** Kaplan–Meier survival curves for MEC2 systemic mouse xenografts. Mice received daratumumab ($n = 7$) or isotype control ($n = 6$) weekly for 4 weeks, starting 1 week after cell inoculation. Then, mice were monitored twice a week for any sign of disease and sacrificed when body weight decreased 15% to 20%. All control mice had to be sacrificed between days 23 and 38. One daratumumab-treated mouse became ill and was sacrificed on day 41. **B,** Body weight changes in the isotype and daratumumab-treated mice. **C,** Cells from lungs and kidneys were isolated and labeled with huCD45/CD19/CD5. The presence of MEC2 cells was evaluated by $CD19^+CD5^-$ (right) cells in the $CD45^+$population (left). Representative flow cytometric density plots for one mouse of each cohort sacrificed at day 30 are shown. **D,** IHC staining of H&E and CD19 of kidneys from isotype and daratumumab-treated mice (magnification, 400×). **E,** Density plots showing the percentage of CLL cells ($CD19^+CD5^+$), T cells ($CD3^+CD56^-$), NK cells ($CD3^-$ $CD56^+$), and monocytes ($CD14^+$). **F,** Total number of $huCD45^+CD19^+CD5^+$ recovered from the spleen of isotype control and daratumumab-treated groups (CLL16: open symbols; CLL17: closed symbols). Statistical differences between groups were assessed by unpaired *t* test.

Macrophages are tissue-resident immune cells that play a critical role not only in maintaining homeostasis and fighting infection but also in the progression of many pathologies including cancer (44). Human macrophages express both activating and inhibitory FcγR and are involved as most prominent effector cell populations in mAb-mediated tumor elimination *in vivo*. We have demonstrated that daratumumab induces phagocytosis of CLL by macrophages both *in vitro* and *in vivo*. In the *in vitro* model, CLL cells and macrophages were cocultured in the presence or absence of daratumumab. In the *in vivo* model, CLL cells were injected into the peritoneum of SCID *beige* mice, which are devoid of NK cells, but possess active macrophages in their peritoneal cavity.

Taken together, these results provide evidence that anti-CD38 therapy with daratumumab may be relevant for $CD38^+$ CLL cases.

We next explored whether these *in vitro* mechanisms translate to *in vivo* tumor growth inhibition. To accomplish this task, we developed 2 approaches. First, we successfully developed an MEC2 tumor model that showed engraftment efficacy of 100% and systemic disease involving mostly lungs, kidney, ovaries, parathyroid glands, enlarged lymph nodes, and bone marrow in a portion of the mice. This model is clinically relevant for CLL with

leukemic infiltrates in isolated organs similar to that reported in patients with CLL (45), presenting aggressive disease or transformation to Ritcher syndrome. In this model, treatment with relevant pharmacologic doses of daratumumab efficiently prevented tumor progression and significantly prolonged survival. Mice treated with daratumumab showed long-term survival even though daratumumab dosing was stopped after 4 weeks and the antibody concentrations in the serum were minimal, suggesting that these mice were free of disease. Second, we developed a CLL-PDX model using NSG mice, to avoid CLL clearance by mouse NKs or phagocytes, and inoculating selected CLL enriched in NKs and monocytes to provide the system with FcγR-bearing effector cells. In this model, daratumumab proved to reduce tumor burden in the mouse spleen, which constitutes the main infiltrated organ in this model (46). These results are the first to provide evidence that anti-CD38 therapy with daratumumab may be relevant for $CD38^+$ CLL.

As described in Introduction, daratumumab activity may extend beyond its effect on the tumor cells, as it shows immunomodulatory effects on CD38-expressing immunosuppressive regulatory T and B cells and myeloid-derived suppressor cells (23). We have also demonstrated additional activities of daratumumab besides ADCC and ADCP in CLL. Daratumumab has the potential to counteract microenvironment-derived signaling in protective cancer niches, such as lymph node and bone marrow. We have demonstrated that daratumumab interferes with *in vitro* cell migration and *in vivo* homing of CLL cells to spleen in NSG mice. Transendothelial migration and organ invasion of malignant cells require proteolytic degradation of the vascular basement membrane and extracellular matrix of lymphoid tissues and MMPs play a key role in these processes. MMP9 is the predominant MMP expressed in CLL and is physiologically regulated by CD49d/CD29 and CXCL12, playing a key role in cell invasion and transendothelial migration (47). Moreover, MMP9 correlates with advanced-stage disease and poor patient survival (48). We have demonstrated that daratumumab significantly reduces CD49d/CD29-mediated adhesion of CLL cells to VCAM-1 and, more importantly, downregulates both constitutive and adhesion-induced MMP9 expression. On the basis of the prominent role of MMP9 on CLL cell invasion, our results indicate that daratumumab treatment may impede CLL tissue infiltration that leads to progressive disease. Thus, in the era of BCR kinase inhibitors, daratumumab immunotherapy opens a new horizon offering unique effects on tumor dissemination against $CD38^+$ CLL cases. In conclusion, our results support daratumumab as a novel therapeutic approach for $CD38^+$ CLL by not only inducing the classical FcγR-mediated cytotoxicity but also harnessing microenvironment-derived survival signaling and blocking CLL dissemination to secondary lymphoid organs.

## Disclosure of Potential Conflicts of Interest

A. Wiestner reports receiving commercial research grants from and is a consultant/advisory board member for Pharmacyclics. P.W.H.I. Parren holds ownership interest (including patents) in Genmab. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A. Matas-Céspedes, A. Vidal-Crespo, V. Rodriguez, N. Villamor, J. Delgado, E. Giné, H. Roca-Ho, P. Pérez-Galán

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A. Matas-Céspedes, A. Vidal-Crespo, V. Rodriguez, E. Campo, A. López-Guillermo, P.W.H.I. Parren, P. Doshi, J.L. van Bueren, P. Pérez-Galán

**Writing, review, and/or revision of the manuscript:** A. Matas-Céspedes, A. Vidal-Crespo, N. Villamor, J. Delgado, E. Giné, E. Campo, D. Colomer, G. Roué, P.W.H.I. Parren, P. Doshi, J.L. van Bueren, P. Pérez-Galán

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** V. Rodriguez, H. Roca-Ho, P. Menéndez, P. Doshi, P. Pérez-Galán

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## Acknowledgments

We thank Jocabed Roldán, Laura Jiménez, and Sandra Cabezas for their technical assistance. We also acknowledge Marije Overdijk for her advice on phagocytosis experiments and Wim Bleeker for his advice on mouse models. We thank Mariona M. Cid for her advice on MMP9 experiments. This work was carried out at the Esther Koplowitz Center, Barcelona.

## Grant Support

Genmab and Janssen pharmaceuticals funded this research. Additional grants that contributed to this work included: grants from the Spanish Ministry of Economy and Competitivity (RYC2009-05134 and SAF11/29326 to P. Pérez-Galán, SAF12/31242 and IPT.2012-0673-010000 to D. Colomer, and PI12/01847 to G. Roué), Integrated Excellence Grant from the Instituto de Salud Carlos III (ISCIII) PIE1313/00033 to E. Campo and P. Pérez-Galán, Redes Temáticas de Investigación Cooperativa de Cáncer from the Instituto de Salud Carlos III (ISCIII), Spanish Ministry of Economy and Competitiveness & European Regional Development Fund (ERDF) "Una manera de hacer Europa" (RD12/0036/0004 to D. Colomer and RD12/0036/0023 to A. López-Guillermo).

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Received August 27, 2015; revised August 29, 2016; accepted September 10, 2016; published OnlineFirst September 16, 2016.

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Published OnlineFirst September 16, 2016; DOI: 10.1158/1078-0432.CCR-15-2095



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*Clin Cancer Res* 2017;23:1493-1505. Published OnlineFirst September 16, 2016.