

# Preclinical Evidence for the Therapeutic Potential of CD38-Targeted Immuno-Chemotherapy in Multiple Myeloma Patients Refractory to Lenalidomide and Bortezomib

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## Abstract

**Purpose:** Novel therapeutic agents have significantly improved the survival of patients with multiple myeloma. Nonetheless, the prognosis of patients with multiple myeloma who become refractory to the novel agents lenalidomide and bortezomib is very poor, indicating the urgent need for new therapeutic options for these patients. The human CD38 monoclonal antibody daratumumab is being evaluated as a novel therapy for multiple myeloma. Prompted with the encouraging results of ongoing clinical phase I/II trials, we now addressed the potential value of daratumumab alone or in combination with lenalidomide or bortezomib for the treatment of lenalidomide- and bortezomib-refractory patients.

**Experimental Design:** In *ex vivo* assays, mainly evaluating antibody-dependent cell-mediated cytotoxicity, and in an *in vivo* xenograft mouse model, we evaluated daratumumab alone or in combination with lenalidomide or bortezomib as a potential therapy for lenalidomide- and bortezomib-refractory multiple myeloma patients.

**Results:** Daratumumab induced significant lysis of lenalidomide/bortezomib-resistant multiple myeloma cell lines and of primary multiple myeloma cells in the bone marrow mononuclear cells derived from lenalidomide- and/or bortezomib-refractory patients. In these assays, lenalidomide but not bortezomib, synergistically enhanced daratumumab-mediated multiple myeloma lysis through activation of natural killer cells. Finally, in an *in vivo* xenograft model, only the combination of daratumumab with lenalidomide effectively reduced the tumorigenic growth of primary multiple myeloma cells from a lenalidomide- and bortezomib-refractory patient.

**Conclusions:** Our results provide the first preclinical evidence for the benefit of daratumumab plus lenalidomide combination for lenalidomide- and bortezomib-refractory patients. *Clin Cancer Res*; 21(12); 2802–10. ©2014 AACR.

See related commentary by Laubach and Richardson, p. 2660

## Introduction

Multiple myeloma, the malignant disease of antibody producing plasma cells, is still considered incurable. Over the past decade, the survival of patients with multiple myeloma has

significantly improved, mainly due to the introduction of the novel immunomodulatory agent lenalidomide, the potent proteasome inhibitor bortezomib, and the application of high-dose conventional therapy with autologous stem cell rescue (1). Nonetheless, virtually all antimyeloma strategies are eventually hampered by the development of drug resistance. The prognosis of patients with multiple myeloma who become refractory to bortezomib and lenalidomide is very poor with an event-free survival and overall survival of only 5 and 9 months, respectively (2). Therefore, especially for this specific group of (multi-)refractory multiple myeloma patients, new approaches that induce long-term tumor regression are urgently needed. In this respect, several new antimyeloma agents hold promise, including next-generation IMiDs (pomalidomide) and proteasome inhibitors (carfilzomib; ref. 3).

On the other hand, the success of anti-CD20 mAbs in the treatment of non-Hodgkin lymphomas and chronic lymphocytic leukemia, illustrated that antibody-mediated immunotherapy can also represent a powerful therapeutic option for hematologic malignancies and stimulated the investigation of antibody therapies for multiple myeloma (4). Daratumumab is a human IgG1 kappa monoclonal antibody that targets CD38, a

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### Translational Relevance

The prognosis of patients with multiple myeloma who become refractory to the novel antimyeloma agents lenalidomide and bortezomib is very poor, indicating the need for new therapeutic strategies for these patients. The human CD38 monoclonal antibody daratumumab is currently being evaluated in phase I/II clinical trials as a novel therapy for multiple myeloma. Here, we demonstrate the *in vitro* as well as *in vivo* susceptibility of malignant cells, derived from lenalidomide- and bortezomib-refractory multiple myeloma patients, to daratumumab. Thus, this novel CD38 antibody may offer new therapeutic perspectives for multidrug refractory multiple myeloma patients. Furthermore, we discovered that lenalidomide, although no longer effective as a therapeutic drug in these patients, can still synergistically potentiate the antimyeloma effects of daratumumab through the activation of natural killer cells. Therefore, the therapeutic perspectives of multidrug refractory patients may be improved by combination therapy with daratumumab and lenalidomide.

type II transmembrane glycoprotein, which is highly and uniformly expressed in multiple myeloma (5). Previously, we have demonstrated that daratumumab induces effective multiple myeloma cell death mainly via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC; ref. 5). We have also shown in *ex vivo* assays that the anti-multiple myeloma efficacy of daratumumab can be potentiated by immunomodulatory agents such as lenalidomide or bortezomib, alone or in combination with other antimyeloma agents (6, 7). *In vivo*, daratumumab controlled or even eliminated multiple myeloma cell line-derived tumors growing in the bone marrow of RAG2<sup>-/-</sup>, $\gamma$ c<sup>-/-</sup> mice (8). In a more advanced *in vivo* model, daratumumab also mediated significant antitumor activity against tumors derived from patients' multiple myeloma cells, growing in a humanized bone microenvironment (8). With these properties, daratumumab monotherapy represents a promising therapeutic strategy for the treatment of multiple myeloma. Daratumumab is currently evaluated in phase I/II studies (9, 10). Encouraging preliminary results of these studies prompted us to evaluate the potential value of daratumumab in lenalidomide- and bortezomib-refractory multiple myeloma patients, who have a very poor prognosis at present. We hypothesized that, despite the drug resistance of the malignancy, the immune system of these patients could still respond to the previously described immunomodulatory effects of lenalidomide (11–14) and bortezomib (15, 16). Therefore, we now evaluated daratumumab alone or in combination with lenalidomide or bortezomib as a potential therapy for lenalidomide- and bortezomib-refractory patients.

In *in vivo* and *ex vivo* assays, we show the susceptibility of lenalidomide- and bortezomib-refractory multiple myeloma cells to daratumumab treatment. Confirming our hypothesis, the combination of daratumumab with lenalidomide or bortezomib significantly enhanced lysis in lenalidomide- and/or bortezomib-refractory patients. Although the combination of daratumumab with bortezomib resulted in additive effects,

lenalidomide synergized with daratumumab through the activation of effector cells, especially natural killer (NK) cells. Finally, in an *in vivo* xenograft model, the combination of daratumumab with lenalidomide, in the presence of human NK cells, effectively delayed the tumorigenic growth of primary multiple myeloma cells from a lenalidomide- and bortezomib-refractory patient, underscoring the potential beneficial anti-multiple myeloma effects of the daratumumab plus lenalidomide combination for lenalidomide- and bortezomib-refractory patients. These results suggest that daratumumab combined with lenalidomide or bortezomib may be an effective therapeutic combination in lenalidomide- and bortezomib-refractory patients.

## Materials and Methods

### Patients

All patient materials were collected after written informed consent as approved by the institutional medical ethical committee. All samples originated from lenalidomide- and bortezomib-refractory patients, who were extensively treated with a median of four preceding regimens that included, but were not limited to, induction therapy with anthracyclines followed by autologous stem cell transplantation, thalidomide, lenalidomide, and bortezomib. Lenalidomide- and bortezomib-refractory disease is defined as progressive disease on lenalidomide- and bortezomib-therapy, no response (less than partial response) to lenalidomide- and bortezomib-therapy, or progressive disease within 60 days of stopping a lenalidomide- and bortezomib-containing regimen, according to the International Uniform Response Criteria for Multiple Myeloma (2). Characteristics of the tested patients with multiple myeloma are summarized in Table 1.

**Table 1.** Baseline characteristics of the tested BM-MNC of patients with multiple myeloma

Parameter	Patients with multiple myeloma, n = 11
Median age, y (range)	64 (42–75)
Sex, male, n (%)	7 (64%)
Type of monoclonal heavy chain	
IgG, n (%)	5 (46%)
IgA, n (%)	2 (18%)
Light chain only, n (%)	4 (36%)
Type of light chain	
Kappa, n (%)	7 (64%)
Lambda, n (%)	4 (34%)
Previous therapy	
Prior lines of therapy, median (range)	4 (2–6)
Prior stem cell transplantation	8 (73%)
Autologous	8 (73%)
Allogeneic	0 (0%)
Prior LEN treatment, n (%)	11 (100%)
LEN refractory status <sup>a</sup> , n (%)	11 (100%)
Prior BOR treatment, n (%)	10 (91%)
BOR refractory status <sup>a</sup> , n (%)	8 (73%)

Abbreviations: BOR, bortezomib; LEN, lenalidomide.

<sup>a</sup>LEN- and/or BOR-refractory disease is defined as progressive disease on LEN- and BOR-therapy, no response (less than partial response) to LEN- and BOR-therapy, or progressive disease within 60 days of stopping a LEN- and BOR-containing regimen, according to the International Uniform Response Criteria for Multiple Myeloma.(2).

### Bone marrow and peripheral blood mononuclear cells

All samples from patients and healthy individuals were collected and stored using protocols/procedures approved by the institutional medical ethical committee in accordance with the declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) from healthy donors and patients with multiple myeloma and bone marrow mononuclear cells (BM-MNC) from multiple myeloma patient BM aspirates were isolated by Ficoll-Hypaque density-gradient centrifugation. Freshly isolated PBMCs were used after culturing with and without lenalidomide (3  $\mu\text{mol/L}$ ; Celgene) for 48 hours as effector cells in classical 4-hour flow cytometry-based cytotoxicity assays, in which multiple myeloma cell lines were used as target cells.

### Bioluminescence imaging–based ADCC assays using luciferase-transduced multiple myeloma cell lines

Luciferase (LUC)-transduced (lenalidomide/bortezomib-resistant) multiple myeloma cells were cocultured with effector cells (freshly isolated PBMC or MACS-enriched PBMC fractions; see below) at the indicated effector to target ratios in the wells of white opaque 96-well plates (Costar) in the presence of a previously determined optimal concentration of daratumumab (0.1  $\mu\text{g/mL}$ ) for 4 hours. The survival of LUC<sup>+</sup>—multiple myeloma cells was then determined by bioluminescence imaging (BLI), 10 minutes after addition of the substrate luciferin (125  $\mu\text{g/mL}$ ; Promega). The percentage multiple myeloma lysis was determined using the following formula: % lysis = 1 – (mean BLI signal in the presence of effector cells and daratumumab/mean BLI signal in the presence of effector cells and control antibody)  $\times$  100%. In some assays, PBMCs were enriched for T cells and monocytes using immunomagnetic MACS beads coated with CD3 and CD14, respectively, following the instructions of the manufacturer. The purity of both T and monocyte fractions were >90%. The negative fractions of CD3-enriched PBMC, which were highly enriched for NK cells (>90%), were also used as effector cells in the *in vitro* and *in vivo* assays.

### Flow cytometry–based *ex vivo* cell lysis assays in BM-MNC

Freshly isolated BM-MNCs, containing 2% to 35% malignant plasma cells as determined by flow cytometry, were immediately used in *ex vivo* experiments. The BM-MNCs, containing the malignant plasma cells, as well as the patient's own effector cells, were incubated with daratumumab (10  $\mu\text{g/mL}$ ; Genmab), lenalidomide (3  $\mu\text{mol/L}$ ; Celgene), and bortezomib (3 nmol/L; Millennium Pharmaceuticals) alone or in combination in RPMI + 10% FBS in 96-well round bottom plates in fully humidified incubators at 37°C, 5% CO<sub>2</sub>-air mixture for 48 hours. The survival of primary CD138<sup>+</sup> multiple myeloma cells was determined by flow cytometry as previously described (7). Sample viability at incubation was more than 90%, as assessed using ToPro-3 (Invitrogen Life Technologies). Percentage lysis of multiple myeloma cells was deduced using the following formula: % lysis cells = 1 – (counts of surviving CD138<sup>+</sup> cells in treated wells/counts of number of surviving CD138<sup>+</sup> cells in control wells)  $\times$  100%.

### Mesenchymal stromal cells

The mononuclear cell fraction from healthy donor BM aspirates was obtained after Ficoll-Hypaque centrifugation and seeded in culture flasks in  $\alpha$ -MEM (Gibco), supplemented with 5% human platelet lysate, penicillin, and streptomycin

(100 U/mL and 100  $\mu\text{g/mL}$ , respectively; both Invitrogen Life Technologies), and 10 IU/mL heparin (Leo Pharma). Expanded mesenchymal stromal cells (MSC) were replated at a confluence of approximately 90%, passaged once, and collected and stored at –196°C for later use.

### Multiple myeloma cell lines and culture

The CD38<sup>+</sup> multiple myeloma cell lines UM9, L363-CD38 cl2.2, LME-1, and RPMI8226 were cultured in RPMI-1640 (Invitrogen Life Technologies), supplemented with 10% FBS (Integro BV) and antibiotics (100 U/mL penicillin, 100  $\mu\text{g/mL}$  streptomycin; both Invitrogen Life Technologies) as previously described (7).

### Antibodies and reagents

Daratumumab was provided by Genmab. IgG1-b12, a human mAb against an innocuous antigen (HIV-1 gp120), was used as an isotype control as previously described (7).

### *In vivo* treatment of primary multiple myeloma tumors growing in humanized bone marrow niches in immunodeficient mice

*In vivo* experiments were conducted in a recently described human multiple myeloma model in the RAG2<sup>–/–</sup> $\gamma\text{c}^{–/–}$  mice (8) after approval of the local Ethical Committee for Animal Experimentation and in compliance with the Dutch Animal Experimentation Act. Briefly, to enable the engraftment of primary multiple myeloma cells, a humanized microenvironment was generated in mice by subcutaneous implantation of ceramic scaffolds that were seeded with human MSC (2  $\times$  10<sup>5</sup> cells/scaffold) and *in vitro* cultured for 7 days in osteogenic medium, containing ascorbic acid and dexamethasone. Eight weeks after implantation, mice received a sublethal irradiation dose (3 Gy, 200 kV, 4 mA) and luciferase-gene–marked primary multiple myeloma cells were injected directly into the scaffolds (1  $\times$  10<sup>6</sup> cells/scaffold). Luciferase transduction of primary multiple myeloma cells was carried out using the lentiviral construct pRRL-cPPT-CMV-Luc2-IRES-GFP-PRE-SIN as described previously (8, 17). Tumor growth was monitored by BLI using a charge-coupled device camera, controlled by Photo Vision software, and analyzed with M3Vision software (Photon Imager; Biospace Laboratory). When tumors became clearly detectable, mice were distributed over the following treatment groups: (i) control, (ii) T-cell depleted PBMC (PBMC-T), (iii) PBMC-T plus lenalidomide, (iv) PBMC-T plus daratumumab, and (v) PBMC-T plus lenalidomide plus daratumumab. Lenalidomide (1 mg/kg) was given in 5 days on 2 days off schedule for 2 weeks (days 49–53 and 56–60) and both daratumumab (8 mg/kg) and PBMC-T (8  $\times$  10<sup>6</sup> cells/mouse) were given on days 49 and 56. PBMC-T was prepared by Ficoll-Hypaque density-gradient centrifugation of buffy coats, and subsequent depletion of T cells by CD3-beads using the Easy-Sep-technology (STEMCELL Technologies).

### Immunophenotyping by flow cytometry

Cell surface expression of all tested antigens was determined by flow cytometry using FITC-, PE-, PerCP-, or APC-conjugated monoclonal antibodies (all from BD Biosciences). Flow cytometry was carried out using a FACS-Calibur device (BD Biosciences); the data were analyzed using CellQuest software.

### Statistical analysis

Differences between groups were analyzed for significance in two-tailed paired Student *t* tests using Prism software (GraphPad Software Inc. version 5). *P* values below 0.05 were considered significant. In case of combinatorial treatments of daratumumab and lenalidomide or bortezomib, the expected lysis values were calculated to test the null hypothesis that there is only an additive effect between daratumumab and the drug (lenalidomide or bortezomib), using the following formula: % expected lysis = (% lysis with daratumumab + % lysis with drug) – % lysis with daratumumab × % lysis with drug (7, 18). The null hypothesis of "additive effects" was rejected, if the observed values were significantly higher (*P* < 0.05) than the expected values.

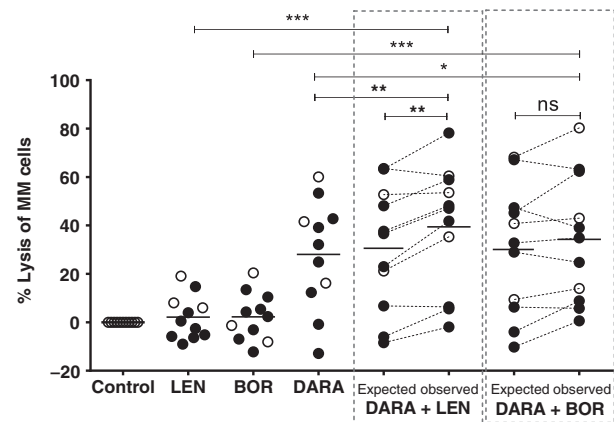
## Results

### Lenalidomide, but not bortezomib, improves daratumumab-mediated ADCC of multiple myeloma cells from lenalidomide- and bortezomib-refractory patients synergistically

We first questioned whether the multiple myeloma cells of lenalidomide- and/or bortezomib-refractory patients would respond to daratumumab alone and whether improvement of multiple myeloma cell lysis is possible when combining daratumumab with lenalidomide or bortezomib in lenalidomide- and/or bortezomib-refractory multiple myeloma patients. To address these questions, we used our previously developed *ex vivo* flow cytometry-based cytotoxicity assay (7). BM-MNC from 11 lenalidomide-refractory patients, of whom 8 were also bortezomib-refractory, were incubated with daratumumab, lenalidomide, and bortezomib alone or in combination to measure the lysis of CD138<sup>+</sup> multiple myeloma cells (Fig. 1). Lenalidomide and bortezomib monotherapy induced virtually no multiple myeloma cell lysis in refractory patients, consistent with the lenalidomide- and/or bortezomib-refractory clinical status of these patients. In contrast, daratumumab induced significant levels of multiple myeloma cell lysis in the BM-MNC from refractory multiple myeloma patients [mean 29.7%, 95% confidence intervals (CI), 13.5%–45.9%]. Nonetheless, there was a large degree of heterogeneity in the lysis induced in lenalidomide- and bortezomib-refractory patients, with less than 30% of multiple myeloma cell lysis in 5 of 11 patients. Remarkably, however, the multiple myeloma cell lysis of 29.7% with daratumumab alone was significantly improved to 39.4% (95% CI, 22.1%–56.7%; *P* = 0.0041) upon combination of daratumumab with lenalidomide, although all tested patients were refractory to lenalidomide. Such an effect was not observed with bortezomib. Assuming drug combinations would be additive, we calculated the expected lysis values, (7, 18) as described in Materials and Methods, and compared them with the observed results. The daratumumab plus lenalidomide combination appeared synergistic, as illustrated by the significant difference between the expected versus observed results, whereas the daratumumab plus bortezomib combination was indeed additive.

### NK activation by lenalidomide contributes to lenalidomide and daratumumab synergism in lenalidomide- and bortezomib-refractory patient multiple myeloma cells

To investigate effector cell contributions to the mechanism of synergy between lenalidomide and daratumumab, we performed standard 4-hour cytotoxicity assays, in which PBMCs from random healthy donors (*n* = 5) were used as effector

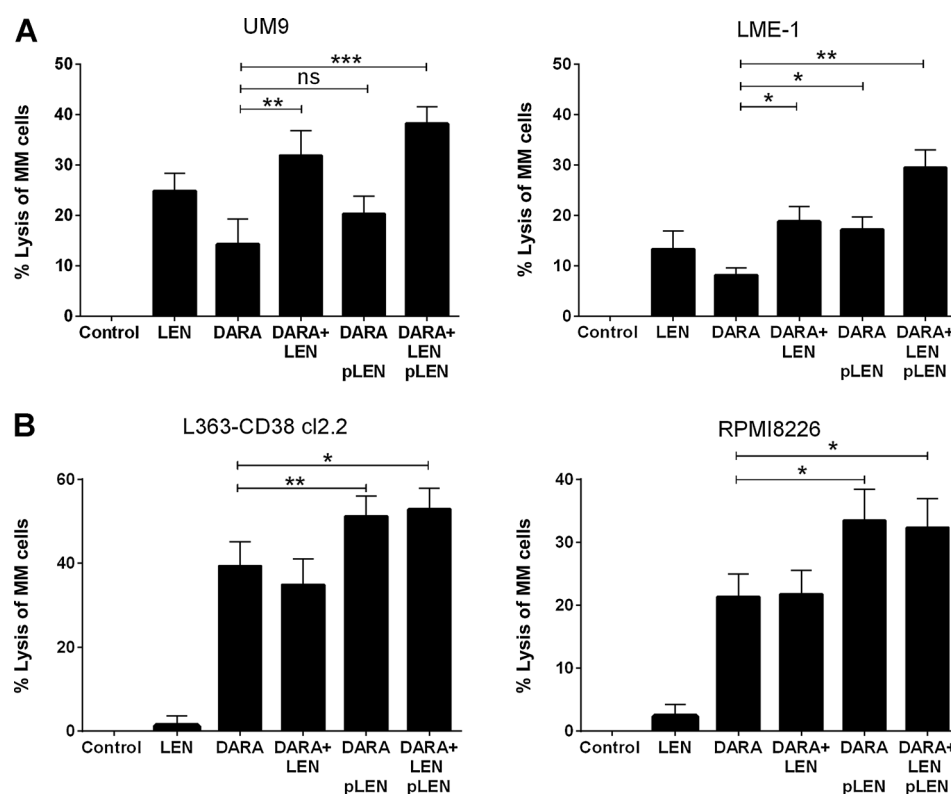


**Figure 1.**

Lenalidomide (LEN), and bortezomib (BOR), significantly improves daratumumab (DARA)-mediated lysis of primary multiple myeloma cells from lenalidomide- and bortezomib-refractory patients. BM-MNC of 11 lenalidomide-refractory multiple myeloma patients, of whom 8 patients were also bortezomib-refractory (the black circles represent the lenalidomide/bortezomib double-refractory patients), were incubated with control antibody (control) or with daratumumab (both at a concentration of 10 μg/mL), alone or in combination with lenalidomide (3 μmol/L) or bortezomib (3 nmol/L) for 48 hours. Surviving multiple myeloma cells were enumerated by FACS-analysis of CD138<sup>+</sup> cells. Percentage lysis of multiple myeloma cells was calculated as indicated in Materials and Methods. Daratumumab and lenalidomide acted synergistically, as illustrated by significant differences between the expected versus observed results, while the combination of bortezomib and daratumumab shows an additive effect, as illustrated by the nonsignificant differences between the expected versus observed results as indicated in Materials and Methods. *P* values were calculated by a paired *t* test; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; ns, not significant.

cells against the lenalidomide-resistant multiple myeloma cell lines L363-CD38 c12.2 and RPMI8226, and the lenalidomide-sensitive cell lines UM9 and LME-1. As expected, lenalidomide alone induced multiple myeloma cell lysis and also enhanced the daratumumab-dependent lysis in the lenalidomide-sensitive cell lines (Fig. 2A). In the lenalidomide-resistant cell lines daratumumab, but not lenalidomide, induced significant multiple myeloma cell lysis (Fig. 2B). Daratumumab-mediated lysis of multiple myeloma cells was not improved when multiple myeloma cells were preincubated with lenalidomide in lenalidomide-resistant cell lines (Fig. 2B). Interestingly, when we pretreated the PBMC (effector cells; Plen) with lenalidomide for 48 hours, the daratumumab-mediated multiple myeloma cell lysis was significantly enhanced, not only in lenalidomide-sensitive, but also in lenalidomide-resistant cell lines (Fig. 2A and B). On the basis of these results, the synergy between lenalidomide and daratumumab appeared to be due to the action of lenalidomide on the effector cells present in PBMC and not via direct effects on the tumor cells.

To identify the effector cell population activated by lenalidomide, we determined the frequencies of various cell subsets in the PBMC of 9 healthy donors, and in BM-MNC of 5 lenalidomide- and bortezomib-refractory multiple myeloma patients. Lenalidomide treatment had no effect on the frequencies of T cells or monocytes, but significantly increased the frequency of CD3<sup>+</sup>CD56<sup>+</sup> NK cells and the activated fraction of CD3<sup>+</sup>CD56<sup>+</sup>CD16<sup>+</sup> NK cells in healthy donor PBMC (Fig. 3A)

**Figure 2.**

Potent capacity of lenalidomide (LEN) to activate ADCC effector cells as explanation for the lenalidomide and daratumumab (DARA) synergy in lenalidomide-resistant multiple myeloma cells. A and B, results of standard 4-hour ADCC performed using PBMC from healthy donors as effector cells. Target cells were the lenalidomide-sensitive multiple myeloma cell lines UM9 and LME-1 in A, and the lenalidomide-resistant cell lines L363-CD38 c12.2 and RPMI8226 in B. Data show mean and SEM of at least five experiments. Control antibody (control) or daratumumab was added in the assay at a concentration of 0.1  $\mu$ g/mL. Lenalidomide was either added in the assay (lenalidomide) and/or preincubated with effector PBMC for 48 hours (pLEN) at a concentration of 3  $\mu$ mol/L. *P* values between the indicated groups were calculated using a paired Student *t* test; \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; ns, not significant.

and in lenalidomide- and bortezomib-refractory multiple myeloma patient BM-MNC (Fig. 3B). To functionally validate these results, we pretreated PBMC from healthy donors and a heavily pretreated lenalidomide- and bortezomib-refractory multiple myeloma patient with lenalidomide or solvent control and used their T-cell-, NK cell-, or monocyte-enriched fractions as effector cells in a 4-hour ADCC assay (Fig. 4A and B). Regardless of the source of the sample, the multiple myeloma cell lysis was mainly mediated by CD3<sup>-</sup>/CD56<sup>+</sup>-enriched NK cell fraction in these assays. Lenalidomide preincubation of PBMC improved the NK cell-mediated multiple myeloma cell lysis, but did not induce or enhance T-cell- or monocyte-mediated multiple myeloma cell lysis, confirming that the synergism between lenalidomide and daratumumab is mainly due to the activation of NK cells.

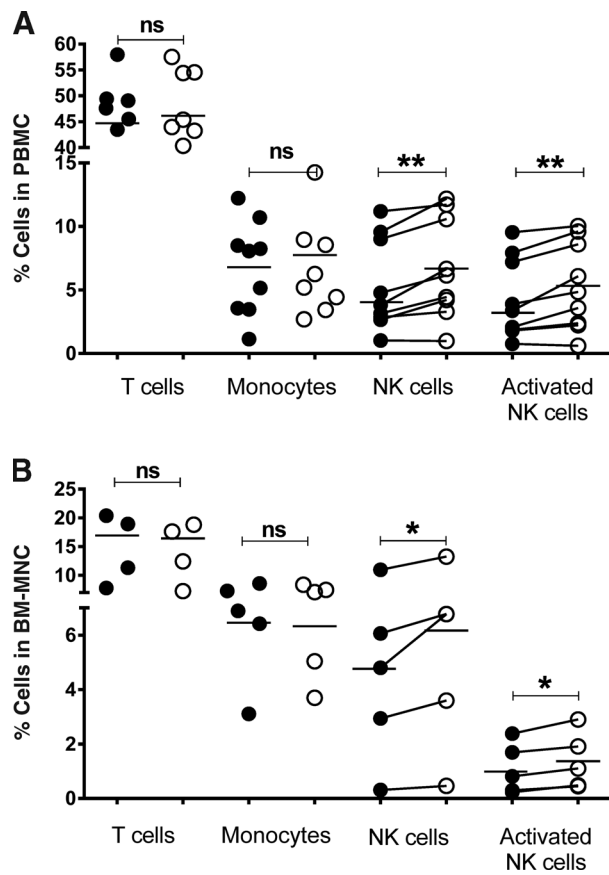
#### Lenalidomide improves responses to daratumumab in humanized mice engrafted with multiple myeloma cells derived from a lenalidomide- and bortezomib-refractory patient

To evaluate the added value of lenalidomide to daratumumab-mediated treatment for lenalidomide- and bortezomib-refractory patients *in vivo*, we used our recently developed model in which primary multiple myeloma cells from patients can be grown in humanized bone scaffolds implanted in RAG2<sup>-/-</sup> $\gamma$ <sub>c</sub><sup>-/-</sup> mice (8). We inoculated the humanized scaffolds with luciferase-marked primary multiple myeloma cells, derived from a lenalidomide- and bortezomib-refractory patient. After confirmation of tumor growth by BLI, mice were treated with daratumumab and lenalidomide alone or in combination (Fig. 5A). To optimally evaluate the effects of lenalidomide and daratumumab, we also

coinjecting the mice with NK cell-enriched (T-cell-depleted) PBMC of a healthy donor in combination with daratumumab and/or lenalidomide as RAG2<sup>-/-</sup> $\gamma$ <sub>c</sub><sup>-/-</sup> mice are devoid of NK cells. As expected, multiple myeloma tumors from the lenalidomide- and bortezomib-refractory patient showed tumor growth after treatment with lenalidomide alone (Fig. 5B and C). Confirming *in vitro* results, treatment with daratumumab alone suppressed the tumor growth significantly. However, only the combination of daratumumab plus lenalidomide was able to reduce the tumor volume (Fig. 5B and C) indicating synergistic effects. Notably, the tumor growth was suppressed/delayed for 6 weeks, while the controls showed a 4-log increase in tumor growth. These results provided the first preclinical *in vivo* evidence that clinically beneficial and sustained anti-multiple myeloma effects can be achieved in lenalidomide- and bortezomib-refractory multiple myeloma patients with daratumumab therapy in combination with lenalidomide.

#### Discussion

Despite significant advances in the management of multiple myeloma over the past decades, the development of drug resistance remains a significant obstacle to the long-term survival of patients with multiple myeloma. In the present study, using well-established *in vivo* and *ex vivo* models, we now show that daratumumab, especially when combined with lenalidomide, can be effective as a targeted treatment for lenalidomide- and bortezomib-refractory patients. Specifically, we demonstrate that the multiple myeloma cells of these patients can be killed by daratumumab-dependent ADCC and these anti-multiple myeloma effects can still be potentiated by lenalidomide



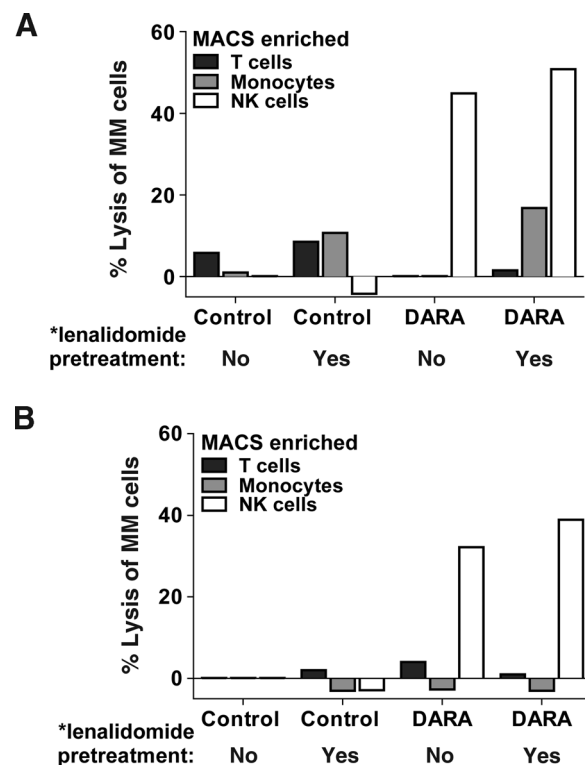
**Figure 3.** Effect of lenalidomide on NK cells in healthy PBMC and BM-MNC derived from lenalidomide- and bortezomib-refractory patients. Effect of 48-hour lenalidomide preincubation on immune cell subsets in PBMC of healthy donors (A) and on BM-MNC of lenalidomide- and bortezomib-refractory multiple myeloma patients' full bone marrow aspirates (B). Results represent percentages of cell subsets in untreated (black circles) and lenalidomide-treated (white circles) samples, determined by FACS-analysis. T cells, monocytes, and NK cells were identified as CD3<sup>+</sup>, CD14<sup>+</sup>, and CD3<sup>+</sup>CD56<sup>+</sup> cells, respectively. Activated NK cells were identified as CD3<sup>+</sup>CD56<sup>+</sup>CD16<sup>+</sup> cells (32, 33). The statistical differences between untreated and treated groups were calculated using a paired Student *t* test; \*, *P* < 0.05; \*\*, *P* < 0.01; ns, not significant. For (activated) NK cells, the paired samples are depicted with a connection-line, for the clarity.

in a synergistic fashion not only *in vitro*, but also in an optimized *in vivo* model.

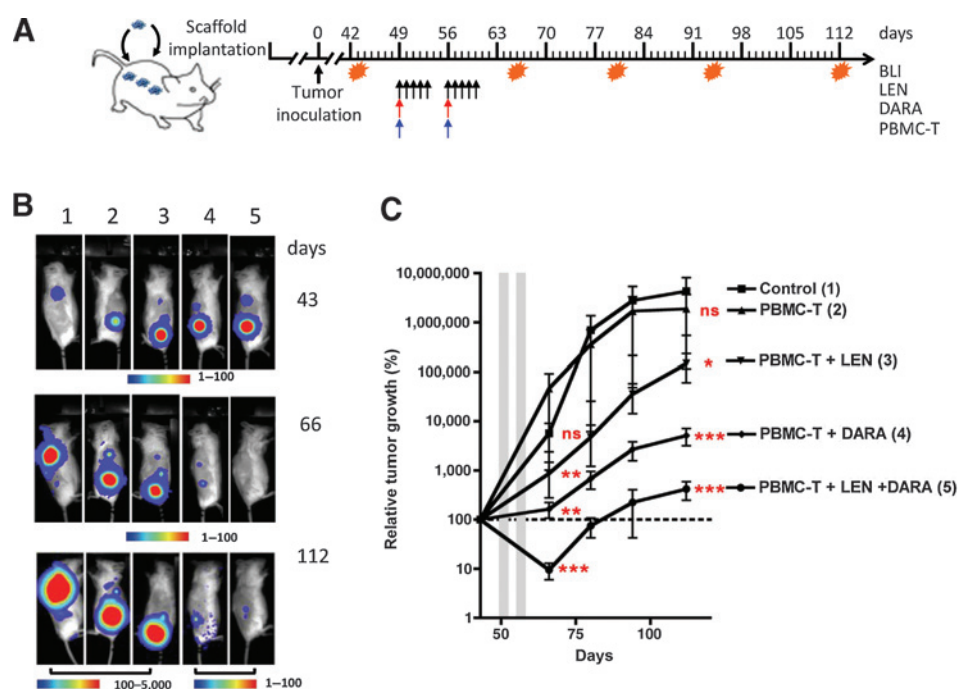
We have previously shown that daratumumab induces effective lysis of multiple myeloma cells not only via ADCC, but also via CDC, via apoptosis upon cross-linking with anti-immunoglobulin G antibody, and via anti-enzymatic activity (5). In our current *in vitro* assays, however, we mainly focused on assays that measure (NK-cell-mediated) ADCC. From this point of view, our results may underestimate the potential therapeutic benefits that can be achieved by combining daratumumab with bortezomib or with lenalidomide. Nonetheless, we think to have measured one of the most relevant parameters because the previously described immunomodulatory effects of lenalidomide and bortezomib are especially beneficial for ADCC. For instance, lenalidomide has been shown to increase the NK cell numbers and activation status of these cells (11, 13). Lenali-

domide has further been shown to upregulate FcγRIIIa (CD16) expression (13), and to inhibit T-regulatory cell function (12). Bortezomib on the other hand, has been described to down-regulate expression of inhibitory NK cell ligands (16) and to upregulate CD95 and TRAILR2 on multiple myeloma tumor cells, (15, 19) whereby enhancing the susceptibility of myeloma cells to NK cell-mediated killing *in vitro*.

The potential benefit of targeting multiple myeloma tumor cells by specific mAbs alone or in combination with other anti-multiple myeloma agents has also been demonstrated by other recent experimental and clinical studies (4, 20–26). For instance, bortezomib was described to synergistically enhance the lysis of ADCC mediated by the CS1 mAb elotuzumab *in vitro* (27). We observed only additive effects of bortezomib combined with daratumumab. The apparent discrepancy between the aforementioned elotuzumab study and ours may be due to the fact that a significant enhancement of elotuzumab-mediated ADCC was only observed at doses of bortezomib ≥ 5 nmol/L (27), while we have specifically chosen a dose of 3 nmol/L for bortezomib, and 3 μmol/L for lenalidomide, because these dose levels are clinically achievable in humans without causing severe side effects and comparable



**Figure 4.** NK cells as mediators of daratumumab (DARA)-dependent ADCC. PBMCs pretreated with lenalidomide for 48 hours at a concentration of 3 μmol/L were indicated (\*). Then MACS-enrichment for monocytes, T cells, and NK cells was performed as described in Materials and Methods. MACS-enriched monocytes, T cells, and NK cells from the PBMC of a healthy donor (A) and the PBMC of a heavily pretreated lenalidomide- and bortezomib-refractory multiple myeloma patient (B) were tested in 4-hour ADCC assays as effector cells. RPMI8226 multiple myeloma cell line was used as target cells. We show a representative example of three performed experiments. See for further details the legend of Fig. 2 and Materials and Methods.



**Figure 5.**

Lenalidomide (LEN) improves response to daratumumab (DARA) in lenalidomide- and bortezomib-refractory multiple myeloma patient-derived tumor cells in an *in vivo* mouse model. A, humanized bone marrow environment scaffolds implanted in  $Rag2^{-/-}\gamma_c^{-/-}$  mice were inoculated with luciferase-marked primary multiple myeloma cells, derived from a lenalidomide- and bortezomib-refractory patient. Mice were monitored biweekly by BLI. When visible tumor growth was observed, mice were treated with T-cell-depleted PBMC (PBMC-T), combined with lenalidomide, or daratumumab, or the respective combination, or vehicle as a control at the indicated time points. B, images of representative mice per treatment group: 1, control; 2, PBMC-T; 3, PBMC-T plus lenalidomide; 4, PBMC-T plus daratumumab; and 5, PBMC-T plus lenalidomide plus daratumumab. Shown are BLI images before (day 43; top), 2 weeks after the initiation of treatment (day 66; middle), and at the end of the experiment (day 112; bottom). C, quantification of the therapeutic intervention. BLI results are expressed as relative tumor growth with the intensity at day 43 (before start of treatment) set to 100% (indicated by dashed line). Lines represent mean BLI signal of at least 12 individual tumors followed in time (control  $n = 12$ , PBMC-T  $n = 16$ , PBMC-T plus lenalidomide  $n = 17$ , PBMC-T plus daratumumab  $n = 13$ , PBMC-T plus lenalidomide plus daratumumab  $n = 15$ )  $\pm$  SEM. The treatment cycles are indicated by the grey bars. The statistical differences between groups directly after treatment and at the end of the experiment are calculated using a Mann-Whitney test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant.

with concentrations found in the serum of patients treated with therapeutic doses of bortezomib and lenalidomide (28, 29). Another possible explanation might be that, depending on the condition, bortezomib mitigates ADCC. For instance, bortezomib was described to disrupt TRAIL expression on multiple myeloma cells and to downregulate the activating NK receptor NKp46, resulting in diminished ADCC activity mediated by NK cells (30, 31).

In contrast with the daratumumab–bortezomib combination, the combination of daratumumab with lenalidomide, dosed at clinically achievable and tolerable doses, showed synergistic effects in lenalidomide- and bortezomib-refractory patients in our *ex vivo* BM assays. We found that the synergism between daratumumab and lenalidomide was mainly mediated through the activation of NK cells. These results confirmed our hypothesis that in the BM-MNC of these multi-refractory patients, despite heavily pretreated with several lines of antimyeloma therapy, the NK cells were still able to respond to the well-described immunomodulatory effects of lenalidomide.

We confirmed the synergistic anti-multiple myeloma effects of the daratumumab plus lenalidomide combination *in vivo* in a setting where primary multiple myeloma cells from a

lenalidomide- and bortezomib-refractory patient were growing in humanized niches in  $RAG2^{-/-}\gamma_c^{-/-}$  mice. In fact, in this model, we have previously shown that daratumumab monotherapy can mediate significant antitumor activity against tumors growing from patient-derived primary multiple myeloma cells (8). However, to study the synergy between daratumumab and lenalidomide, we needed to optimize the model, because the  $RAG2^{-/-}\gamma_c^{-/-}$  mice lack NK cells, which according to our *in vitro* results, appeared necessary to establish the synergistic effect (Fig. 4A and B). Therefore, we coinjected NK cell-enriched, T-cell-depleted PBMC together with lenalidomide and daratumumab in the mice and showed indeed a significant improvement of daratumumab-mediated anti-multiple myeloma effect with lenalidomide. Although in this *in vivo* setting, the PBMCs (NK cells) are not autologous to the multiple myeloma tumor, these results indicate that an optimal preclinical testing of ADCC-inducing mAbs in  $RAG2^{-/-}\gamma_c^{-/-}$  and  $NOD/scid/\gamma_c^{-/-}$  (NSG)-based xenotransplant models will highly benefit from such an NK cell repletion approach. Although NK cell repletion can be performed by several ways, our NK cell enrichment method, via T-cell depletion, can become a standard procedure because it is convenient, effective, and will not induce T-cell-mediated

xenogeneic graft-versus-host disease. However, it needs to be noted that for optimal preclinical testing of mAbs, the *in vivo* models may need further adjustments. For instance, NSG-based models may also need complement injections, because these heavily immunodeficient mice not only lack NK cells, but also complement, which often is required for optimal tumor lysis by mAbs.

Collectively, we provide evidence that the combination of lenalidomide with daratumumab increases the lysis of multiple myeloma cells in a synergistic fashion in lenalidomide- and bortezomib-refractory multiple myeloma patients. To date this multirefractory multiple myeloma patient group has a very poor prognosis. Our findings warrant the testing of this combination approach in clinical trials for these multirefractory multiple myeloma patients.

### Disclosure of Potential Conflicts of Interest

J.M. Bakker, P.W.H.I. Parren, and J.J. Lammerts-van Bueren are employees of and have ownership interest (including patents) in Genmab. H.M. Lokhorst reports receiving a commercial research grant from and is a consultant/advisory board member for Genmab. N.W.C.J. van de Donk reports receiving commercial research grants from Celgene and Janssen Pharmaceuticals. T. Mutis reports receiving a commercial research grant from Genmab. No potential conflicts of interest were disclosed by the other authors.

### References

- Barosi G, Merlini G, Billio A, Boccadoro M, Corradini P, Marchetti M, et al. SIE, SIES, GITMO evidence-based guidelines on novel agents (thalidomide, bortezomib, and lenalidomide) in the treatment of multiple myeloma. *Ann Hematol* 2012;91:875–88.
- Kumar SK, Lee JH, Lahuerta JJ, Morgan G, Richardson PG, Crowley J, et al. Risk of progression and survival in multiple myeloma relapsing after therapy with IMiDs and bortezomib: a multicenter international myeloma working group study. *Leukemia* 2012;26:149–57.
- van de Donk NW, Lokhorst HM, Dimopoulos M, Cavo M, Morgan G, Einsele H, et al. Treatment of relapsed and refractory multiple myeloma in the era of novel agents. *Cancer Treat Rev* 2011;37:266–83.
- van de Donk NW, Kamps S, Mutis T, Lokhorst HM. Monoclonal antibody-based therapy as a new treatment strategy in multiple myeloma. *Leukemia* 2012;26:199–213.
- de Weers M, Tai YT, van der Veer M, Bakker JM, Vink T, Jacobs DC, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol* 2011;186:1840–8.
- van der Veer M, de Weers M, van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. Towards effective immunotherapy of myeloma: enhanced elimination of myeloma cells by combination of lenalidomide with the human CD38 monoclonal antibody daratumumab. *Haematologica* 2011;96:284–90.
- van der Veer M, de Weers M, van Kessel B, Bakker JM, Wittebol S, Parren PW, et al. The therapeutic human CD38 antibody daratumumab improves the anti-myeloma effect of newly emerging multi-drug therapies. *Blood Cancer J* 2011;1:e41.
- Groen RW, Noort WA, Raymakers RA, Prins HJ, Aalders L, Hofhuis FM, et al. Reconstructing the human hematopoietic niche in immunodeficient mice: opportunities for studying primary multiple myeloma. *Blood* 2012;120:e9–e16.
- Lokhorst HM, Plesner T, Gimsing P, Nahi H, Lisby S, Richardson P. Daratumumab, a CD38 monoclonal antibody study in advanced multiple myeloma; an open-label, dose escalation followed by open-label extension in a single-arm phase I/II study. *EHA Meeting Abstracts* 2013 2013;2013:S576.
- Plesner T, Lokhorst HM, Gimsing P, Nahi H, Lisby S, Richardson P. Daratumumab, a CD38 Monoclonal Antibody in Patients with Multiple Myeloma - Data From a Dose-Escalation Phase I/II Study [abstract]. In: *Proceedings of the 54th ASH Annual Meeting and Exposition*; 2012 Dec 8–11; Atlanta, GA. Washington, DC: ASH; 2012. Abstract nr73.
- Davies FE, Raje N, Hideshima T, Lentzsch S, Young G, Tai YT, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* 2001;98:210–6.
- Galustian C, Meyer B, Labarthe MC, Dredge K, Klaschka D, Henry J, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. *Cancer Immunol Immunother* 2009;58:1033–45.
- Hayashi T, Hideshima T, Akiyama M, Podar K, Yasui H, Raje N, et al. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. *Br J Haematol* 2005;128:192–203.
- van de Donk NW, Gorgun G, Groen RW, Jakubikova J, Mitsiades CS, Hideshima T, et al. Lenalidomide for the treatment of relapsed and refractory multiple myeloma. *Cancer Manag Res* 2012;4:253–68.
- Lundqvist A, Abrams SI, Schrupp DS, Alvarez G, Suffredini D, Berg M, et al. Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. *Cancer Res* 2006;66:7317–25.
- Shi J, Tricot GJ, Garg TK, Malaviarachchi PA, Szmania SM, Kellum RE, et al. Bortezomib down-regulates the cell-surface expression of HLA class I and enhances natural killer cell-mediated lysis of myeloma. *Blood* 2008;111:1309–17.
- Geuze RE, Prins HJ, Oner FC, van der Helm YJ, Schuijff LS, Martens AC, et al. Luciferase labeling for multipotent stromal cell tracking in spinal fusion versus ectopic bone tissue engineering in mice and rats. *Tissue Eng Part A* 2010;16:3343–51.
- Greco WR, Bravo G, Parsons JC. The search for synergy: a critical review from a response surface perspective. *Pharmacol Rev* 1995;47:331–85.
- Hallett WH, Ames E, Motarjemi M, Barao I, Shanker A, Tamang DL, et al. Sensitization of tumor cells to NK cell-mediated killing by proteasome inhibition. *J Immunol* 2008;180:163–70.
- Hsi ED, Steinle R, Balasa B, Szmania S, Draksharapu A, Shum BP, et al. CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. *Clin Cancer Res* 2008;14:2775–84.
- Jakubowiak AJ, Benson DM, Bensinger W, Siegel DS, Zimmerman TM, Mohrbacher A, et al. Phase I trial of anti-CS1 monoclonal antibody elotuzumab in combination with bortezomib in the treatment of relapsed/refractory multiple myeloma. *J Clin Oncol* 2012;30:1960–5.

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22. Lonial S, Vij R, Harousseau JL, Facon T, Moreau P, Mazumder A, et al. Elotuzumab in combination with lenalidomide and low-dose dexamethasone in relapsed or refractory multiple myeloma. *J Clin Oncol* 2012;30:1953–9.
23. Tai YT, Li XF, Catley L, Coffey R, Bretkreutz I, Bae J, et al. Immunomodulatory drug lenalidomide (CC-5013, IMiD3) augments anti-CD40 SGN-40-induced cytotoxicity in human multiple myeloma: clinical implications. *Cancer Res* 2005;65:11712–20.
24. Veitonmaki N, Hansson M, Zhan F, Sundberg A, Lofstedt T, Ljungars A, et al. A human ICAM-1 antibody isolated by a function-first approach has potent macrophage-dependent antimyeloma activity *in vivo*. *Cancer Cell* 2013;23:502–15.
25. Zonder JA, Mohrbacher AF, Singhal S, van RF, Bensinger WI, Ding H, et al. A phase 1, multicenter, open-label, dose escalation study of elotuzumab in patients with advanced multiple myeloma. *Blood* 2012;120:552–9.
26. Deckert J, Wetzel MC, Bartle LM, Skaletskaya A, Goldmacher VS, Vallee F, et al. SAR650984, a novel humanized CD38-targeting antibody, demonstrates potent antitumor activity in models of multiple myeloma and other CD38+ hematologic malignancies. *Clin Cancer Res* 2014; 20:4574–83.
27. Rhee van F, Szmania SM, Dillon M, van Abbema AM, Li X, Stone MK, et al. Combinatorial efficacy of anti-CS1 monoclonal antibody elotuzumab (HuLuc63) and bortezomib against multiple myeloma. *Mol Cancer Ther* 2009;8:2616–24.
28. Bringhen S, Gay F, Pautasso C, Cerrato C, Boccadoro M, Palumbo A. Evaluation of the pharmacokinetics, preclinical, and clinical efficacy of lenalidomide for the treatment of multiple myeloma. *Expert Opin Drug Metab Toxicol* 2012;8:1209–22.
29. Richardson PG, Schlossman RL, Weller E, Hideshima T, Mitsiades C, Davies F, et al. Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. *Blood* 2002;100:3063–7.
30. Feng X, Yan J, Wang Y, Zierath JR, Nordenskjold M, Henter JI, et al. The proteasome inhibitor bortezomib disrupts tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and natural killer (NK) cell killing of TRAIL receptor-positive multiple myeloma cells. *Mol Immunol* 2010;47:2388–96.
31. Wang X, Ottosson A, Ji C, Feng X, Nordenskjold M, Henter JI, et al. Proteasome inhibition induces apoptosis in primary human natural killer cells and suppresses NKp46-mediated cytotoxicity. *Haematologica* 2009;94:470–8.
32. Leibson PJ. Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity* 1997;6:655–61.
33. Shereck E, Satwani P, Morris E, Cairo MS. Human natural killer cells in health and disease. *Pediatr Blood Cancer* 2007;49:615–23.