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Monocytes and Granulocytes Reduce CD38 Expression Levels on Myeloma Cells in Patients Treated with Daratumumab

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Abstract

Purpose: Daratumumab treatment results in a marked reduction of CD38 expression on multiple myeloma cells. The aim of this study was to investigate the clinical implications and the underlying mechanisms of daratumumab-mediated CD38 reduction.

Experimental Design: We evaluated the effect of daratumumab alone or in combination with lenalidomide-dexamethasone, on CD38 levels of multiple myeloma cells and nontumor immune cells in the GEN501 study (daratumumab monotherapy) and the GEN503 study (daratumumab combined with lenalidomide-dexamethasone). *In vitro* assays were also performed.

Results: In both trials, daratumumab reduced CD38 expression on multiple myeloma cells within hours after starting the first infusion, regardless of depth and duration of the response. In addition, CD38 expression on nontumor immune cells, including natural killer cells, T cells, B cells, and monocytes, was also reduced irrespective of alterations in their absolute numbers during therapy. In-depth analyses revealed that CD38 levels of multiple myeloma cells were only reduced in the presence of complement or effector cells, suggesting that the rapid elimination of CD38^{high} multiple myeloma cells can contribute to CD38 reduction. In addition, we discovered that daratumumab–CD38 complexes and accompanying cell membrane were actively transferred from multiple myeloma cells to monocytes and granulocytes. This process of trogocytosis was also associated with reduced surface levels of some other membrane proteins, including CD49d, CD56, and CD138.

Conclusions: Daratumumab rapidly reduced CD38 expression levels, at least in part, through trogocytosis. Importantly, all these effects also occurred in patients with deep and durable responses, thus excluding CD38 reduction alone as a mechanism of daratumumab resistance.

The trials were registered at www.clinicaltrials.gov as NCT00574288 (GEN501) and NCT1615029 (GEN503). *Clin Cancer Res; 23(24); 7498–511. ©2017 AACR.*

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Introduction

The anti-CD38 monoclonal antibody, daratumumab, is well tolerated and has robust single-agent activity in multiple myeloma. Combined analysis of the GEN501 and Sirius clinical studies showed that daratumumab at a dose of 16 mg/kg induced a partial response (PR) or better in 31% of the heavily pretreated patients including complete response in 5% (1, 2). Preclinical studies have shown that the immunomodulatory drug (IMiD) lenalidomide improves daratumumab-mediated killing of multiple myeloma cells (3). Based on these results, a phase I/II study (GEN503) was initiated, which demonstrated that daratumumab combined with lenalidomide-dexamethasone (DRd) induced rapid, deep, and durable responses (4). Altogether, this formed the rationale for a phase III study of lenalidomide-dexamethasone with or without daratumumab in relapsed/refractory multiple myeloma, which showed improved outcome for the three-drug compared with the two-drug combination (5). Similarly, addition of daratumumab to bortezomib-dexamethasone markedly improved response rate and progression-free survival (6).

The mechanisms implicated in daratumumab-mediated killing of tumor cells include the activation of potent cytotoxic immune effector functions including complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis, antibody-dependent cellular cytotoxicity (ADCC; refs. 3, 7–10), as well as a direct apoptotic effect (11). Another important mechanism of action includes the elimination of CD38-positive immune-suppressor cells, which may contribute to an improved host-antitumor immune response (12).

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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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Translational Relevance

The CD38-targeting antibody, daratumumab, is well tolerated and has marked activity in multiple myeloma as single agent and in combination with other anti–multiple myeloma agents. Although response to daratumumab monotherapy is partly dependent on baseline CD38 expression levels on the tumor cells, daratumumab treatment also results in a marked reduction of CD38 expression on multiple myeloma cells. In this study, we show that daratumumab-mediated CD38 reduction is an early event, already observed after the first infusion. Furthermore, CD38 reduction also occurs on nonmalignant cells, and both in the presence and absence of lenalidomide. The mechanism of CD38 reduction probably involves both elimination of $CD38^{high}$ multiple myeloma cells and transfer of daratumumab–CD38 complexes and accompanying cell membrane from multiple myeloma cells to monocytes and granulocytes. Importantly, CD38 reduction is observed in every patient including those with deep and durable responses, thereby excluding the sole CD38 reduction as a mechanism of daratumumab resistance.

We have previously shown that extent of CDC and ADCC in pretreatment samples was associated with clinical response to daratumumab as single agent (13). We also demonstrated that response to daratumumab is partly dependent on baseline CD38 expression levels on the tumor cells (13). In addition, increased expression of CD38, either by transducing multiple myeloma cells with the human CD38 gene or by treating cells with all-*trans* retinoic acid, significantly enhanced daratumumab-mediated killing (13, 14). Furthermore, another group recently reported that lenalidomide and pomalidomide also increase CD38 expression on multiple myeloma cell lines (15, 16), and suggested that IMiD-induced CD38 upregulation could contribute to the observed synergy between daratumumab and IMiDs.

Unexpectedly, however, daratumumab treatment also results in a marked reduction of CD38 expression on multiple myeloma cells (13, 14). Not only the clinical implications, but also the underlying mechanisms of daratumumab-mediated CD38 reduction on multiple myeloma cells, the effect of daratumumab on CD38 expression of nontumor cells, and precise kinetics of CD38 reduction are currently unknown. Furthermore, although lenalidomide increases CD38 expression (15, 16), it is also unknown whether lenalidomide can prevent the daratumumab-mediated CD38 reduction. We therefore set out to thoroughly address these relevant issues by performing *in vitro* assays, as well as flow cytometric analysis of bone marrow (BM) and blood samples from patients treated with daratumumab alone or in combination with lenalidomide.

We show that daratumumab-mediated CD38 reduction on multiple myeloma cells is an early event, which also occurs on nonmalignant cells, and both in the presence or absence of lenalidomide. Highly important, CD38 reduction occurred in every patient including those with deep and durable responses, thus excluding the sole CD38 reduction as a mechanism of daratumumab resistance. Our analyses reveal that the rapid CD38 downregulation occurs only in the presence of effector cells and, to a lesser extent, complement, suggesting that rapid elimination of $CD38^{high}$ multiple myeloma cells can partly explain this phenomenon. In addition, we discovered that CD38 reduction in the presence of effector cells mainly occurs through the active transfer of daratumumab–CD38 complexes and accompanying cell membrane from multiple myeloma cells to monocytes and granulocytes, in a process often designated as trogocytosis. Remarkably, this active membrane transfer process was also associated with reduced expression levels of other membrane proteins including adhesion molecules that play an important role in multiple myeloma biology.

Materials and Methods

Patients and protocols

Data on expression levels of CD38 on natural killer (NK) cells, B cells, T cells, and monocytes were derived from 17 relapsed or refractory multiple myeloma patients treated with daratumumab monotherapy (16 mg/kg) in the GEN501 study (NCT00574288) and from 9 patients treated in part 2 of the GEN503 study [daratumumab 16 mg/kg in combination with lenalidomide-dexamethasone (DRd); NCT1615029; refs. 1, 2]. In addition, in GEN503 patients, CD38 expression on multiple myeloma cells was determined before start of therapy and approximately 16 weeks after initiation of treatment, and at the time of progression.

Briefly, in the GEN501 study, patients had multiple myeloma requiring systemic therapy and relapsed from or refractory to at least two prior therapies (1). In part 2 of the GEN503 study, patients refractory to lenalidomide were excluded and patients with $\geq$1 prior line of therapy were included (4). In both studies, patients had age $\geq$18 years; life expectancy $\geq$3 months; Eastern Cooperative Oncology Group performance status of $\leq$2; and measurable disease. Exclusion criteria included other malignancies; uncontrolled infections; cardiovascular and respiratory conditions; or meningeal involvement of multiple myeloma.

For the analysis of CD38 expression on multiple myeloma cells directly after the first daratumumab infusion, we obtained blood samples from 8 patients before and immediately after the first infusion of daratumumab and prior to administration of any combinational treatment.

Study site ethics committees or Institutional Review Boards approved the protocols, which were conducted according to the principles of the Declaration of Helsinki, the International Conference on Harmonization, and the Guidelines for Good Clinical Practice. All patients gave written informed consent.

Antibodies and reagents

Daratumumab was provided by Janssen Pharmaceuticals. Human IgG1-b12 (Genmab), a human mAb against an innocuous antigen (HIV-1 gp120), was used as an isotype control as described previously (13).

Multiple myeloma and lymphoma cell lines and culture

Multiple myeloma cell lines (UM9 and UM9-CD38) and the lymphoma cell line (Daudi) were cultured in RPMI 1640 (Invitrogen), supplemented with 10% FBS (Lonza) and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin; both Life Technologies) as previously described (17). UM9 was obtained after prolonged *in vitro* culture of the BM aspirate of a multiple myeloma patient. Daudi was purchased from the American Tissue Culture Collection (ATCC). Mycoplasma

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testing was performed every month by using real-time PCR (Microbiome), and cell lines were authenticated by partial HLA typing carried out maximal 6 months before the most recent experiment.

Generation of the UM9 cell line with higher CD38 expression

The multiple myeloma cell line UM9 was transduced with the human CD38 gene to obtain CD38 expression levels comparable with primary myeloma cells. For this, the amphotropic Phoenix packaging cell line (Phoenix Ampho) was transfected, using calcium phosphate precipitation, with the pQCXIN vector in which the gene encoding human CD38 was inserted. This cell line is referred to as UM9-CD38 (14).

Flow cytometric analysis of BM and blood samples from patients treated with daratumumab

BM-localized multiple myeloma cells were identified and analyzed for CD38 expression levels by staining 1×10^6 cells with CD38 V450, CD138 PercP-Cy5.5, CD56 Pe-Cy7 (all Becton Dickinson), HuMax-003 FITC (this antibody binds to an epitope distinct from the epitope bound by daratumumab; Genmab/Janssen Pharmaceuticals; HuMax-003 FITC was used for analysis of CD38 expression in sequential samples obtained before, during, and after treatment with daratumumab), CD45 Pacific Orange (Invitrogen), CD19 APC-A750 (Beckman Coulter; Immunotech) combined with cytoplasmic staining for immunoglobulin light chains using monoclonal anti-kappa APC (Becton Dickinson), and polyclonal anti-lambda PE (DAKO). These samples were analyzed within 24 hours from the time the BM aspirate was collected. As previously described, expression levels of CD38 were stable during the first 48 hours after BM sampling (13).

Quantitation of circulating multiple myeloma cells and analysis of their CD38 expression level were performed by the incubation of 35×10^6 cells with sufficient amounts of antibodies in the combinations described above for 30 minutes at room temperature in the dark. Cells were then washed with PBS, suspended in 2 mL of PBS/human serum albumin, and directly analyzed by flow cytometry. Multiple myeloma cells were enumerated by using the calculated ratio of multiple myeloma cells and B cells in the sample and the established absolute number of B cells in the same blood sample using single-platform analysis. These samples were analyzed within 24 hours from the time the blood sample was collected.

CD38 expression on immune cell subsets was determined on cryopreserved peripheral blood mononuclear cells (PBMC), isolated by density-gradient centrifugation. At the time of analysis, thawed PBMCs were resuspended in FACS medium (PBS/0.05% Azide/0.1% HSA). A total of 2×10^5 cells were stained with relevant fluorescent-conjugated monoclonal antibodies to identify by multicolor flow cytometry various immune cell populations and their CD38 expression. The following antibodies were used: CD45 KO, CD16 PE, CD56 PC7 (all Beckman Coulter), CD3 V450, CD19 APC-H7, CD14 PerCP, CD4 APC-H7, CD8 PerCP-Cy5.5, CD14 PE, CD44 APC-H7, CD54 APC, CD138 PE (all Becton Dickinson), CD138 APC (IQP), and CD49d PE (Biolegend). All samples were additionally stained with the anti-CD38 antibody, HuMax-003 FITC, which binds to an epitope distinct from the epitope bound by daratumumab.

Flow cytometry was performed using a 3-laser Canto II flow cytometer (Becton Dickinson). Fluorescent labeled beads (CS&T beads; Becton Dickinson) were used to monitor the performance of the flow cytometer and verify optical path and stream flow. This procedure enables controlled standardized results and allows the determination of long-term drifts and incidental changes within the flow cytometer. No changes were observed which could affect the results. Compensation beads were used to determine spectral overlap, and compensation was automatically calculated using Diva software.

Statistical analysis

Comparisons between variables were performed using two-tailed (paired) Student *t* test, or Mann–Whitney *U* test or Wilcoxon matched-pairs signed-rank test in case the data do not follow a normal distribution. *P* values below 0.05 were considered significant.

Additional methods are presented in Supplementary Data.

Results

CD38 expression is reduced on multiple myeloma cells during DRd treatment

We have recently demonstrated that daratumumab monotherapy rapidly reduces CD38 expression levels on multiple myeloma cells (13). Because lenalidomide increases CD38 expression on multiple myeloma cell lines (15, 16), we hypothesized that lenalidomide may prevent the daratumumab-mediated reduction in CD38 and thereby improve daratumumab's efficacy to kill multiple myeloma cells. Therefore, we analyzed CD38 expression on BM-localized multiple myeloma cells from GEN503 patients treated in our center (clinical characteristics are shown in Supplementary Table S1). Sequential BM samples were obtained from 6 of 9 patients. We used an anti-CD38 monoclonal antibody, HuMax-003-FITC, that binds to a different epitope than daratumumab (12). This excluded the possibility that binding of daratumumab masked the detection of CD38.

Sixteen weeks after the first daratumumab infusion, the multiple myeloma cells of DRd-treated patients had significantly lower CD38 expression levels, compared with baseline values [median fluorescence intensity (MFI) CD38: 9,220 vs. 60,516; $P = 0.0313$; Fig. 1A]. Similarly, at the time of progression during DRd treatment, multiple myeloma cells had low CD38 expression levels.

We also analyzed CD38 expression on circulating multiple myeloma cells in the same subset of GEN503 patients. Peripheral blood clonal plasma cells were detected before start of DRd treatment in 5 of 9 patients (56%). In 2 patients, these cells were still detectable after 1 week of DRd treatment and also displayed lower CD38 expression levels, compared with baseline ($P = 0.078$; Fig. 1B). Only in 1 patient, circulating multiple myeloma cells remained detectable and showed low CD38 expression throughout the whole treatment period, even 3 months after stopping daratumumab due to disease progression (Fig. 1C).

Daratumumab with or without lenalidomide reduces CD38 expression levels on normal white blood cells

To determine whether daratumumab-mediated CD38 reduction is specific for multiple myeloma cells or also occurs on nonmalignant cells, we evaluated CD38 expression levels of PBMC subsets during treatment of patients with single-agent daratumumab (GEN501, $n = 17$; clinical characteristics are shown in Supplementary Table S2) or DRd (GEN503, $n = 9$).

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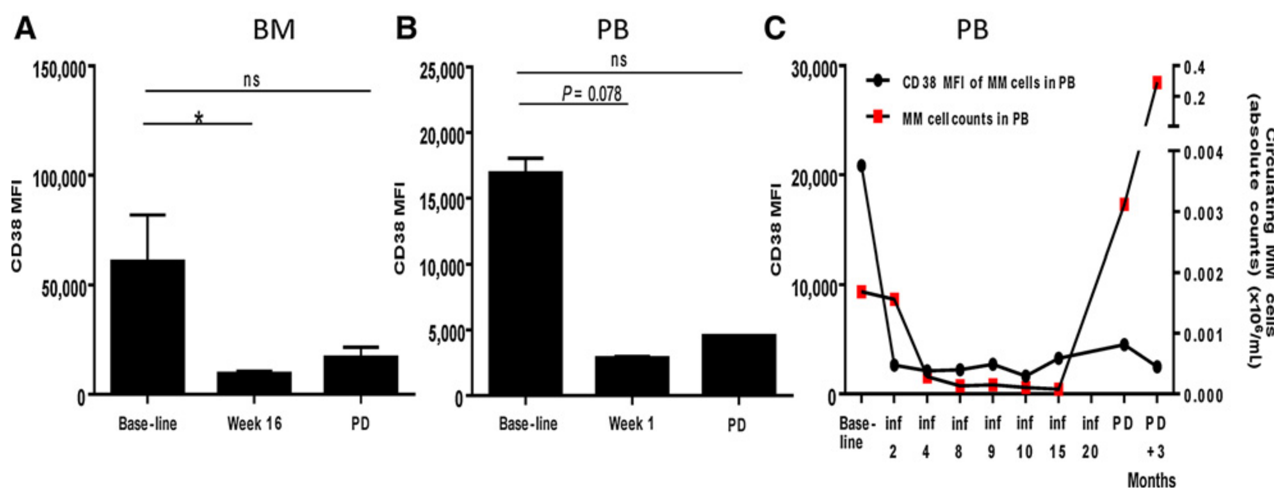

Figure 1.

Treatment with daratumumab plus lenalidomide-dexamethasone is associated with reduced CD38 expression on multiple myeloma (MM) cells. **A,** CD38 expression was measured on BM-localized multiple myeloma cells in a subset of GEN503 patients before start of treatment (baseline; $n = 6$), 16 weeks after the initiation of daratumumab plus lenalidomide-dexamethasone treatment ($n = 6$), and also at the time of progression (PD) during daratumumab plus lenalidomide-dexamethasone therapy ($n = 3$). **B,** CD38 expression was also determined in patients with circulating multiple myeloma cells. Before start of treatment, 5 of 9 patients had circulating tumor cells, whereas 1 week after the first infusion, these cells were only detected in 2 patients. In 1 patient, we were able to determine CD38 levels on circulating tumor cells at the time of progression (PD). **C,** Only in 1 patient, circulating MM cells were detectable during the whole treatment period allowing the longitudinal measurement of CD38 expression and absolute numbers. CD38 expression was determined by using HuMax-003-FITC, which binds to a different epitope compared with daratumumab, thereby excluding the possibility that binding of daratumumab masked the detection of CD38. Data are presented as mean ± SEM. *P* values between the indicated groups were calculated using a paired Student *t* test; *, $P < 0.05$; ns, not significant. Abbreviation: PB, peripheral blood.

These immune cell subsets expressed varying levels of CD38 with NK cells having higher expression, compared with B cells, T cells, or monocytes. As previously described, the absolute counts of these immune cell subsets were differentially affected by daratumumab monotherapy with a rapid decrease in NK cells but increase in $CD8^+$ T cells (12, 18), whereas there was no detectable effect on monocyte, $CD4^+$ T-cell, or B-cell counts. Although daratumumab had different effects on the absolute numbers of the PBMC subsets, we observed a significant and uniform reduction of CD38 expression levels on all these immune cells. The CD38 reduction was already observed after 1 week of treatment, whereas 6 months after the last daratumumab infusion, CD38 levels were restored to baseline levels (Fig. 2A and B).

In the 9 DRd-treated patients, we observed a reduction in absolute numbers of NK cells and B cells, whereas monocyte, $CD4^+$ T-cell, and $CD8^+$ T-cell counts did not change. Similar to daratumumab monotherapy, a rapid reduction of CD38 levels was also observed for all PBMC subsets in DRd-treated patients (Fig. 2A and B). No posttreatment samples were available to assess recovery of CD38 levels after DRd therapy was stopped.

CD38 levels are reduced directly after the first infusion

To gain further insight into the kinetics of CD38 reduction, we analyzed CD38 expression levels on PBMC subsets and circulating multiple myeloma cells directly after the end of the first infusion. We therefore obtained blood samples from a different group of patients treated with daratumumab ($n = 8$). Median duration of their first infusion was 6 hours and 55 minutes (range, 6 hours 30 minutes–8 hours 30 minutes). Importantly, directly after the first infusion, CD38 levels were already markedly reduced on circulating multiple myeloma cells, which were detected in 3 of these patients. CD38 levels were also significantly reduced on NK cells, B cells, and $CD4^+$ T cells. There was also CD38 reduction on $CD8^+$ T cells, but this did not reach statistical significance. No change in CD38 levels was observed on monocytes (Fig. 2C and D).

Loss of CD38 from the multiple myeloma cell membrane reduces CDC and ADCC

We previously showed that extent of both CDC and ADCC is associated with CD38 expression levels (13, 14). We therefore evaluated the effect of CD38 downmodulation on CDC and ADCC efficiency in serial BM samples obtained from patients who had achieved PR with daratumumab monotherapy. As expected, CD38 loss reduced daratumumab-mediated CDC and ADCC against multiple myeloma cells, even though these patients had persistent response (Supplementary Fig. S1).

CD38 reduction on multiple myeloma cells is only observed after addition of complement or effector cells

To analyze the mechanism of CD38 reduction, we determined CD38 levels on multiple myeloma cell lines treated with daratumumab in the presence or absence of effector cells or complement. CD38 expression was not reduced bv daratumumab in the absence of effector cells or complement (Fig. 3A). In the presence of complement, the CD38 expression of only Daudi and UM9-CD38 cells was sufficient to induce CDC. Remarkably, the surviving cells after CDC had reduced CD38 levels (Fig. 3B), suggesting that CDC selectively eliminated multiple myeloma cells with high CD38 expression in these cell lines. Supporting this idea, daratumumab did not affect CD38 expression levels in the UM9 cell line, whose CD38 expression levels were already too low to induce CDC. In the

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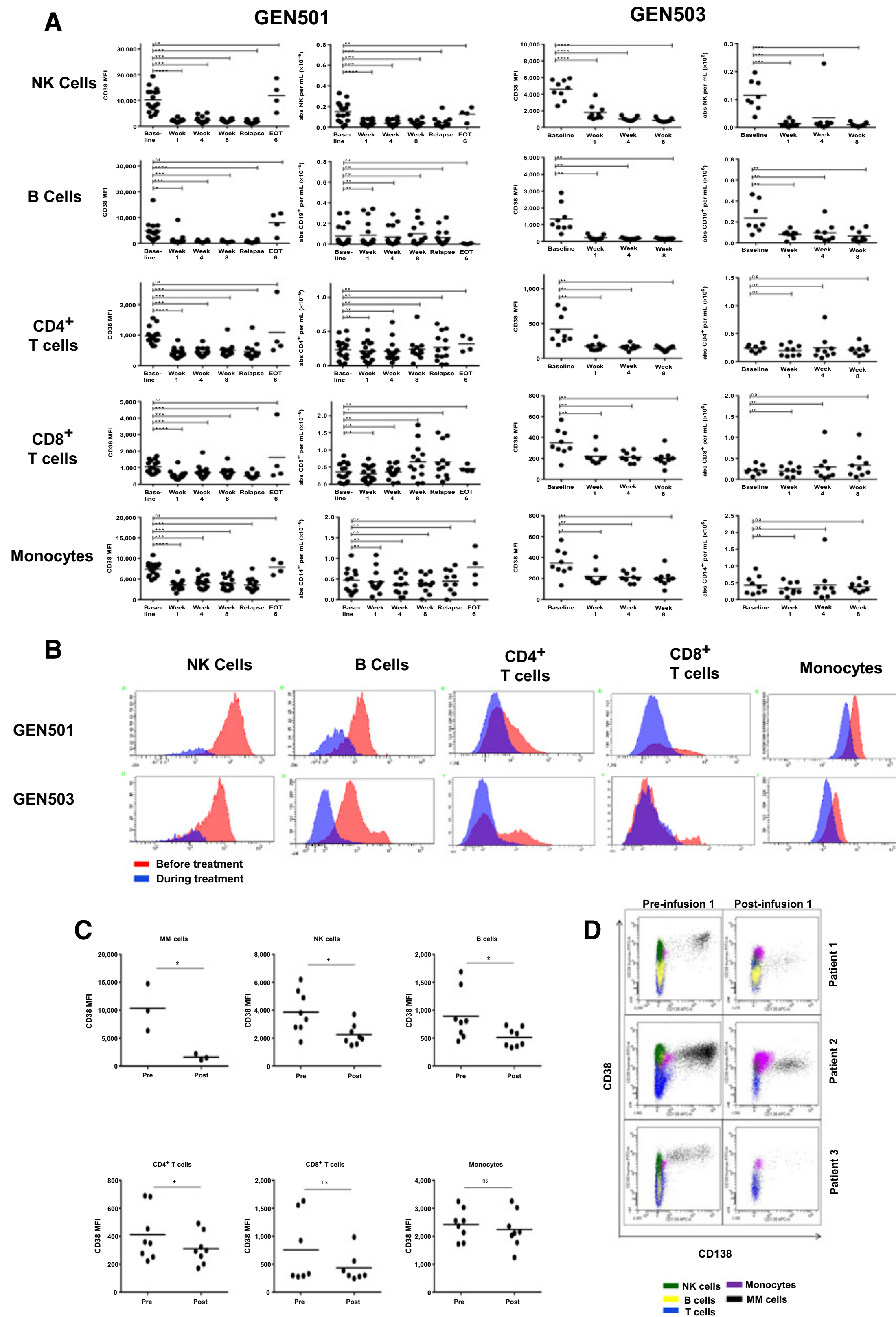

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presence of healthy PBMCs as effector cells, all cell lines were to a certain extent susceptible to ADCC. At the end of the ADCC assay, all remaining cells, which could not be killed by ADCC, had lower CD38 expression (Fig. 3C). Western blot analysis confirmed the flow cytometry results (Supplementary Fig. S2).

CD38 reduction on primary multiple myeloma cells is most pronounced in the presence of effector cells

We also analyzed the effect of daratumumab on CD38 expression in *ex vivo* CDC and ADCC assays with BM aspirates from multiple myeloma patients. Similar to the experiments with cell lines, there was a significant CD38 reduction on primary multiple myeloma cells that survived after treatment with daratumumab in CDC or ADCC assays (Fig. 3D). However, despite similar levels of lysis, the CD38 reduction was more pronounced in ADCC as compared with CDC assays (64.5% vs. 27.6% reduction, respectively). It seemed therefore likely that apart from selection of multiple myeloma cells with lower CD38 levels, effector cells had additional effects on CD38 expression.

Monocytes and granulocytes contribute to CD38 reduction

We next analyzed which immune cell subsets were responsible for the reduction of CD38 levels on multiple myeloma cells. We therefore carried out 4-hour ADCC assays using UM9 cells and unseparated PBMCs or T-cell–, NK-cell–, or monocyte-enriched PBMC fractions as effector cells. Because granulocytes are separated from the PBMCs during processing of whole blood, we also incubated UM9 cells with purified granulocytes. Killing of UM9 cells was predominantly mediated by NK cells and, to a lesser degree, monocytes, but not granulocytes. However, CD38 reduction was mainly mediated by monocytes and granulocytes, but not by NK cells or T cells (Fig. 4A). Similarly, depletion of monocytes from PBMCs abrogated the daratumumab-mediated CD38 reduction on NK cells (Supplementary Fig. S3).

Daratumumab is transferred from UM9 cells to monocytes and granulocytes

Because monocytes and granulocytes contributed significantly to CD38 reduction of daratumumab-treated multiple myeloma cells, we questioned whether they could take up daratumumab–CD38 complexes from multiple myeloma cells in the absence of phagocytosis. To this end, we incubated UM9 cells with AF488-labeled daratumumab or IgG-b12 control antibody, and subsequently cocultured these cells with human leukocytes. Before the incubation, UM9 cells were also intracellularly stained with CellTrace Violet, which enabled us to clearly distinguish UM9 cells from nonstained leukocytes. Gating on leukocytes with a low Violet signal also excluded cells that had engulfed complete UM9 cells (these cells have higher CellTrace Violet signal; Fig. 4B). In line with our previous experiments, gating on different leukocyte populations with low CellTrace Violet signal showed that monocytes, and to a lesser extent granulocytes, were strongly capable of taking up AF488-labeled daratumumab, compared with T cells (no increase) or NK cells (small increase). There was no uptake of IgG-b12 by the different leukocyte subsets. Altogether, these results showed the capacity of monocytes and granulocytes to take up daratumumab–CD38 complexes, even without phagocytosis of whole multiple myeloma cells.

We also evaluated the presence of the daratumumab–CD38 complex in monocytes by using an intracellular staining of human IgG. In concordance with the previous experiments, human IgG was detected in monocytes after their coculture with daratumumab-opsonized UM9 cells. Concomitantly, we observed a significant reduction of the signal for human IgG on daratumumab-opsonized UM9 cells. No such changes were detected after coculture of monocytes with UM9 cells incubated with fully human IgG-b12 control antibody, or in the absence of monocytes (Supplementary Fig. S4). To exclude that the increase in human IgG in monocytes could be caused by passive binding of unwashed daratumumab, monocytes were also incubated with a cell-free supernatant from the last washing step of daratumumab-opsonized UM9 cells. No human IgG was detected in these monocytes after this incubation, ruling out passive take up of unbound daratumumab by monocytes.

Uptake of AF488-labeled daratumumab by monocytes is inhibited by polyclonal human IgG

To evaluate the possible role of Fcγ receptors on monocyte-mediated CD38 reduction, we blocked the Fcγ receptors on monocytes by pretreating them with or without polyclonal human IgG. As expected, the daratumumab-AF488 signal of UM9 cells was significantly lowered after a 2-hour coculture by untreated monocytes, with a concomitant increase of the signal in monocytes (Fig. 5A). Such changes were partly reversed after treatment of monocytes with polyclonal human IgG, indicating that monocytes were partly dependent on Fcγ receptors for their ability to reduce the daratumumab-AF488 signal on UM9 cells.

Membrane fragments are transferred to acceptor cells together with daratumumab-AF488

We also evaluated whether effector cells take up the daratumumab–CD38 complex selectively or together with parts of the

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Figure 2.

Daratumumab alone or combined with lenalidomide-dexamethasone reduces CD38 expression levels on normal white blood cell subsets irrespective of changes in their absolute numbers. **A,** Changes in CD38 expression levels and absolute numbers (abs) of various white blood cell populations during daratumumab monotherapy (GEN501 study; $n = 17$) or daratumumab combined with lenalidomide and dexamethasone (GEN503 study; $n = 9$). **B,** Representative flow cytometry histogram overlays depicting cell surface expression of CD38 on various white blood cell populations from a representative patient treated with daratumumab in the GEN501 study and a representative patient treated with daratumumab plus lenalidomide-dexamethasone in the GEN503 trial at different time points: before the first daratumumab infusion (red histogram) and during daratumumab treatment (blue histogram). **C,** CD38 expression levels of various white blood cell subsets were measured both before (pre) and directly after the end of the first infusion (post) in a different group of patients treated with daratumumab in our institution ($n = 8$). In 3 these 8 patients, circulating multiple myeloma (MM) cells were also detected and analyzed for CD38 expression levels. Median duration of the first infusion was 6 hours and 55 minutes. **D,** Flow cytometry dot plots from the 3 patients who had circulating multiple myeloma cells showing CD38 and CD138 expression on the different PBMC subsets and the circulating multiple myeloma cells before and directly after the first daratumumab infusion. CD38 expression was determined by using HuMax-003-FITC, which binds to a different epitope compared with daratumumab, thereby excluding the possibility that binding of daratumumab masked the detection of CD38. Data are presented as mean ± SEM. P values between the indicated groups were calculated using a paired Student t test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; ns, not significant.

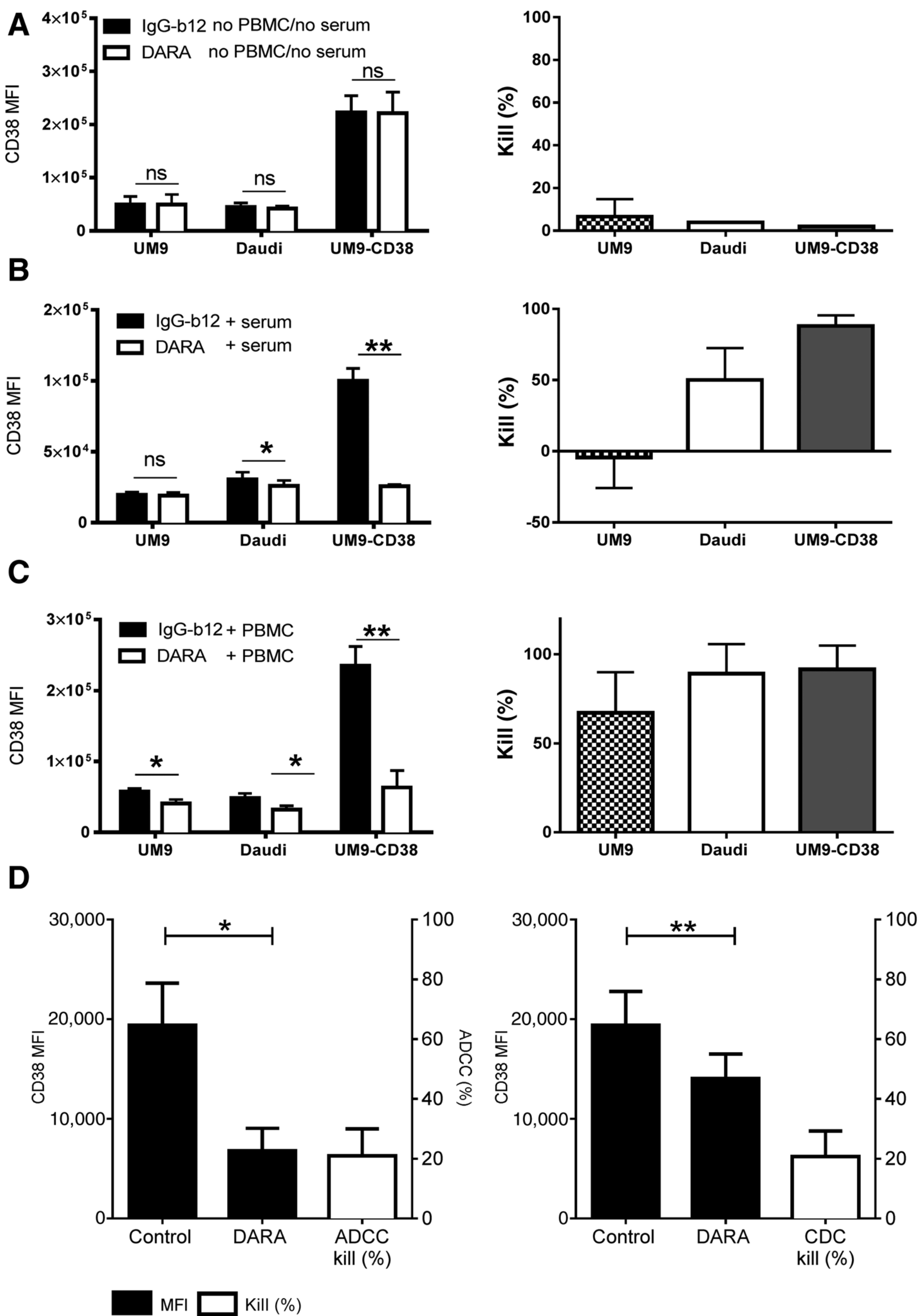

Figure 3.
Daratumumab-mediated CD38 reduction on multiple myeloma cell lines is only observed in the presence of effector cells or complement. UM9, UM9-CD38, and Daudi cells were treated with daratumumab (10 μg/mL) or IgG-b12 control antibody in the absence of complement or effector cells (**A**), in the presence of pooled unheated human serum as a source of complement (**B**), or in the presence of freshly isolated PBMCs from healthy donors as effector cells (**C**). The left plot shows for each of these conditions the MFI of CD38 on surviving multiple myeloma cells treated with daratumumab or control IgG-b12 antibody. The right plot shows for each condition the percentage lysis, which was determined and calculated as described in the Materials and Methods. ADCC and CDC assays were performed as described in Materials and Methods. **D,** *Ex vivo* ADCC and CDC assays were performed with BM aspirates from 7 and 10 multiple myeloma patients, respectively. CDC and ADCC assays were performed as described in Materials and Methods. The black bars show MFI of CD38 on surviving multiple myeloma cells as determined by flow cytometry, and white bars show the percentage of multiple myeloma cell lysis mediated by daratumumab in ADCC (left) or CDC assays (right). Data are presented as mean ± SEM. *P* values between the indicated groups were calculated using a paired Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ns, not significant. Abbreviation: DARA, daratumumab.

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multiple myeloma cell membrane. To this end, we labeled the UM9 cell surface with the membrane dye PKH-26 before opsonization with daratumumab-AF488. Coculture of PKH-26–stained UM9 cells with monocytes led to the concurrent increase of both the daratumumab-AF488 signal and the PKH-26 cell membrane signal on monocytes (Fig. 5B). Similar to the previous experiments, the addition of monocytes to daratumumab-opsonized UM9 cells also resulted in a significant decrease of daratumumab-AF488 signal on UM9 cells (Supplementary Fig. S5). No such changes were observed when membrane-stained UM9 cells were incubated with IgG-b12 (Fig. 5B). Altogether, this suggested that CD38–daratumumab complexes were transferred together with parts of the cell membrane from daratumumab-opsonized UM9 cells to monocytes.

Confocal scanning laser microscopy studies supported the results obtained with flow cytometry. UM9 cells were membrane-labeled with PKH-26 (red) and opsonized with daratumumab-AF488 (green), and then mixed with monocytes, which were intracellularly labeled with CellTrace Violet (blue). This resulted in transfer of red UM9 membrane components and green CD38–daratumumab–AF488 complexes to monocytes (Fig. 5C; Supplementary Fig. S6). Uptake was predominantly mediated by monocytes in the proximity of UM9 cells, whereas other monocytes showed less or no increase in

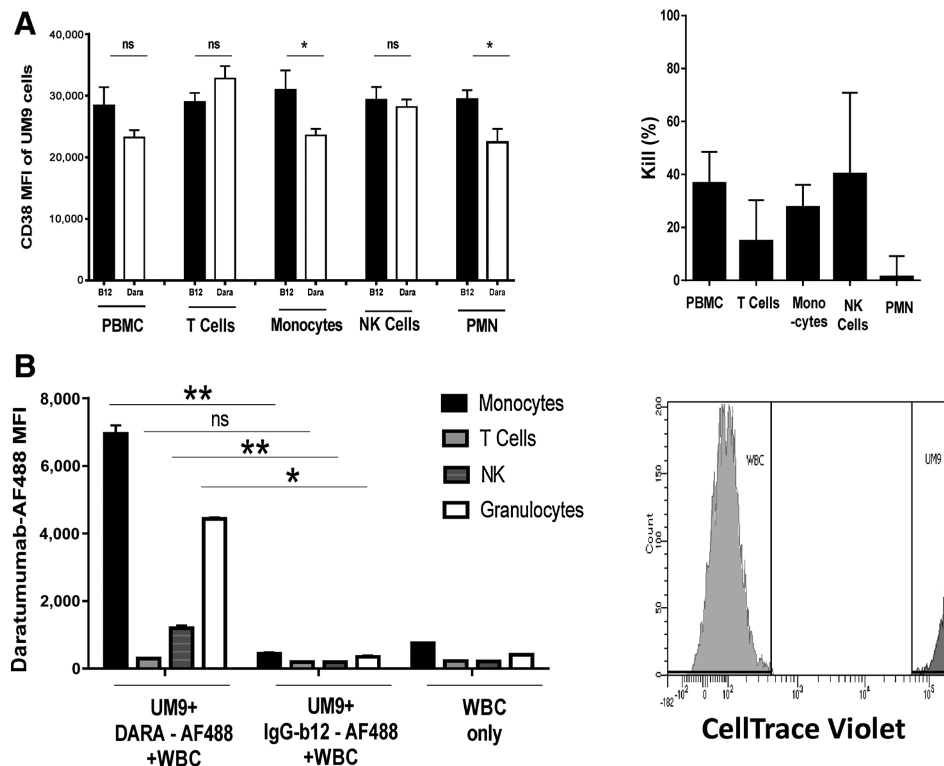

Figure 4.

Monocytes and granulocytes reduce CD38 expression of multiple myeloma (MM) cells. **A,** UM9 cells were cocultured with PBMCs or with purified white blood cell populations, isolated from healthy donor buffy coats as described in the Materials and Methods. The left plot shows the MFI of CD38 on surviving UM9 cells treated with 10 μg/mL daratumumab or IgG-b12 control antibody in the presence of the different effector cells. The right plot shows the lysis of the UM9 cells mediated by the different white blood cell populations. **B,** UM9 cells were stained with CellTrace Violet and then opsonized with daratumumab or IgG-b12 control antibody, which were both labeled with AF488. These UM9 cells were subsequently cocultured with white blood cells. Uptake of AF488 signal by white blood cell subsets was analyzed by flow cytometry. The left plot shows the MFI values for the AF488 signal of the different white blood cell subsets. Gating on cells with a negative signal for CellTrace Violet ensured that the increase in AF488 signal of PBMC was not caused by phagocytosis of complete UM9 cells (right). Data are presented as mean ± SEM. *P* values between the indicated groups were calculated using a paired Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ns, not significant. Abbreviations: PMN, polymorphonuclear leukocytes (granulocytes); WBC, white blood cells.

daratumumab-AF488 or cell membrane signal, suggesting that direct contact is required for the transfer of CD38–daratumumab complexes to monocytes.

Loss of other proteins from the multiple myeloma cell surface

Because parts of the cell membrane from daratumumab-opsonized multiple myeloma cells were transferred to acceptor cells, we evaluated whether this process resulted in reduction of other cell membrane proteins. Remarkably, we observed not only CD38 reduction, but also a significant decrease in CD138 levels on daratumumab-treated UM9 and UM9-CD38 cells in the presence of PBMCs. Similarly, this membrane transfer process reduced CD19 expression on Daudi cells. Importantly, in the presence of complement, there was no reduction of CD138 or CD19 (Fig. 6A).

Next, we investigated the effect of daratumumab on other surface molecules, whereby we focused on the major adhesion molecules (19). UM9 and UM9-CD38 cells, treated for 2 hours with daratumumab in the presence of monocytes, had a significant reduction of CD49d (α4-integrin, a subunit of VLA-4), CD54 (ICAM-1), and CD44 (HCAM). These cell lines have very low expression of CD56, and its levels were only reduced in UM9-CD38 cells. Similarly, daratumumab resulted in reduced expression of CD49d and CD54 on Daudi cells in the presence of monocytes. CD44 was not detected on Daudi cells. Again in the presence of complement, expression levels of these membrane proteins were unaffected or only modestly reduced (Fig. 6B).

To estimate the *in vivo* relevance of these *in vitro* findings, we analyzed the expression levels of the same molecules on circulating and BM-localized multiple myeloma cells from patients treated with daratumumab monotherapy or DRd. We confirmed that directly after the first infusion, there was not only reduction of CD38 expression on circulating multiple myeloma cells, but also a reduction of CD138 and CD56 in both responding and non-responding patients (Fig. 6C). Similarly, a longitudinal analysis of BM samples showed a marked reduction of CD56 expression levels with daratumumab monotherapy (GEN501) and with DRd (GEN503; Fig. 6D). Finally, we analyzed the expression of other adhesion molecules in 2 patients. In both patients, daratumumab monotherapy led not only to reduced levels of CD38, but also of CD56, CD138, and CD49d, whereas CD54 and CD44 levels were only slightly reduced or modestly increased (Fig. 6E).

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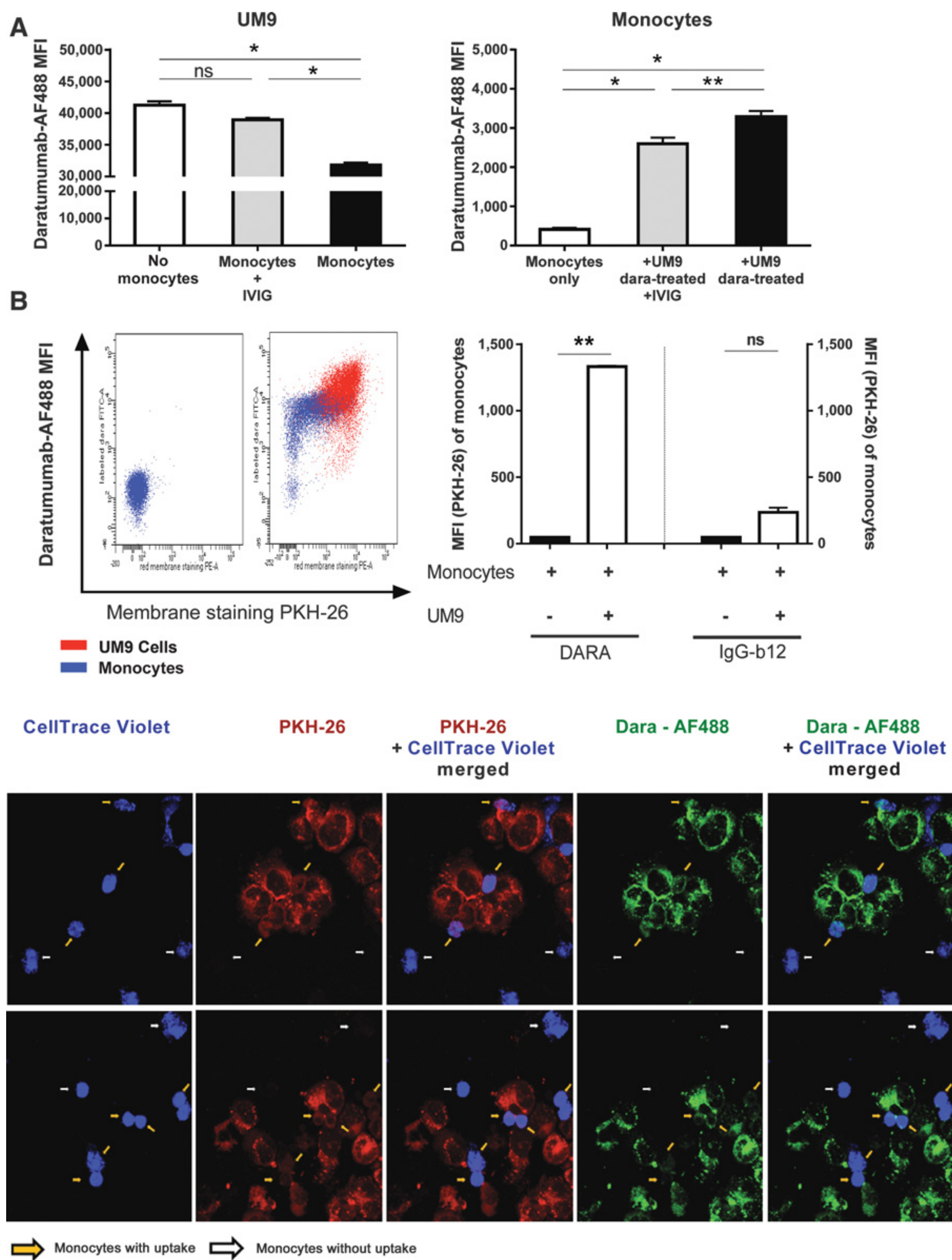

Figure 5.

Transfer of membrane fragments from UM9 cells to monocytes. **A,** UM9 cells were stained with CellTrace Violet and then opsonized with AF488-labeled daratumumab. These UM9 cells were subsequently cocultured for 2 hours with monocytes, which were pretreated with or without polyclonal human IgG to block Fcγ receptors. The signal for AF488–daratumumab on monocytes and UM9 cells was analyzed by flow cytometry. Shown are the MFI values for the AF488 signal. Pretreatment of monocytes with polyclonal IgG partially prevented the loss of AF488-labeled daratumumab on UM9 cells. (*Continued on the following page.*)

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Discussion

We previously showed that daratumumab as single agent results in decreased CD38 expression levels on multiple myeloma cells from relapsed/refractory multiple myeloma patients (13). Here, we demonstrate that CD38 expression is also rapidly, and to a similar extent, reduced on both BM and peripheral blood multiple myeloma cells from patients treated with DRd. This indicates that the synergy between lenalidomide and daratumumab cannot be explained by the abrogation of CD38 downregulation, and that other mechanisms, including effector cell activation by lenalidomide, play a role (20, 21). Interestingly, we also show that daratumumab alone or in combination with lenalidomide-dexamethasone reduces CD38 expression on nontumor immune cells. Importantly, the CD38 reduction on tumor cells and immune cells occurs within hours after initiation of the daratumumab infusion and is present during the whole period of treatment as well as several months after daratumumab has been stopped because of development of progressive disease. Four to 6 months after the last infusion, CD38 expression increases again (13), which can be explained by the continued presence of daratumumab in the circulation during the first months after the last infusion due to the approximately 21-day half-life of daratumumab (22). Importantly, we and others showed that daratumumab-mediated CD38 reduction can also be observed by using other techniques than flow cytometry, including cytometry by time-of-flight (CyTOF; ref. 23) and Western blotting (24).

In the absence of effector cells or complement, daratumumab did not affect CD38 levels on multiple myeloma cells. This suggests that direct internalization of daratumumab-bound CD38 is likely not a key mechanism of CD38 reduction during daratumumab treatment. In contrast, in the presence of complement or effector cells, surviving multiple myeloma cells had lower CD38 expression, suggesting that the mechanism whereby daratumumab reduces CD38 expression probably includes selection of cells with lower CD38 levels, whereas tumor cells with higher CD38 expression are eliminated. Indeed, the level of CD38 expression is an important determinant of daratumumab-mediated CDC and ADCC (14). However, selection cannot be the sole explanation, because CD38 reduction was observed not only in patients with marked reduction in tumor load, but also in those with stable or progressive disease (13). Similarly, CD38 expression was reduced on immune subsets irrespective of whether their frequencies were reduced, increased, or unaffected by therapy. This indicates that next to selection, other mechanisms are involved in CD38 reduction.

Indeed, in a series of experiments, we demonstrate the transfer of the CD38–daratumumab complex from multiple myeloma cells to monocytes and granulocytes, which also occurred in the absence of evident phagocytosis of tumor cells. Furthermore, CD38 reduction on multiple myeloma cells by monocytes could be partially blocked by pretreatment of monocytes with polyclonal IgG, which indicates that the monocyte-mediated CD38 reduction is in part Fcγ receptor dependent. In addition, we show that daratumumab transfers not only CD38–daratumumab complexes from multiple myeloma cells to acceptor cells, but also membrane fragments. This provides an explanation why daratumumab in the presence of effector cells also reduced the expression of several other membrane proteins including CD49d, CD54, CD56, CD138, and CD44 on multiple myeloma cells, whereas the expression of these proteins was mostly unaffected by daratumumab in the presence of complement. We made similar observations in samples from patients treated with daratumumab, including the rapid reduction of CD49d, CD138, and CD56. Importantly, daratumumab does not reduce expression of all membrane proteins. Indeed, we previously showed that CD46 expression is unaffected by daratumumab, whereas CD55 and CD59 expression levels increase at the time of progression during daratumumab monotherapy (13). These findings are compatible with the transfer of membrane-bound organized molecular structures or may suggest that proteins located in close proximity to the CD38–daratumumab complex are removed as innocent bystanders when the daratumumab–CD38 complexes are taken up by effector cells. Indeed, CD38 is physically associated with CD49d and CD44 on the CLL cell surface membrane (25–27). Similarly, CD38 associates with CD19 in normal and malignant B cells (28).

Altogether, the mechanisms of daratumumab-mediated CD38 reduction probably include both selection of cells with lower CD38 expression and uptake of CD38–daratumumab complexes by monocytes and granulocytes in the process of trogocytosis. Daratumumab-mediated CD38 reduction was more evident on primary multiple myeloma cells, when compared with cell lines. This is most likely related to lower baseline CD38 expression levels on multiple myeloma cell lines. Indeed, CD38 reduction was more pronounced in the UM9-CD38 cell line with CD38 expression levels comparable with primary multiple myeloma cells, when compared with the parental cell line UM9. Similarly, trogocytosis by rituximab is also more efficient with high CD20 expression levels (29). The relative contribution of trogocytosis to CD38 reduction is currently unknown. Trogocytosis is probably the most important mechanism for loss of CD38 on multiple myeloma cells and immune cells whose frequencies increase or remain stable, whereas probably both selection and trogocytosis

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(*Continued.*) At the same time, the increase in AF488-labeled daratumumab on monocytes was significantly lower in monocytes pretreated with polyclonal IgG compared with untreated monocytes. **B,** Monocytes were incubated with or without daratumumab-AF488 or IgG-b12-AF488–treated UM9 cells. The cell membrane of these UM9 cells was also stained with PKH-26. After a 2-hour coincubation, monocytes experienced a significant increase of both PKH-26 and AF488-labeled daratumumab. The uptake of PKH-26 by monocytes was only observed when UM9 cells were opsonized with daratumumab, but not if they were opsonized with the IgG-b12 control antibody. The left plot shows representative flow cytometry dot plots [left dot plot: monocytes only; right dot plot: membrane-stained (PKH-26) and daratumumab-AF488–opsonized UM9 cells in the presence of monocytes; monocytes are depicted in blue; UM9 cells are depicted in red]. The right plot shows the MFI of the cell membrane dye PKH-26 on monocytes treated under different conditions. Data are presented as mean ± SEM. P values between the indicated groups were calculated using a paired Student t test; *, $P < 0.05$; **, $P < 0.005$; ns, not significant. **C,** Membrane-stained (PKH-26) and daratumumab-AF488–opsonized UM9 cells were incubated with CellTrace Violet–labeled monocytes. Confocal scanning laser microscopy shows the transfer of both daratumumab-AF488 (green) and cell membrane (red) from UM9 cells to CellTrace Violet–labeled monocytes (blue) in two representative cytospins. Monocytes (yellow arrows), which are in close proximity to UM9 cells, generally take up both AF488-labeled daratumumab and membrane fragments from UM9 cells, whereas monocytes (white arrows), which are not attached to UM9 cells, are generally negative for both AF488-daratumumab and membrane staining. Abbreviation: DARA, daratumumab.

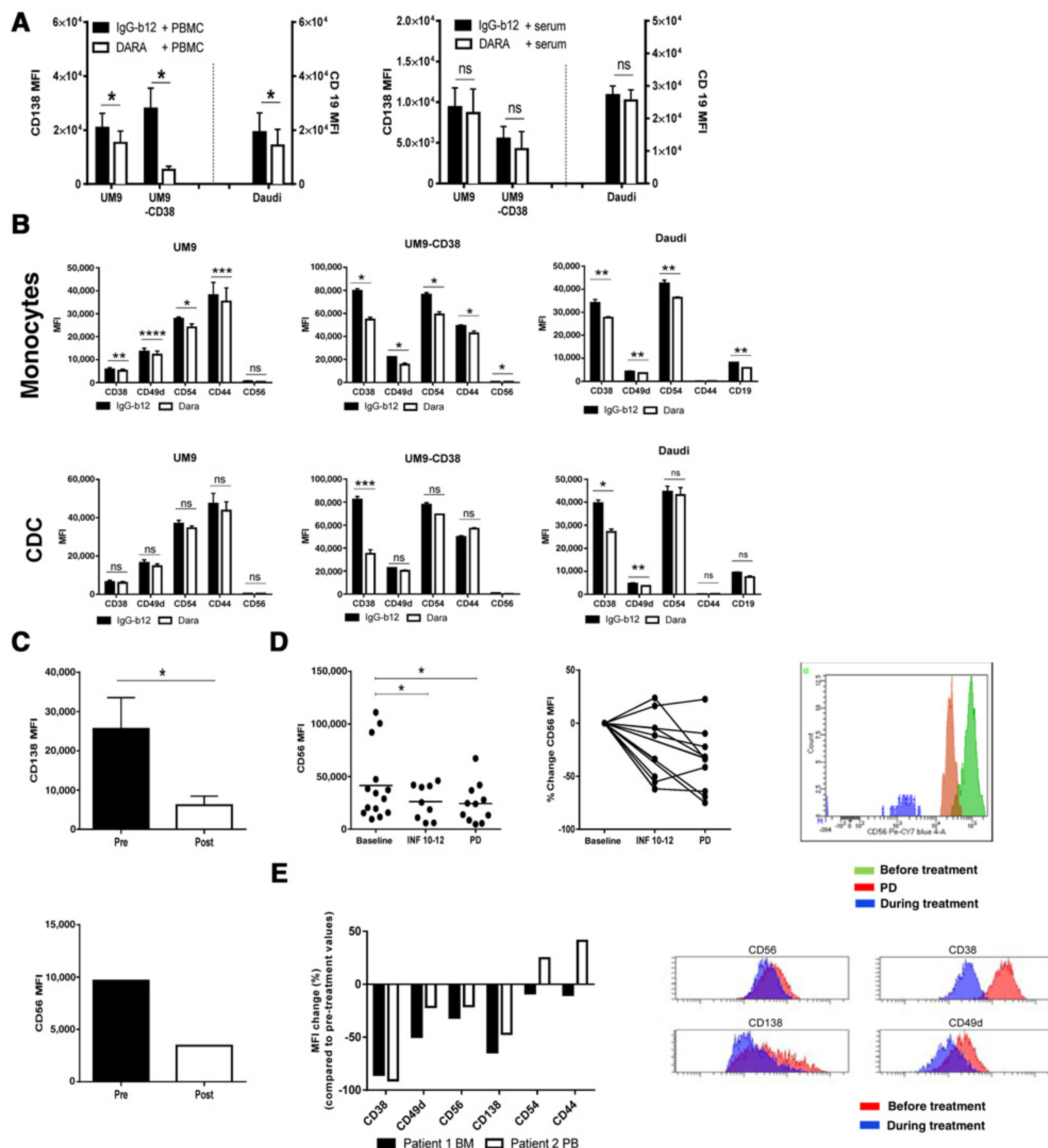

Figure 6.

Several cell membrane proteins also decrease on multiple myeloma cells exposed to daratumumab in the presence of effector cells. **A,** Cell lines were incubated with daratumumab (10 μg/mL) or IgG-b12 control antibody (10 μg/mL) in the presence of pooled unheated human serum as source of complement or freshly isolated PBMCs as effector cells, as described in Materials and Methods. Cells were then harvested to determine CD138 (UM9, and UM9-CD38 cells) or CD19 (Daudi) expression levels on surviving cells by flow cytometry. **B,** The effect of daratumumab on several other membrane proteins known to be expressed on multiple myeloma and lymphoma cells (CD44, CD49d, CD54, and CD56) was tested in the presence of monocytes (top) or pooled unheated human serum as a source of complement (bottom), as described in Materials and Methods. After a 2-hour incubation, cells were harvested to determine expression levels of CD38, CD44, CD49d, CD54, and CD56 on surviving cells by flow cytometry. *P* values between the indicated groups were calculated using a paired Student *t* test. **C,** Expression of CD138 on circulating multiple myeloma cells was determined before (pre) and directly after the first infusion of daratumumab (post) in 3 patients. The circulating tumor cells were CD56 positive in 1 of these 3 patients, and expression levels of this protein were also analyzed before and directly after the first daratumumab infusion. (*Continued on the following page.*)

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contribute to reduction of CD38 expression on multiple myeloma cells and immune cells whose numbers decrease as a result of daratumumab treatment.

Trogocytosis is characterized by the process whereby chelated ligands on the donor cell along with sections of its plasma membrane are pinched off and taken up by the acceptor cell (30, 31). Similar to daratumumab, several other antibodies including epratuzumab (anti-CD22), daclizumab (anti-CD25), IPH2101 (anti-KIR), and rituxumab (anti-CD20) have the ability to downregulate their target proteins by trogocytosis, which was also accompanied by loss of other membrane proteins (32–38). For example, rituximab treatment leads to loss of not only CD20, but also CD19, CD21, CD22, and CD79b from the B-cell surface (32, 35, 37). Thus, as a consequence of trogocytosis, tumor cells may have altered levels of critical phenotypic markers, which could confound flow-cytometric assessment of residual tumor cells during the course of antibody-based therapy.

Our data suggest that trogocytic transfer of CD38 and daratumumab from the multiple myeloma cell surface to effector cells reduces CDC and ADCC, thereby compromising the therapeutic efficacy of daratumumab. However, CD38 reduction seems to be a uniform response in all patients treated with daratumumab, including those with sustained clinical responses and those with increasing depth of response over time (13). Therefore, we expect that although CD38 reduction via trogocytosis is associated with impaired classic Fc-dependent immune effector mechanisms, it may not be *per se* associated with escape from daratumumab treatment. Importantly, we have recently discovered that daratumumab has also other mechanisms of action including an improved host-antitumor immune response resulting from eradication of CD38-positive immune-suppressor cells (12), which may be important for persistent tumor control. Furthermore, the continuous pressure by daratumumab to maintain multiple myeloma cells in a $CD38^{-/low}$ state may contribute to effective immune surveillance at another level, because CD38 ectoenzymatic activity is implicated in production of immunosuppressive adenosine in the BM microenvironment (39–41). Indeed, it was recently shown that tumor-associated CD38 inhibits T-cell function (42). Moreover, daratumumab-mediated trogocytosis may impair the ability of multiple myeloma cells to interact with the protective BM microenvironment by reducing expression of several adhesion proteins on multiple myeloma cells, including CD38 which also functions as adhesion molecule (43). Finally, even the process of trogocytosis itself may lead to death of antibody-opsonized tumor cells as a result of membrane damage as well as loss of the target antigen and other important molecules (44). All these possibilities need to be investigated in future studies.

It is also important to understand under which conditions monocytes or granulocytes engulf whole daratumumab-opsonized multiple myeloma cells or only internalize small amounts of material from the surface of the opsonized cell. Saturation or exhaustion of effector cell–based killing mechanisms (45), limited contact time between multiple myeloma cell and effector cell, or physical constraints by surrounding cells may favor trogocytosis over phagocytosis. Furthermore, polarized localization of antigen/antibody complexes facilitates their transfer by trogocytosis (46, 47). Interestingly, it was recently demonstrated that binding of immobilized daratumumab to CD38 results in redistribution of the CD38 molecules and formation of distinct polar aggregates (48, 49). Therefore, capping of CD38–daratumumab on multiple myeloma cells could lead to removal of the cap by acceptor cells and thus prevent the destruction of these cells by whole-cell phagocytosis. Alternatively, *in vitro* these polar aggregates can also be shed as microvesicles, which can subsequently interact with immune cells (49).

Our findings also suggest that daratumumab resistance is not necessary mediated by loss of CD38 expression, and that other mechanisms underlying acquired resistance exist. Indeed, we previously showed that development of daratumumab-resistant disease was associated with increased expression of the complement inhibitors CD55 and CD59 on multiple myeloma cells, or the outgrowth of subpopulations with high expression levels of complement inhibitors (13). Changes in frequency and activity of effector cells may also contribute to development of resistance. In this respect, NK cells have high CD38 expression and are rapidly reduced after infusion of daratumumab (18), which may impair multiple myeloma cell killing. However, responding and nonresponding patients have similar NK-cell reductions (18). The impact of specific mutations and activation status of signaling pathways to the development of daratumumab resistance is currently unknown.

Preliminary evidence suggests that retreatment of patients with daratumumab is feasible and effective (50). However, it is currently unclear whether a daratumumab-free interval is required in order to allow CD38 expression levels to return to baseline on remaining multiple myeloma cells. Another strategy is to maintain daratumumab and add other therapeutic agents to potentiate any anti–multiple myeloma effects of daratumumab. Furthermore, all-trans retinoic acid (ATRA) has been shown to restore CD38 expression on daratumumab-resistant multiple myeloma cells, which resulted in improved CDC and ADCC (13). A clinical study is currently evaluating the combination of ATRA and

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(*Continued.*) **D,** Longitudinal data representation of CD56 expression levels on BM-localized multiple myeloma cells from 14 patients treated in GEN501 and GEN503 studies with CD56-positive multiple myeloma cells from whom sequential BM samples were available. There was marked reduction of CD56 expression levels during therapy but also at the time of progression (PD). The reduction was most prominent in patients with high CD56 expression. The left plot shows the absolute MFI values; the middle plot shows the percentage change of MFI for each individual patient; and the right plot shows a representative flow cytometry histogram overlay depicting cell surface expression of CD56 on multiple myeloma cells at different time points: before start of treatment (green histogram), during daratumumab treatment (blue histogram), and at the time of progressive disease (PD; red histogram). **E,** Paired BM samples (patient 1; treated in GEN501) and paired peripheral blood samples (patient 2; treated in GEN501) were used to analyze expression levels of CD38, CD49d, CD44, CD54, CD138, and CD56 on multiple myeloma cells by flow cytometry. The first sample was obtained before start of daratumumab treatment, and the second sample was obtained 4 weeks after the first daratumumab infusion. Shown is the percentage change in MFI of the different membrane proteins, as compared with baseline values. Flow cytometry histogram overlays depict surface expression of CD38, CD56, CD49d, and CD138 on BM-localized multiple myeloma cells from patient 1 [before the first daratumumab infusion (red histogram) and 4 weeks after the first daratumumab infusion (blue histogram)]. Data are presented as mean ± SEM. *P* values between the indicated groups were calculated using a paired Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$; ns, not significant. Abbreviations: DARA, daratumumab; PB, peripheral blood.

daratumumab in daratumumab-refractory multiple myeloma patients.

In conclusion, we show that daratumumab-mediated reduction of CD38 on multiple myeloma cells and nontumor cells occurs within hours, and also in the presence of lenalidomide-dexamethasone. The mechanisms of CD38 reduction probably involve both selection of tumor cells with lower CD38 levels and trogocytic transfer by monocytes and granulocytes. Importantly, because CD38 reduction is a uniform response in all patients, including those with deep and durable responses, our analyses seem to exclude CD38 reduction via trogocytosis as a major mechanism of daratumumab resistance.

Disclosure of Potential Conflicts of Interest

S. Zweegman is a consultant/advisory board member for and reports receiving commercial research grants from Celgene, Janssen, and Takeda. R.W.J. Groen reports receiving commercial research grants from Janssen Research and Development. T. Plesner reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Janssen. H.M. Lokhorst is a consultant/advisory board member for Janssen. A.K. Sasser is an employee of and has ownership interests (including patents) at Genmab. T. Mutis reports receiving commercial research grants from Genmab Utrecht, Gilead, and Janssen Pharma. N.W.C.J. van de Donk reports receiving speakers bureau honoraria from, is a consultant/advisory board member for, and reports receiving commercial research grants from Janssen Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: J. Krejcik, T. Plesner, H.M. Lokhorst, T. Mutis, N.W.C.J. van de Donk

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