

Long-term CD38 saturation by daratumumab interferes with diagnostic myeloma cell detection

Monoclonal antibodies emerge as a new treatment option for patients with multiple myeloma, significantly broadening our therapeutic landscape in this disease. CD38 is a valuable target in the treatment of multiple myeloma since this antigen is over-expressed on malignant plasma cells.^{1,2} Recently, the monoclonal CD38 antibody daratumumab has been licensed for the treatment of patients resistant to both proteasome inhibitors and immunomodulatory drugs. This antibody shows considerable efficacy even in the monotherapy setting of heavily pre-treated patients, with an overall response rate of about 30%, as well as in combination with bortezomib and lenalidomide.³⁻⁵ As we are entering a new era of antibody targeting in myeloma, a few considerations on practical patient management have been raised and deserve our attention, such as blood transfusions during CD38-directed treatment and correct determination of complete remission in the presence of therapeutic antibody concentrations in plasma.⁶⁻⁹

Here, we present *in vitro* and *in vivo* data illustrating that interference of daratumumab with diagnostic CD38 antibodies in flow cytometry represents another practical issue relevant for the management of patients with multiple myeloma who have received CD38-directed treatment.

We report on a 55-year old patient with 17p-deleted, International Staging System (ISS) stage II multiple myeloma and almost complete bone marrow infiltration, treated at our institution with high-dose melphalan and autologous stem cell support followed by allogeneic transplant (Figure 1A). During the treatment course, this patient relapsed and received a total of eight daratumumab infusions until clinical progression. Six weeks after the last daratumumab dose, we drained a new pleural effusion. Flow cytometry showed 58% of cells with positivity for CD138 and CD56. CD38 was negative on these cells with standard diagnostic CD38 antibodies and CD45 was fully expressed, contrasting substantially with this patient's CD45/CD38⁺ myeloma immunophenotype at diagnosis and at first relapse after allogeneic transplant (Figure 1B). Yet, cytology and clinical history of daratumumab treatment allowed us to correctly diagnose myelomatous pleural effusion in this patient with progressive disease.

Although our patient had discontinued daratumumab six weeks prior to flow cytometry analysis, we suspected that the lack of CD38 detection was due to competition of the CD38 detection antibody with daratumumab. CD38 loss as a mechanism of resistance in this daratumumab-refractory patient appeared unlikely since CD38 was equally undetectable on immune cells within the pleural effusion (Figure 1B). To discriminate potential CD38 antigen/epitope loss from antibody competition, we stained these cells with a CD38 multi-epitope antibody from Cytognos reported to bind independently of daratumumab¹⁰ and with a non-cross-reactive CD38 nanobody JK36. These stainings clearly showed preserved CD38 antigen expression on the malignant plasma cells (Figure 1C), confirmed by Western blot analysis (Figure 1D). CD38 next-generation sequencing did not reveal any evidence of point mutations or splice variants leading to epitope loss that could explain antibody resistance and failed CD38 detection on these cells. Since the myeloma cells showed full saturation of CD38 with antibody despite daratumumab resistance, we investigated the expression of complement regulators CD55 and CD59 that impair complement-

dependent cytotoxicity of daratumumab.^{11,12} Especially CD55 was highly expressed on the myeloma cells, potentially accounting for clinical resistance in this case (Figure 1E).

The finding of CD38 saturation by daratumumab six weeks after termination of daratumumab treatment was rather unexpected given this patient's progressive disease with high cell turnover since the last antibody infusion. Since parallel receptor-saturation studies were not performed in the context of previous daratumumab trials,³⁻⁵ only estimates from a kinetic model, but no *in vivo* target saturation data, were available. According to this model, daratumumab plasma concentrations of 236 µg/mL are necessary to achieve 99% CD38 saturation *in vivo*.¹³⁻¹⁵ Performing the cell line saturation assay with dilutions of patient plasma, we determined a daratumumab concentration of 11.5 µg/mL in our patient six weeks after the last infusion, when pre-existing and newly generated myeloma cells showed full CD38 saturation with antibody. This concentration was expected based on pharmacokinetic data from the clinical trials (peak levels, half-life, target-mediated clearance),⁴ but clearly challenged the target saturation model. In line with this, we found that daratumumab completely saturates cell surfaces of CD38^{high} and CD38^{low} myeloma cell lines at minimal concentrations of only 0.2 to 0.8 µg/mL (exemplarily shown in Figure 2A). Interestingly, after thorough washing of daratumumab-treated KMS-12-BM cells and subsequent cell line expansion, loss of CD38 antibody saturation corresponded approximately to the rate of cell division, indicating that daratumumab stably persists on the membrane with only little turnover by internalization of antibody-bound CD38 (Figure 2B). Therefore, typical daratumumab peak plasma levels of 915 µg/mL after initial weekly dosing can be expected to over-saturate body CD38 for several months after antibody discontinuation.

Taken together, we found surprisingly long persistence of daratumumab on the cell surface of myeloma cells *in vitro* and *in vivo*. Since all standard monoclonal diagnostic CD38 antibodies bind to epitopes overlapping with the daratumumab binding site, this may interfere with CD38 detection by flow cytometry for several months after the last antibody infusion representing a diagnostic challenge, especially in the context of minimal residual disease, where phenotypic deviations are of special importance. It may also be a relevant diagnostic pitfall if other phenotypic shifts have occurred or if other typical surface markers are lacking, as may happen with CD138 in samples that have been stored for a few hours prior to flow cytometry. Therefore, diagnostic labs should be aware of this issue, they need to be informed by the clinician on daratumumab pre-treatment, and should use detection anti- or nanobodies with differential epitope recognition in these cases. The HuMax-003 antibody from Janssen¹¹ or our nanobody JK36 (although neither are currently commercially available) circumvent this issue and ensure reliable CD38 detection by flow cytometry. The commercially available CD38 multi-epitope antibody from Cytognos is less suitable according to our data due to generally lower mean fluorescence intensities and partial cross-reactivity with daratumumab (Figure 1C).

In addition, our data may indicate inaccuracy of the published target saturation model with potential implications for rational daratumumab dosing schedules. The 16 mg/kg dosing has shown superior outcomes and is certainly advisable due to relevant target-mediated clearance in patients with high tumor load at treatment initiation.⁴ However, *in vivo* target saturation studies should be incor-

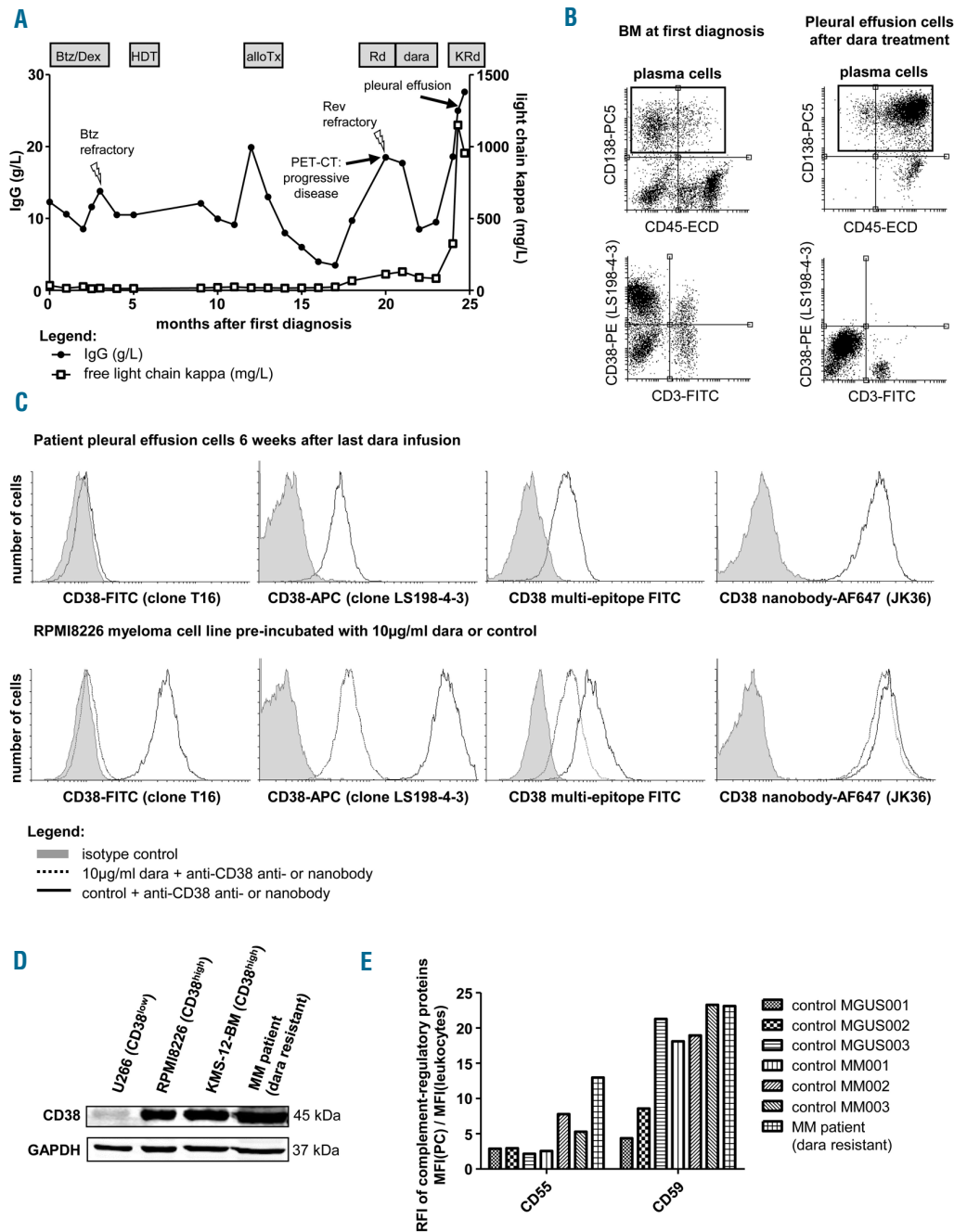


Figure 1. Long-term CD38 saturation by daratumumab interfering with flow cytometry myeloma detection in a patient with daratumumab-refractory disease.

(A) Clinical history and treatment response of index patient. (B) Flow cytometry of bone marrow (BM) cells at diagnosis and cells from pleural effusion six weeks after last daratumumab infusion showing negativity for CD38. Immunophenotype at first relapse after autologous transplant (directly preceding daratumumab treatment) corresponded to initial phenotype at diagnosis. Flow cytometry was performed using antibodies from Beckman Coulter as indicated on a FC500 flow cytometer (Beckman Coulter). (C) Flow cytometry staining of viable cells from pleural effusion of myeloma patient or RPMI8226 myeloma cell line pre-incubated with 10 µg/mL daratumumab. Stainings were performed with two conventional CD38-recognizing antibodies (Beckmann Coulter), JK36 nanobody or CD38 multi-epitope antibody (Cytognos) on a FACSCalibur flow cytometer (BD). JK36 nanobody was purified from transfected HEK cells and conjugated to Alexa647, as described elsewhere (*W. Fumey et al., 2017, manuscript in preparation*). Complete negativity of daratumumab exposed cells with CD38 antibody clone T16 points to a fully overlapping epitope of these two antibodies. Staining with clone LS198-4-3 resulted in a minimal shift compared to the isotype control, suggesting a lesser degree of steric hindrance of these two antibodies. (D) Western Blot analysis of CD38 expression in cells from pleural effusion. The CD38 antibody clone EPR4106 (Abcam), anti-GAPDH antibody (Santa Cruz Biotechnology) and secondary antibodies IRDye® 680RD Goat anti-Rabbit IgG and IRDye® 800CW Goat anti-Mouse IgG (both from LI-COR, Bad Homburg, Germany) were used and visualized with Odyssey Clx Imaging System (LI-COR). CD38^{low} and CD38^{high} myeloma cell lines served as a reference. (E) Expression of complement-regulators CD55 and CD59 on myeloma cells from pleural effusion relative to healthy leukocytes. Plasma cell to healthy leukocytes CD55 and CD59 expression ratios are shown for representative patients with untreated myeloma or monoclonal gammopathy of undetermined significance (MGUS). Myeloma and MGUS patients consented to the use of their biological material for this study as approved by the Ethikkommission der Ärztekammer Hamburg (vote PV4767). Btz: bortezomib; Dex: dexamethasone; HDT: high-dose therapy (melphalan); alloTx: allogeneic stem cell transplant; Rd: lenalidomide (Rev, Revlimid) plus low-dose dexamethasone; dara: daratumumab; K: carfilzomib; PET-CT: positron emission tomography-computed tomography; RFI: relative fluorescence intensity.

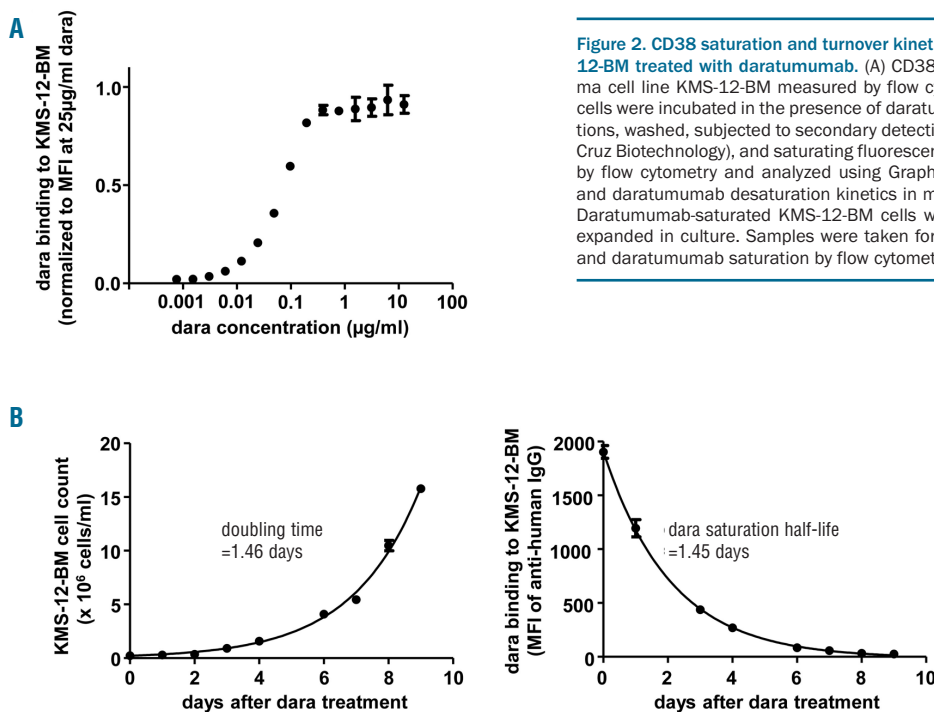


Figure 2. CD38 saturation and turnover kinetics in myeloma cell line KMS-12-BM treated with daratumumab. (A) CD38 saturation kinetics in myeloma cell line KMS-12-BM measured by flow cytometry. Briefly, KMS-12-BM cells were incubated in the presence of daratumumab at various concentrations, washed, subjected to secondary detection (anti-human IgG PE, Santa Cruz Biotechnology), and saturating fluorescence intensities were evaluated by flow cytometry and analyzed using GraphPad Prism5. (B) Proliferation and daratumumab desaturation kinetics in myeloma cell line KMS-12-BM. Daratumumab-saturated KMS-12-BM cells were washed three times and expanded in culture. Samples were taken for determination of cell counts and daratumumab saturation by flow cytometry.

ported into future clinical trials to determine if, after initial loading, longer antibody dosing intervals (e.g. 8-12 weeks, as in CD20 targeting) may be sufficient to reach sustained CD38 saturation.

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