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Comparison of CD38 antibodies in vitro and ex vivo mechanisms of action in multiple myeloma

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CD38, a transmembrane glycoprotein, is widely expressed on multiple immune cell populations.\textsuperscript{1,2} High expression of CD38 on myeloma cells makes it a target of choice for therapeutic antibodies targeting cell surface molecules in multiple myeloma (MM).\textsuperscript{2} CD38 functions as a receptor for CD31 and as an ectoenzyme catalyzing the reaction between NAD\textsuperscript{+} and NADP\textsuperscript{+} to generate cyclic ADP ribose (ADPR), NAADP, and ADPR.\textsuperscript{3}

Daratumumab, a human IgG1κ mAb targeting CD38, eliminates MM cells through several direct mechanisms: antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and apoptosis,\textsuperscript{4-7} as well as immunomodulatory mechanisms.\textsuperscript{2,8} Other CD38-targeting mAbs in clinical development, isatuximab (ISA) and TAK-079, are reported to act similar to daratumumab.\textsuperscript{9,10} It remains unclear how the pleiotropic mechanisms of CD38 targeting mAbs collectively impact tumor cytolysis and exhibit anti-tumor effects in a comprehensive ex vivo immune milieu. Here, we report results of mechanistic comparison studies of 3 CD38-targeting mAbs: daratumumab and analogs of ISA and TAK-079 (generated based on the published antigen-binding fragment sequences for ISA\textsuperscript{11} and TAK-079\textsuperscript{12}, respectively).

To assess antibody binding, CD38-expressing Daudi and LP-1 tumor cells were coated with daratumumab, ISA analog, or TAK-079 analog antibodies at varying concentrations. Cells were washed and stained with Live/Dead\textsuperscript{®} (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 647–conjugated goat anti-human Fc (Jackson ImmunoResearch, West Grove, PA, USA), and binding was analyzed by flow cytometry on a FACS Celesta (BD Biosciences, San Diego, CA, USA). CD38 expression was measured using CD38 (clone HIIT2) PerCp-Cy5.5 (BioLegend, San
Diego, CA, USA). All 3 CD38 mAbs (daratumumab, ISA analog, and TAK-079 analog) demonstrated similar relative binding to the target cells, which is in line with earlier findings of the binding properties of the 3 mAbs.\(^5\)

CDC activity of the 3 CD38 mAbs was tested on multiple cell lines with a range of CD38 surface expression and CDC sensitivity levels (Supplementary Figure 1A).\(^13\) In Daudi, LP-1, and MOLP-8 cells, daratumumab resulted in higher levels of CDC activity compared with the other CD38 mAbs, with a more pronounced difference seen in Daudi cells (Supplementary Figure 1B). In contrast, LP-1 and MOLP-8 cells were susceptible to CDC activity with all 3 CD38 mAbs. However, in LP-1 cells, daratumumab exhibited higher maximal cytotoxicity versus ISA and TAK-079 analogs and lower half maximal effective concentration (EC50) versus TAK-079 (Supplementary Figure 1C). Similarly, in MOLP-8 cells, daratumumab exhibited higher maximal cytotoxicity versus ISA analog and lower EC50 versus TAK-079 analog.

In ADCC assays (E:T ratio 50:1) using PBMC as effectors, all 3 CD38 mAbs induced similar levels of target cell death (Supplementary Figure 2A). Compared to Daudi cells, MOLP-8 and LP-1 cells were less susceptible to ADCC activity. In ADCP assays (E:T ratio 4:1) using monocyte-derived M2c macrophages as effectors, all 3 CD38 mAbs induced similar levels of target cell phagocytosis as detected by pHrodo labeling of target cells after 4 hours (Supplementary Figure 2B). Daudi cells and MOLP-8 cells were phagocytosed by M2c macrophages by all 3 CD38 antibodies. However, LP-1 cells were relatively the most resistant to phagocytosis.
Apoptosis was assessed in the presence and absence of FcR crosslinking. Phosphatidylserine translocation to the cell surface was induced by ISA analog in the absence of FcR crosslinking as measured by annexin V staining, similar to previously published reports (Figure 1A). Neither daratumumab or TAK-079 analog could elicit annexin V staining in the absence of cross-linking. However, in the presence of cross-linking, all 3 CD38 mAbs could induce annexin V staining in Daudi cells. To probe the level of cell death over time, we used a 5-day cytotoxicity assay to detect metabolically active cells (Figure 1B and 1C). All 3 CD38 mAbs elicited comparably high levels of cell death in the presence of the FcR crosslinker and low levels in its absence. Minimal activation-induced cell death (AICD) was observed with LP-1 (Figure 1D) or MOLP-8 cells (Figure 1D).

Daratumumab has been shown to reduce CD38 expression levels partly by trogocytosis. To assess trogocytosis, we utilized Daudi cells as targets and THP-1 cells as effectors. Time-dependent loss of CD38 mAb staining on Daudi cells was correlated with a gain of signal on THP-1 cells (Supplementary Figure 3A). Daratumumab with a silent Fc did not mediate loss of CD38 on cell surfaces, indicating the effect is Fc dependent. Membrane dye was transferred in addition to CD38 (Supplementary Figure 3B), and imaging showed comparable efficiency of target transfer from Daudi to THP-1 cells among all 3 CD38 mAbs (Supplementary Figure 3C). In the absence of THP-1, there was a negligible loss of signal for all 3 mAbs observed on Daudi cells. The lysosome-associated membrane protein 1 (CD107a), co-localized with CD38 in effector cells, suggesting that CD38 is degraded after trogocytosis (Supplementary Figure 3D). All 3 CD38 mAbs showed comparable results, suggesting that all can mediate trogocytosis.
We aimed to compare cytotoxicity of the CD38 mAbs ex vivo utilizing all mechanisms of action. The cumulative effect of the CD38 mAbs was compared using europium labeled LP-1 and MOLP-8 cells in the presence of whole blood from healthy donors containing both effector cells and complement. Within the assay, daratumumab demonstrated a significantly higher maximal cytotoxicity than comparator mAbs in LP-1 cells ($P<0.0001$ for both comparisons) and MOLP-8 cells ($P=0.0016$ for both comparisons; Figure 2). Moreover, the EC50 was significantly lower for daratumumab versus TAK-079 analog in both cell lines ($P<0.0001$) and was lower than ISA analog in MOLP-8 cells ($P=0.0008$). Similar trends were seen with a 24-hour assay using flow cytometry as a read-out.

Bone marrow samples from untreated newly diagnosed patients, containing tumor cells and autologous immune effector cells, were obtained commercially to compare the cumulative impact of the mechanisms of action of CD38 mAbs ex vivo. Depletion of the CD19$^-$CD20$^-$CD38$^+$CD138$^+$ MM cells were measured by flow cytometry after 3 days in the presence of CD38 mAbs and human complement (Figure 3A). Daratumumab elicited higher percent cytotoxicity of the CD38$^+$CD138$^+$ MM cells compared with ISA and TAK-079 analogs (Figure 3B). CD38 was detected using HuMab, which was developed to not compete with daratumumab. It also did not compete with ISA analog or TAK-079 analog. Data was similar with gating on CD19$^-$CD20$^-$CD138$^+$CD27$^{dim}$ cells (Figure 3C).

The results from this study add to the literature on the mechanism of action (MOA) of CD38 mAbs and can provide insight into potential clinical differences that may be seen among the agents. We confirmed all 3 mAbs bind to CD38 at a similar level. Additionally, all 3 mAbs
demonstrated CDC, ADCC, ADCP, AICD, and trogocytosis MOA. Although most mechanisms were similar among the 3 mAbs, daratumumab demonstrated higher CDC activity, and in the presence of human serum (which allows all possible MOAs for antibody activity), daratumumab showed stronger depletion of MM cells.

The cell lines tested varied in their sensitivity to different effector functions, partly due to differing expression levels of CD38 and complement inhibitory proteins. Regardless, daratumumab had greater CDC activity across cell lines compared to ISA analog and TAK-079 analog. In contrast to a report published by Jiang et al.,\textsuperscript{9} we did not observe AICD in MOLP-8 cells. Although it is unclear why this was observed, one possibility is that the MOLP-8 cells used in our study had lower CD38 levels or were more resistant to AICD. The difference between the results from the 24-hour annexin V staining and the 5-day CellTiter Glo assay in the absence of crosslinking suggests that the impact of AICD over time without crosslinking is minimal; cells may be able to recover and continue to proliferate. However, in the presence of crosslinking, AICD resulted in more effective tumor killing by all 3 CD38 mAbs in Daudi cells. The structural differences between daratumumab and ISA have previously been hypothesized to account for the differing interactions with FcR crosslinking.\textsuperscript{15} Neither MOLP-8 nor LP-1 cells were susceptible to AICD in this study.

The ex vivo assay using healthy donor blood and MM cell lines was performed within 3 hours. At this timepoint, ADCC, CDC, and ADCP were the major mechanisms responsible for MM cell ablation. Whole blood contains endogenous complement, NK cells, and monocytes, which function as effector cells. This assay was repeated at 24 hours with similar results using absolute
cell counts by flow cytometry of the labeled MM cell lines. In the MM patient samples, which contain endogenous NK cells and monocytes, the superiority of daratumumab killing was maintained even after 3 days. It is likely that daratumumab had the highest maximal cytotoxicity because of its superior CDC activity.

Our study has several limitations. First, daratumumab was compared with analogues of comparators, ISA and TAK-709. Second, not all anti-tumor mechanisms of CD38 mAbs, including direct inhibition of enzymatic activity, were tested in this study. Last, observed differences in CDC were tested in blood and findings may vary in the setting of a bone marrow compartment in multiple myeloma patients.

In conclusion, daratumumab and surrogate analogs of ISA and TAK-079 have generally similar MOAs. It remains to be determined in clinical trials if these in vitro differences lead to differences in clinical benefit.
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Data sharing policy

The data sharing policy of Janssen Pharmaceutical Companies of Johnson & Johnson is available at https://www.janssen.com/clinical-trials/transparency. As noted on this site, requests for access to the study data can be submitted through the Yale Open Data Access (YODA) Project site at http://yoda.yale.edu.

Disclosures

MK was an employee of Janssen and owns stock/shares in Johnson & Johnson. NJB received research funding from Celgene; served in a consulting or advisory role for AbbVie, Amgen, Celgene, Janssen, Sanofi, and Takeda; and had travel, accommodations, or other expenses paid or reimbursed by Celgene and Janssen. FM has received research support from Janssen, Celgene, Tusk Therapeutics, and Centrose, and served on advisory boards for Centrose, Tusk Therapeutics, Janssen, Takeda, and Sanofi. BdG is an employee of Genmab. JS, JR, and CK are employees of Janssen and own stock/shares in Johnson & Johnson. AB and KB are employees of Janssen. NWCJvdD received research funding from Amgen, Bristol-Myers Squibb, Celgene,
Janssen, and Novartis; and served in a consulting or advisory role for Amgen, Bayer, Bristol-Myers Squibb, Celgene, Janssen, Novartis, Servier, and Takeda.
References


Figure legends

Figure 1. AICD activity of CD38 mAbs, in the absence and presence of crosslinker, at 24 hours\(^a\) in Daudi cells by (A) annexin V staining and (B) at 5 days\(^b\) by CellTiter Glo Assay; no activity was seen in (C) LP-1 cells at 5 days\(^b\) by CellTiter Glo Assay and (D) MOLP-8 cells at 48 hours\(^a\) by annexin V staining and at 5 days\(^b\) by CellTiter Glo Assay.

Daudi cells were plated with or without crosslinker (AffiniPure F(ab')\(_2\) Fragment Goat Anti-Human IgG, Fc\(\gamma\) fragment specific, in which endotoxin was removed; Jackson ImmunoResearch, West Grove, PA, USA). Antibody dilutions were added and incubated for 24 hours (annexin V staining) or 5 days (CellTiter Glo Luminescent Cell Viability Assay). After 24 hours, cells were washed and stained with Live/Dead (Invitrogen) followed by annexin V (BioLegend, San Diego, CA, USA) and analyzed by flow cytometry (FACSCanto). After 5 days, CellTiter Glo reagent was prepared according to the manufacturer’s recommendations and added to a second plate to assess viability. Luminescence was measured and normalized to “plate max” and “plate min”. This analysis was also conducted using MOLP-8 cells, with 48-hour (annexin V staining) or 5-day (CellTiter Glo) incubation.

\(^a\)Data are a summary of 3 independent experiments performed in duplicate.

\(^b\)Data are a summary of 3 independent experiments performed in triplicate.

mAbs, monoclonal antibodies; AICD, activation-induced cell death; XL, crosslinking.
Figure 2. Daratumumab demonstrated higher cytotoxicity than comparator CD38 mAbs in whole blood assays with (A) LP-1 and (B) MOLP-8 cells.

MM cell lines LP-1 or MOLP-8 were labeled with DELFIA® Europium solution (PerkinElmer, Pittsburgh, PA, USA) according to the manufacturer’s protocol. Healthy donor blood samples were added with a titration of daratumumab, ISA analog, TAK-079 analog, or isotype control. Europium release was measured after 3-hour incubation. Percent cytotoxicity = [(experimental lysis – min lysis)/(max lysis – min lysis)] × 100.

Daudi cells did not effectively label with Europium in this assay, which is a phenomenon that has been previously described.

Data are shown as representative experiments (LP-1, n = 6 donors; MOLP-8, n = 5 donors).

mAb, monoclonal antibody; MM, multiple myeloma.

Figure 3. Daratumumab depletes MM cells in patient samples as depicted by (A) counts of CD38+CD138+, (B) percent depletions of CD138+CD38+, and (C) percent depletion of CD27dimCD138+ MM cells.a

PBMCs or bone marrow mononuclear cells from MM patients were obtained from MM patients, according to the guidelines of the Ethics Committee of the Discovery Life Sciences (Huntsville, AL, USA) and in compliance with Declaration of Helsinki protocols. Cells were thawed and measured for viability/density and 200,000 live cells were seeded to assay plates. MM patient cells were treated with daratumumab, ISA analog, or TAK-079 analog at specified concentrations in the presence of 10% human complement. After 3 days, MM cell numbers were measured using Precision Count Beads™ (BioLegend) and by gating on live CD19CD20−
CD138^CD38^ (HuMab; does not compete with tested CD38 mAbs). The percent cytotoxicity was determined relative to the corresponding IgG1 control. Complement was present in each experiment. Data are shown as representative experiment at 10 µg/mL treatment.

^aPBMC or BMM from MM patients (n = 5 donors).

MM, multiple myeloma; PBMC, peripheral blood mononuclear cell; BMM, bone marrow–derived macrophage.
Supplementary Appendix

Supplementary Results

Supplementary Figure 1. (A) MOLP-8 cells showed higher CD38 expression levels compared with LP-1 and Z-138 cells; Daratumumab demonstrated higher CDC activity than comparator CD38 mAbs in Daudi, LP-1, and MOLP-8 cells assessed by (B) percent cytotoxicity and (C) EC50.

<table>
<thead>
<tr>
<th></th>
<th>Daratumumab</th>
<th>ISA analog</th>
<th>TAK-079 analog</th>
<th>Daratumumab vs ISA analog</th>
<th>Daratumumab vs TAK-079 analog</th>
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<td><strong>Daudi</strong></td>
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<tr>
<td>Max, µg/mL; 95% CI</td>
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<td>–</td>
<td>10.27; 4.04-16.50</td>
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<tr>
<td>EC50, µg/mL; 95% CI</td>
<td>0.14; 0.11-0.19</td>
<td>–</td>
<td>0.49; 0.23-1.01</td>
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<tr>
<td><strong>LP-1</strong></td>
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<td>Max, µg/mL; 95% CI</td>
<td>98.31; 97.07-99.55</td>
<td>77.98; 73.99-81.97</td>
<td>82.40; 77.20-87.61</td>
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<td>0.42; 0.34-0.52</td>
<td>0.1339</td>
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<tr>
<td><strong>MOLP-8</strong></td>
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<td>Max, µg/mL; 95% CI</td>
<td>73.33; 64.26-82.41</td>
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<td>0.81; 0.34-1.96</td>
<td>0.3054</td>
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CD38 expression was measured on MOLP-8, LP-1, and Z-138 cells using CD38 (clone HIIT2) PerCp-Cy5.5 (BioLegend, San Diego, CA, USA). CDC was analyzed by a CellTiter Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) in Daudi, LP-1, and MOLP-8 cells, according to the manufacturer’s protocol. Cells were plated at 10,000 cells/well. Human complement (CompTech, Tyler, TX, USA) and diluted antibodies were added and incubated for 2 hours. CellTiter Glo reagent was added, luminescence was measured on a SpectraMax® M5 spectrophotometer (Molecular Devices, LLC, San Jose, CA, USA), according to the manufacturer’s protocol. Percent cytotoxicity = [(experimental lysis – spontaneous lysis)/(max lysis – spontaneous lysis)] × 100.

aData are a summary of 3 independent experiments.

bWe were unable to model ISA analog in Daudi cells. Due to the high level of divergence between the daratumumab and TAK-079 analog modeled curves, P values were not able to be calculated. However, the 95% CIs of max and EC50 values that do not overlap and the large difference between max values indicate a statistically significant difference between the max and EC50 values of daratumumab and TAK-079 analog.

CDC, complement-dependent cytotoxicity; EC50, half maximal effective concentration; max, maximum; CI, confidence interval.
Supplementary Figure 2. All 3 CD38 mAbs demonstrated similar levels of (A) ADCC\textsuperscript{a} and (B) ADCP\textsuperscript{b} activity in Daudi, LP-1, and MOLP-8 cells.

A calcein-release assay using human peripheral blood mononuclear cells (PBMCs) from healthy donors as effector cells was used to assess ADCC of Daudi, LP-1, and MOLP-8 cells. Cells were labeled with calcein-acetoxy methyl (10 μM; Thermo Fisher, Waltham, MA, USA) and seeded at
10,000 cells/well. Diluted antibodies were added to cells and incubated for 15 minutes at room temperature. PBMCs were obtained from 3 donors and seeded at an effector:target (E:T) ratio of 50:1. Calcein release was measured by fluorescence after 3 hours. Percent cytotoxicity = 
\[
\frac{\text{experimental lysis} - \text{spontaneous lysis}}{\text{max lysis} - \text{spontaneous lysis}} \times 100
\]

Macrophage phagocytosis of tumor cells was measured by ADCP using monocyte-derived macrophages as effector cells and pHrodo® Red (Life Technologies, Carlsbad, CA, USA)–labeled Daudi, LP-1, and MOLP-8 cells. Healthy donor CD14+ monocytes were isolated from PBMCs (EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion, STEMCELL Technologies, Vancouver, BC, Canada) and differentiated into M2c macrophages by culturing with macrophage colony-stimulating factor (25 ng/mL; R&D Systems, Minneapolis, MN, USA) for 7 days and IL-10 (40 ng/mL; R&D Systems) for the final 3 days. Target cells were labeled with pHrodo Red and seeded with M2c macrophages at an E:T ratio of 4:1, along with mAbs. After 4 hours of incubation, cells were collected and stained with Live/Dead® and CD11b. ADCP was evaluated by flow cytometry (FACSCanto™; BD Biosciences, CA, USA). Percent phagocytosis = (pHrodo Red+CD11b+ cells/pHrodo Red+ cells) × 100.

a Three donors were tested in duplicate for all cell lines; data are shown as a representative experiment.

b 4 assays for Daudi and 2 assays for LP-1 and MOLP-8; data are shown as a representative experiment.

mAbs, monoclonal antibodies; ADCC, antibody-dependent cellular-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis.
Supplementary Figure 3. All 3 CD38 mAbs showed comparable results, suggesting that all are participating in the same MOA, consistent with trogocytosis. (A) CD38 signal on Daudi is lost in a time-, effector cell-, and FcR-dependent manner. Data are normalized to the 0-hour timepoint and demonstrate percent reduction. (B) Membrane dye is transferred in addition to CD38. (C) Comparable efficiency of target transfer from Daudi to THP-1 cells among the comparators. (D) CD107a co-localizes with CD38 in effector cells, suggesting that CD38 is degraded after trogocytosis.

Human monocytic THP-1 cells were labeled with carboxyfluorescein succinimidyl ester (Thermo Fisher) or PHK26 proliferation dye (Sigma-Aldrich, St. Louis, MO, USA). Daudi cells were coated with Alexa Fluor 647–conjugated daratumumab, ISA analog, or TAK-079 analog (10 µg/mL). Cells were cultured at an E:T ratio of 1:1 for 2 hours and washed and stained with...
Hoechst dye (Thermo Fisher) and CD107a (BioLegend). Data were acquired using ImageStream® X Mark II flow cytometer and INSPIRE® software (both Amnis, Seattle, WA, USA). Three-thousand events (60× resolution) were collected to evaluate signal intensity in cell populations. Image analysis was performed in IDEAS 6.2 (Amnis). Data were normalized to 0-hour incubation to account for labeling efficiency differences.

mAb, monoclonal antibody; MOA, mechanism of action; MFI, mean fluorescence intensity; BF, bright field; CFSE, carboxyfluorescein succinimidyl ester.