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CD319 (SLAMF7) an Alternative Marker for Detecting Plasma Cells in the Presence of Daratumumab or Elotuzumab

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Abstract

Background: Daratumumab is an anti-CD38 immunotherapeutic drug that has increasingly been used to treat patients with heavily pre-treated and relapsed/refractory multiple myeloma. In so doing, the detection of CD38 antigen on plasma cells by flow cytometry is impeded. We hypothesized that alternative markers can be used in place or in addition to CD38 when detecting plasma cells post-treated with daratumumab.

Methods: A total of 16 alternative markers were tested using 22 bone marrow aspirates from patients with plasma cell neoplasm. The ability of selected markers to discern plasma cells from other hematopoietic cells were evaluated. The stability of tested markers when stored at 4°C or 25°C after T = 0, 24, 48, and 72 hours was also established. Finally, selected markers were incorporated into a panel used for monitoring multiple myeloma measurable residual disease to test their utility to identify plasma cells in the presence of daratumumab and/or elotuzumab (anti-CD319) drugs.

Results: Out of the 16 tested markers, CD319, CD54, CD229, CD317, and p63 were expressed by >90% of the plasma cells. Only CD319, CD54, and CD229 achieved 100% detection sensitivity. Further analysis showed that CD319 was better than CD229 and CD54 at resolving plasma cells from background hematopoietic cells, with CD54 being the worst (resolution metric, mean ± SD: CD319 (2.04 ± 0.86); CD229 (1.47 ± 0.45); and CD54 (1.22 ± 0.60)). CD229 was expressed by >90% of T lymphocytes, whereas CD319 was expressed preferentially by the CD8+

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CONFLICT-OF-INTEREST

Philip McCarthy: Advisory Board/Consulting: BlueBird Biotech, Bristol-Myers Squibb, Celgene, Fate Therapeutics, Janssen, Juno, Karyopharm, Magenta Therapeutics, Sanofi, and Takeda; Honoraria: BlueBird Biotech, Bristol-Myers Squibb, Celgene, Fate Therapeutics, Janssen, Juno, Karyopharm, Magenta Therapeutics, Sanofi, and Takeda;

Jens Hillengass: Advisory Board: Adaptive Biotechnologies, Amgen, Bristol-Myers Squibb, Celgene, GlaxoSmithKline, Janssen, and OncoTracker.

The other authors have no competing financial interest to declare.

T cells and less frequently in CD4+ T cells. Additionally, CD229 was found on > 60% of B and NK cells, as well as minor subsets of monocytes and granulocytes. CD319 was expressed on most NK cells and a minor subset of B cells, granulocytes, and monocytes. Even though CD229 and CD319 were expressed by different leukocyte subsets, their expression levels were highest on plasma cells. The expression of CD138 on plasma cells was significantly lower after storage at 4°C, while the expression levels of CD38, CD229, and CD319 remained stable at 4°C or 25°C. Using limiting dilution experiments, the treatment of cells with daratumumab severely impeded the detection of CD38 antigen on plasma cells, whereas elotuzumab treatment did not block detection of CD319 on plasma cells.

Conclusions: CD319 is a suitable alternative to CD38 for identifying plasma cells. Our results showed that a panel used for monitoring multiple myeloma measurable residual disease could be modified by using CD319 alone or in combination with CD38 to detect PCs in daratumumab or elotuzumab treated patients.

Keywords

Measurable Residual Disease; Multiple Myeloma; Flow Cytometry; CD319; Resolution Metric; Daratumumab; Elotuzumab

INTRODUCTION

Plasma cell (PC) neoplasms are a heterogeneous group of mature B cell diseases that are typically characterized by the presence and accumulation of abnormal PCs, which results in the excess production of monoclonal immunoglobulin and/or light chains found in the serum and/or urine (1). Contemporary advances in therapeutic intervention have driven deeper responses in multiple myeloma (MM) patients, thus requiring more sensitive modalities that can be used to reliably detect and quantify remnant neoplastic cells that survived treatment. Due to its high patient applicability, excellent sensitivity, and encouraging results from various clinical trials, multiparametric flow cytometry has been recognized as an indispensable tool to supplement the diagnosis, classification, and monitoring of the disease (2–4).

In 2008, the European Myeloma Network (EMN) recommended the inclusion of CD38, CD138, and CD45 as crucial gating markers for identifying PCs, with the primary gate being CD38 bright vs. CD138+. The heavy reliance on these two markers for the absolute identification of PCs, however, poses several major limitations. First, up to 20% of abnormal PCs were found to have lower surface density of CD38, resulting in staining intensity that is similar to that of normal B cell precursors (e.g. hematogones) and activated T lymphocytes (5). Similar to CD38, the expression level of CD138 on PCs can be heterogeneous and the antigen can be shed from the cell surface. Our preliminary studies and reports by independent investigators showed that the expression of CD138 was down-modulated when cells were Ficolled and/or cryopreserved (6–8).

Most importantly, progress in contemporary immunotherapeutic interventions that employ antigen-specific drugs can impede detection of marker targeted by flow cytometry. Daratumumab, an anti-CD38 IgG1 kappa human monoclonal antibody that binds to a unique

CD38 epitope with high affinity, can block the detection of CD38 antigen by conventional flow cytometry. Daratumumab induces potent antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity of CD38-expressing myeloma cells in the absence of stromal cells in the bone marrow (9). The pharmacological plasma concentrations of daratumumab can be maintained well above 10 μg/ml for a minimum of 6 months post-infusion. Elotuzumab is a humanized IgG1 immuno-stimulatory monoclonal antibody targeting CD319. The humanized anti-CS1 monoclonal antibody elotuzumab (Empliciti) can activate the antibody-dependent cell-mediated cytotoxicity and natural cytotoxic (cell mediated response) functions of NK cells against myeloma cells (10). It has been approved by the FDA for use in combination with lenalidomide and dexamethasone for treating patients with relapsed/refractory MM following one to three prior therapies (11,12). Concentrations of 100 – 200 μg/mL are achievable in vivo (12). Currently, there are no reports investigating the interference of elotuzumab administration with the detection of CD319 on PCs by flow cytometry.

Several markers have been postulated and tested in recent years to replace CD38 and CD138 for the identification of PCs (13,14). For example, a study conducted by Pojero and colleagues showed that CD229 can be used for the detection of PCs, while CD54 and CD319 were less specific markers that could be expressed by other myeloid cells (13). CD269 is another marker that has been shown to be PC specific and is more robust that CD138 when stored at unfavorable conditions. Another interesting antigen is p63, which is expressed intracellularly and present in both normal and neoplastic PCs (15).

Although these antigens have been previously documented to be expressed by PCs, none of these studies objectively evaluated the ability of antibodies to resolve PCs directly from other hematopoietic cell populations. Additionally, there is a lack of studies using these alternative markers as gating parameters in samples from patients treated with antibody-based immunotherapies. Finally, no studies have been attempted to incorporate these markers into the International Clinical Cytometry Society/European Society for Clinical Cell Analysis consensus panel used for monitoring MM MRD.

These observations provided us with the impetus to test if alternative antigens can be used to replace CD38 and/or CD138 for the identification of PCs. First, we conducted an exhaustive literature search to identify a list of relevant markers that are suitable to discriminate PCs from other hematopoietic cells. We evaluated the ability of these antigens to distinguish PCs from other hematopoietic cells. We also conducted stability testing to follow the expression patterns of selected antigens at different temperatures. Finally, we evaluated the best performing markers to monitor MM MRD in the presence of daratumumab and/or elotuzumab.

MATERIALS AND METHODS

Bone marrow aspirate

The Roswell Park Comprehensive Cancer Center Institutional Review Board approved this study (IRB# MODCR00000319). A total of 22 sodium heparinized bone marrow samples with PC neoplasms, as determined by flow cytometric and/or histopathological assessments,

received by Roswell Park clinical flow cytometry laboratory between 11/15/18 and 11/21/19 were used after all requested testing had been performed. Samples from thirteen males and nine females were included. The median age was 68.5 years (range 51 – 87) and the median PC count based on morphological assessment was 19% (range 1 – 88%). For antigen stability testing, RPMI 1640 (Thermo Fisher Scientific, Waltham, MA; Cat #10–040-CV) was added to the fresh bone marrow cells and the diluted samples were stored at 4°C or 25°C for up to 72 hours.

Flow cytometry

All bone marrow aspirates were processed within 24 hours of collection. The viability of all samples prior to testing was >95% based on LIVE/DEAD™ Fixable Aqua Cell Stain (Thermo Fisher Scientific; Cat #L34957). The bone marrow cells were filtrated using a 70 μm cell strainer (Millipore Sigma; St. Louis, MO; Cat #Z742103), washed once using Flow Cytometric buffer (FCM; Leinco Technologies, Inc, St Louis, MO; contain 0.5% Bovine serum albumin, 0.1% sodium azide, and 0.04 g/L tetrasodium EDTA in phosphate-buffered saline), and resuspended to 1×10^7 cells/mL. One hundred microliters of the washed bone marrow cells were stained according to the antibody panels outlined in Table 1 for 30 minutes at room temperature. The comprehensive list of antibody sources, specificities, and fluorochrome conjugates can be found in Supplementary Table 1. All antibodies were tittered on receipt and used at its saturating concentration. The cells were lysed for 5 minutes using 2 mL of ACK Lysing Buffer (Thermo Fisher Scientific; Cat #A10492–01), washed using 3 mL of FCM buffer, and centrifuged at 540 x *g* for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 500 μL of FCM buffer for acquisition.

For labeling p63 intracellular antigen, after surface-labeling the cells were fixed using 100 μL of 2% methanol-free formaldehyde (Polysciences, Warrington, PA; Cat #04018–1) for 10 minutes at room temperature. The cells were washed once using 3 mL of FCM buffer, centrifuged at 540 x *g* for 5 minutes, and the residual volume was resuspended using 100 μL of Permeabilization Medium B (diluted 1:4; Thermo Fisher Scientific; Cat #GAS002S-100). A saturating amount of anti-human p63 FITC reagent was added to the cells and incubated for 30 minutes at room temperature. The cells were washed using 3 mL of FCM buffer and centrifuged at 540 x *g* for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 500 μL of FCM buffer. At minimum of 100,000 total cells with a median PC count of 3,017 (range: 1,006 – 30,156) were acquired using BD's LSRFortessa flow cytometer. Instrument performance was checked daily by recording fluorescence intensity with Cytometer, Setup, and Tracking calibration beads (BD Biosciences; Sane Jose, CA; Cat #655051).

Statistical analysis

All flow cytometric analysis presented in this study were performed using WinList™ version 9.1 (Verity Software House, Brunswick, ME). To assess the stability of markers post-storage, an ANOVA test followed by Tukey's test for post-hoc analysis was used to compare the fold change in detection intensity for each marker. Similarly, an ANOVA test followed by Tukey's post-hoc analysis was performed to compare different gating approaches for detecting PCs post-treatment using daratumumab and/or elotuzumab. In

both instances, p values < 0.05 were considered as statistically significant. To assess the impact of daratumumab and elotuzumab treatment on the detection of tested antigen, fold change comparing intensity of markers before and after treatment were established. The 95% confidence interval was calculated after taking sample size, p-value (≤ 0.05), and standard deviation into consideration.

Daratumumab and elotuzumab

DARZALEX® (daratumumab; an anti-CD38 drug) and EMPLICITI® (elotuzumab; an anti-CD319 drug) were generously provided by the Department of Pharmacy at Roswell Park Comprehensive Cancer Center. Prior to performing surface staining, the bone marrow cells were incubated for 30 minutes at 25°C with a range of drug concentrations, which were prepared by diluting the drugs with 1X phosphate-buffered saline.

RESULTS

CD54, CD229, and CD319 are expressed on all PCs

A total of 16 antigens were tested using 13 bone marrow aspirates from patients with PC neoplasms. These antigens were selected based on previous work published by independent investigators demonstrating positive expression by PCs. Most of these markers play biological roles as adhesion molecules, chemokines, Fc receptors, costimulatory molecules, and the signaling lymphocytic activation molecule family. CD29, CD40, CD47, CD48, CD54, CD229, CD269, CD317, CD307, CD353, CD84, CD150, CD244, CD319, and CD352 were detected using phycoerythrin (PE), p63 was conjugated to fluorescein isothiocyanate (FITC) for detection (Table 1; Panels 1 and 2). The frequency of PCs (defined as CD38br, CD138+, and CD45+/−) expressing these antigens was calculated and summarized in Table 2.

Of the 16 tested markers, CD319, CD54, CD229, CD317, and p63 were expressed by >90% of the PCs. Only CD319, CD54, and CD229, however, achieved 100% detection sensitivity in all 13 cases tested. Consequently, we considered these antigens as top-tier alternatives for replacing CD38 and/or CD138 to identify PCs and further analysis was restricted to them.

CD229 and CD319 resolve PCs better than CD54

The ability of a marker to resolve positive population from the negative population are affected by its staining intensity (e.g. MFI), as well as the spread of the intensity (e.g. how heterogeneous is the cell expressing the marker). As conventional metrics such as stain index and signal-to-noise ratio do not account for the spread of the data for both negative and positive cell populations, they often overestimate the ability of a marker to resolve positive cells from the negative cells. For example, while CD138 is often brightly stained with BV421 on PCs, its expression can be very heterogeneous on PCs (e.g. high CV) but this phenomenon is not reflected by stain index and signal-to-noise ratio. Therefore, we turned to using resolution metric (Rd) as our method of choice for determining the ability of the tested marker to resolve PCs from other hematopoietic cells (16). The R_d can be calculated using the formula below shown below:

$$\textit{Resolution Metric, } Rd = \frac{\text{(Median MFI of Positive Cells)} - \text{(Median MFI of Negative Cells)}}{\text{Total Robust Standard Deviation of (Positive Cells+Negative Cells)}}$$

Using this formula, the median fluorescence intensity of the positive cells (e.g. PCs) and negative cells (e.g. all leukocytes devoid of PCs), as well the variances for each population, were considered. A higher R_d value indicates better separation between PCs from background events. Using a total of 13 bone marrow aspirates from individuals with PC neoplasms, our results demonstrated CD319 was better than both CD229 and CD54 at resolving PCs from background hematopoietic cells (Table 3; mean Rd ± SD: CD319 (2.04 ± 0.86); CD229 (1.47 ± 0.45); and CD54 (1.22 ± 0.6)). Because CD54 had a lower R_d value than CD229 and CD319, we eliminated it from further investigations.

CD229 and CD319 expression on other hematopoietic cells

To profile the expression of CD229 and CD319 on leukocytes, we created a 10-color panel that incorporated the detection of antigens commonly used for delineating T cells, B cells, NK cells, and monocytes (Table 1; Panel 1). A total of 10 samples from patients with PC disease were tested. Although CD38 was found to be expressed by other leukocyte subsets (Figure 1A; top left), notably on most NK cells (mean ± SD: 70.4% ± 17.4%) and monocytes (99.6% ± 0.58%), its expression intensity was about 10 times higher on PCs compared to NK cells and monocytes (Figure 1B; top left; median fluorescence intensity ± SD; PCs: 1,269 ± 832; NK cells: 76 ± 45; monocytes: 126 ± 34). CD138 was expressed mostly on PCs (Figure 1A; top right), with their expression intensity about 100-fold higher than on all other cell populations (Figure 1B; top right).

As shown in the bottom row of Figure 1A, 94.7% ± 4.2% of T lymphocytes expressed CD229, whereas CD319 was expressed preferentially by the CD8+ T cells (82.2% ± 12.7%) as opposed to CD4 T cells (18.2% ± 12.4%). CD229 was found on about 60% of the B and NK cells, and to a much lesser extent on monocytes (17.5% ± 13.2%) and granulocytes (8.5% ± 10%). CD319 was expressed on all NK cells (97.4% ± 2.3%) and some B cells (10.2% ± 6.5%), granulocytes (11.5% ± 12.6%), and monocytes (45.8% ± 22.5%). Even though CD229 and CD319 were expressed by different leukocyte subsets, their expression levels were highest in PCs when compared to all major leukocyte populations (Figure 1B; bottom row).

CD229 and CD319 are stably expressed on PCs

While many bone marrow specimens are processed promptly post-collection (e.g. within a few hours), there are times when the samples are delayed between collection and staining. We tested the stability of CD38, CD138, CD229, and CD319 by recording their fluorescence intensities on PCs stored in RPMI 1640 at 4°C and 25°C over a 72-hour period. An aliquot of cells was retrieved at 0, 24, 48, and 72 hours for staining and flow cytometric analysis (Table 1; Panel 3).

Our results demonstrated that the surface density of CD138 was diminished on PCs when stored for as short as 24 hours; the loss was exacerbated if the cells were stored at 4°C (Table 4; Supplementary Figure 1). The expression level of CD319 on PCs was relatively

stable with no significant changes, except a slight increase was observed when stored at 25°C for 72 hours. Though the expression of CD229 did not significantly change when stored at 25°C, there was a trend towards higher intensity when stored at 4°C. The expression of CD38 showed a decrease, though not statistically significant when stored at 25°C; its expression was better preserved when stored at a lower temperature.

Plasma cells cannot be detected with antibodies to CD38 after daratumumab treatment

Patient samples were pre-treated with concentrations of daratumumab ranging from 1 μg/mL to 1,000 μg/mL Our results demonstrate the detection of CD38 antigen on PCs with clone T16 was blocked by 93% and >99% at 1 μg/mL and 10 μg/ml, respectively (Figure 2A; top row).

We hypothesized that daratumumab may be targeting the same epitope recognized by clone T16. Therefore, we stained the RPMI 8226 myeloma line with 10 different CD38 clones conjugated to FITC in the presence of daratumumab. We found only the CD38 multiepitope reagent could partially restore the detection of CD38 in the presence of 10 μg/mL daratumumab, but the intensity was diminished by more than 50% when compared to untreated samples (Figure 2B).

Plasma cells can be detected using antibodies to CD319 even after treatment with elotuzumab

Anti-CD319 clone 162.1 retained its capability to resolve PCs from background cell populations in samples treated ex vivo with doses of elotuzumab ranging from 1 μg/mL to 1,000 μg/ml (Figure 2A; bottom row). We reasoned elotuzumab was not targeting the same epitope recognized by CD319 clone 162.1, we subsequently tested the only other commercialized available antibody to CD319 (clone 124514; BD Biosciences) and found that elotuzumab did not adversely affect the ability of clone 235614 to resolve PCs from background cells (Supplementary Figure 2).

Impact of daratumumab and elotuzumab on surface antigens

The ICCS/ESCCA consensus panel used for monitoring MRD in MM patients utilizes a two tube, eight-color system that simultaneously evaluates the expression of CD38, CD138, CD45, CD19, CD56, CD27, CD117, and CD81 in one tube (e.g. Tube 1), and cKappa and cLambda replacing CD117 and CD81 in another tube (e.g. Tube 2) (17). We created a 10-color flow cytometric panel to test if the administration of daratumumab or elotuzumab would block the expression of CD229, CD319, along with the other antigens in Tube 1 of the consensus panel (Table 1; Panel 4). The staining intensity of these markers were recorded in bone marrow samples from 5 patients with PC neoplasms after treatment with either phosphate-buffered saline (untreated sample), 10 μg/mL of daratumumab, or 100 μg/mL of elotuzumab.

We found that the intensities of CD319, CD229, and CD38 were significantly lower in daratumumab treated vs. untreated samples, whereas no statistical differences were seen for CD56, CD138, CD117, CD27, CD81, CD19, and CD45 (Figure 3A). In samples treated with elotuzumab, the expression levels of CD319 and CD229 were significantly lower than

the untreated samples, whereas the expression levels of CD56, CD138, CD117, CD27, CD81, CD19, CD45, and CD38 showed no significant differences (Figure 3B).

Combining CD38 and CD319 into the same detection channel identifies PC in daratumumab-treated and –untreated samples

We investigated whether CD319 could be combined with CD38 in the same detection channel to circumvent the difficulty detecting PCs using anti-CD38 monoclonal antibodies in daratumumab-treated patients. We excluded CD229 from this study due to its lower staining intensity on PCs (10 times dimmer than CD138 or CD319) and potential interference by hematopoietic cells expressing CD229. Bone marrow aspirates from patients with PC neoplasms (n = 3) were pre-treated with phosphate-buffered saline (untreated samples), 10 μg/mL of daratumumab, 100 μg/mL of elotuzumab, or both daratumumab and elotuzumab. The cells were labeled according to antibody combination described in Table 1; Panel 5.

The number of recovered PCs was calculated and normalized to the untreated samples. As shown in Table 5 (Gating Approach #1), PCs were rarely detected using the conventional CD38br, CD138+, CD45 gating strategy after the cells were pre-incubated with daratumumab. When CD319+, CD138+, CD45 (Table 5, Gating Approach #2) or CD38/CD319+, CD138+, CD45 (Table 5, Gating Approach #3) were used, the numbers of detected PCs were comparable to untreated samples. All gating strategies yielded similar numbers of PCs when treated with elotuzumab. Using CD38 and CD319 in the same channel to detect and enumerate PCs yielded statistically similar results in untreated, daratumumab-treated, elotuzumab-treated, and daratumumab/elotuzumab-treated samples.

As the treatment of daratumumab *ex vivo* and *in vivo* could potentially imposed different impacts on the detection of various antigens, we further tested if the combination of CD38/CD319+, CD138+, CD45 could be employed to detect PCs using marrow aspirates from MM patients who received daratumumab as treatment. As illustrated by Figure 4A - 4C which is representative of the 4 different patient samples tested, CD319 when combined with CD138 and CD45 could be used to detect PCs when the detection of CD38 was blocked. In the presence of daratumumab, CD319 used alone or in combination with CD38 produced significantly higher R_d values than when CD38 was used alone (Figure 4D; R_d: CD319 PE vs. CD38 PE: 1.76 ± 0.15 vs 0.52 ± 0.10, $p < 0.001$; CD38/CD319 PE vs. CD38 PE: 1.65 ± 0.12 vs. 0.52 ± 0.10, $p < 0.001$).

DISCUSSION

Multiple studies employing different MRD methodologies have demonstrated that MRD-positivity is an adverse prognostic surrogate for progression-free survival and overall survival. This led the International Myeloma Working Group to define MRD-negativity by flow cytometry as an important response criterion in 2016 (18). Antibody-based immunotherapy (e.g. daratumumab) has been used in patients with pre-treated and relapsed/refractory disease and is currently being investigated in the frontline setting as well. This has significantly interfered with our ability to detect PCs by flow cytometry and increased

the reliance on CD138 and CD45 (19,20). CD138, however, is a labile marker that can be negatively modulated when samples are Ficolled or stored for a prolonged period.

In this study, we systematically tested 16 alternatives to CD38 and CD138 for detecting PCs by flow cytometry. Our results demonstrated that CD319 was the best candidate. CD319 was expressed by all PCs in every patient sample, yielded the best resolution between PCs and background hematopoietic cells, and was stably expressed on PCs even after storage at 4°C for up to 72 hours. CD319 (clone 162.1) retained its capability to resolve PCs pre-incubated with daratumumab and/or elotuzumab, as opposed to CD38 which could not be detected in the presence of daratumumab at therapeutic levels. Finally, we showed that CD319 could be readily combined with CD38 for use in a panel to monitor MM MRD.

Studies published by independent investigators have shown that CD229 was expressed by most PCs (21–23). Atanackovic et al. demonstrated that CD229 was expressed on 11 myeloma cell lines, including CD138-, CD229+ cell lines (21). Tembhare et al. demonstrated that CD229 expression was consistently brighter on PCs when compared to other bone marrow cells and more uniformly expressed than CD138 (22). As CD229 is not an exclusive PC marker with a lower surface density than CD138, these authors recommended using CD229 in combination with at least one additional marker to improve the specificity of PC detection. Our results concur with these findings.

Pojero et al. explored the utility of CD54, CD229, and CD319 to identify PCs. In this study, only CD229 provided good discrimination between PCs and all other bone marrow cells, whereas CD54 and CD319 showed limited utility due to the overlapping staining intensities on PCs with myeloid cells (13). These authors found that the combination of CD54 or CD319 with CD138 as gating parameters failed to identify all PCs in a substantial number of bone marrow samples. In our study, we found that CD54 was expressed virtually by all hematopoietic cells, including T cells, B cells, NK cells, monocytes and granulocytes, with monocytes expressing CD54 at almost the same level as PCs (data not shown). This prompted us to eliminate CD54 from further investigation because it lacks the specificity required for detecting PCs. In the study conducted by Pojero et al., CD319 was conjugated to FITC, CD54 to PE, and CD229 to APC, whereas in our study all of the antibodies targeting the evaluated antigens (except p63) were conjugated to PE, thus providing a more appropriate and objective comparison.

This study is limited by the small number of samples tested. Thus, we did not stratify the analyses based on the different types of PC neoplasms. Currently, there is no evidence that different PC neoplasms express CD229 and CD319 differently, however, it would be interesting to determine if the levels of CD229 and/or CD319 could be used as prognostic markers. In this regard, CD138-, CD229+ myeloma cells are thought to be therapy-resistant and a source of disease relapse (23). Additionally, it will be worth comparing the expression of CD229 and CD319 on normal and abnormal PCs. As indicated by our cell line study, the CD38 multiepitope reagent can partially restore the detection of CD38 antigen when cells were pre-incubated with daratumumab. The staining intensity, however, was reduced by approximately 50%, due to partial cross-reactivity with daratumumab. Instead of looking for

a partial fix, we strove to identify markers that were not affected by current antibody-based immunotherapies.

In summary, we tested 16 antigens expressed on PCs that could serve as alternative markers to CD38 and CD138. We demonstrate that CD229 and CD319 are suitable targets for this purpose, with CD319 being superior due to its higher resolution metric and brighter expression intensity. As the ability of CD319 to discern PCs was minimally impacted by daratumumab and elotuzumab treatment, we recommend evaluating CD319 in combination with CD38 in any panel to monitor MM MRD in patient receiving these therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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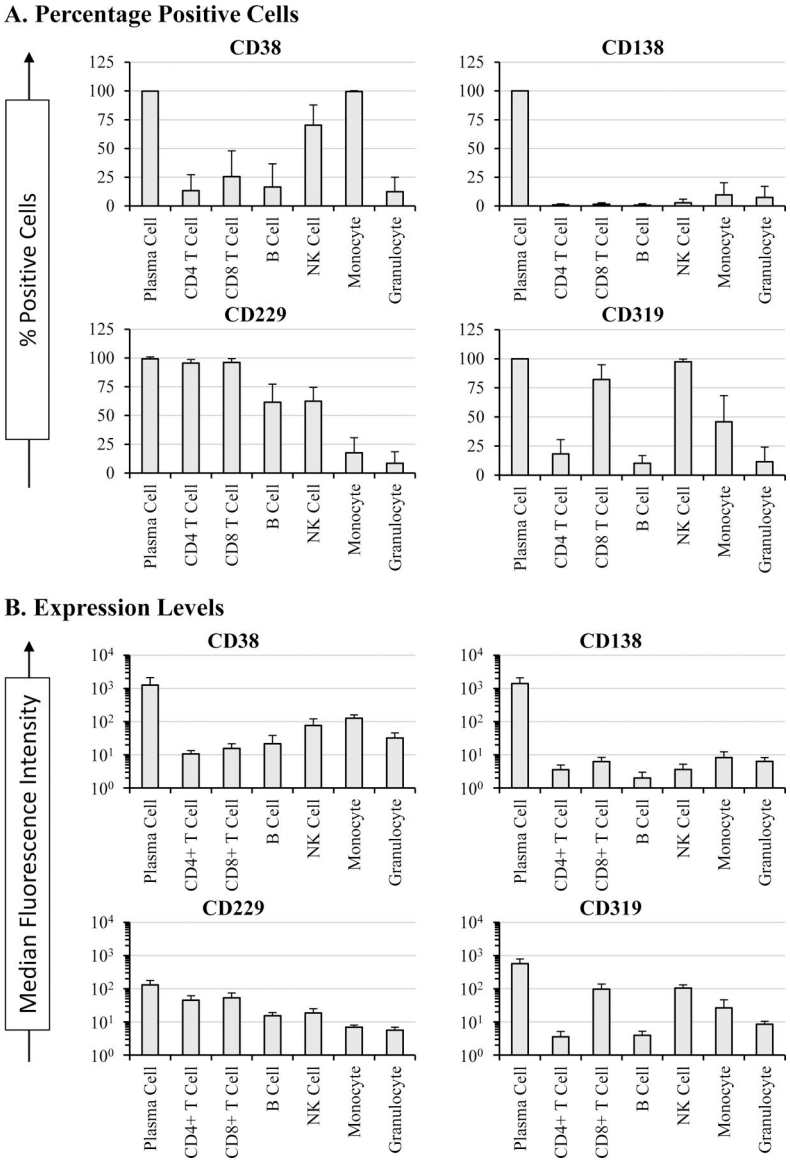

Figure 1. CD229 and CD319 were expressed by plasma cells and other hematopoietic cells.

A total of 10 bone marrow aspirates from patients with plasma cell neoplasms were tested for the expression of CD38, CD138, CD229, and CD319 on various leukocyte subpopulations. Plasma cells were defined by strong CD38 and positive CD138 expression. The rest of the analyzed leukocyte subsets were devoid of plasma cells and were defined as follows: **CD4 T Cells**: CD45br, SSC-A lo, CD3+, CD4+; **CD8 T Cells**: CD45br, SSC-A lo, CD3+, CD8+, CD56-; **B Cells**: CD45br, SSC-A lo, CD19+; **NK Cells**: CD45br, SSC-A lo, CD56+; **Monocytes**: CD45br, SSC-A mid, CD14+; **Granulocytes**: CD45dim, SSC-A hi, CD3-, CD14-, CD19-, CD56-. (A) The percentage of positive cells expressing these markers was evaluated and (B) the intensity of their expression levels was compared. Error bars represent standard deviation of 10 patient samples.

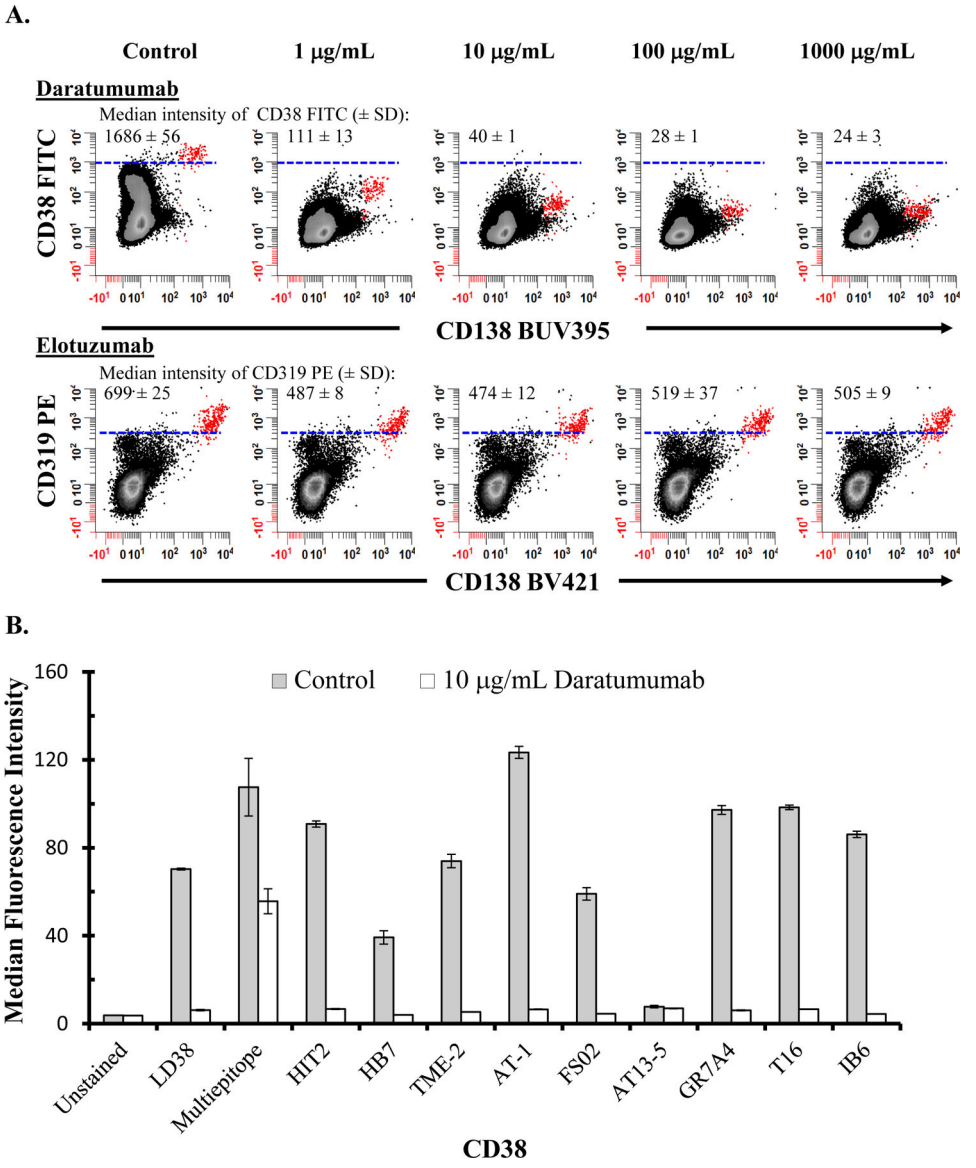

Figure 2. Detection of CD319 was not affected by elotuzumab treatment whereas the detection of CD38 was impeded by daratumumab.

(A) A dilution study was performed to quantify the staining intensity of CD38 and CD138 on plasma cells (red population) after the bone marrow was treated ex vivo with either daratumumab (anti-CD38) or elotuzumab (anti-CD319). The drug concentrations were selected to mimic their pharmacokinetics in patient plasma, with the physiologic dose (e.g. saturated plasma concentration) estimated to be 10 μg/mL for daratumumab and 100 – 200 μg/mL for elotuzumab. The detected expression intensity of CD38 dropped significantly upon daratumumab treatment whereas the detection of CD319 was minimally affected by elotuzumab treatment. The analysis was performed in triplicate on 2 bone marrow samples from patients with plasma cell neoplasms. (B) CD38 detection of RPMI 8226 myeloma cell line using different antibody clones before (gray histograms) and after treatment with 10 μg/mL of daratumumab for 30 minutes (white histograms). Error bars represent ±1 standard deviation of 2 replicated experiment.

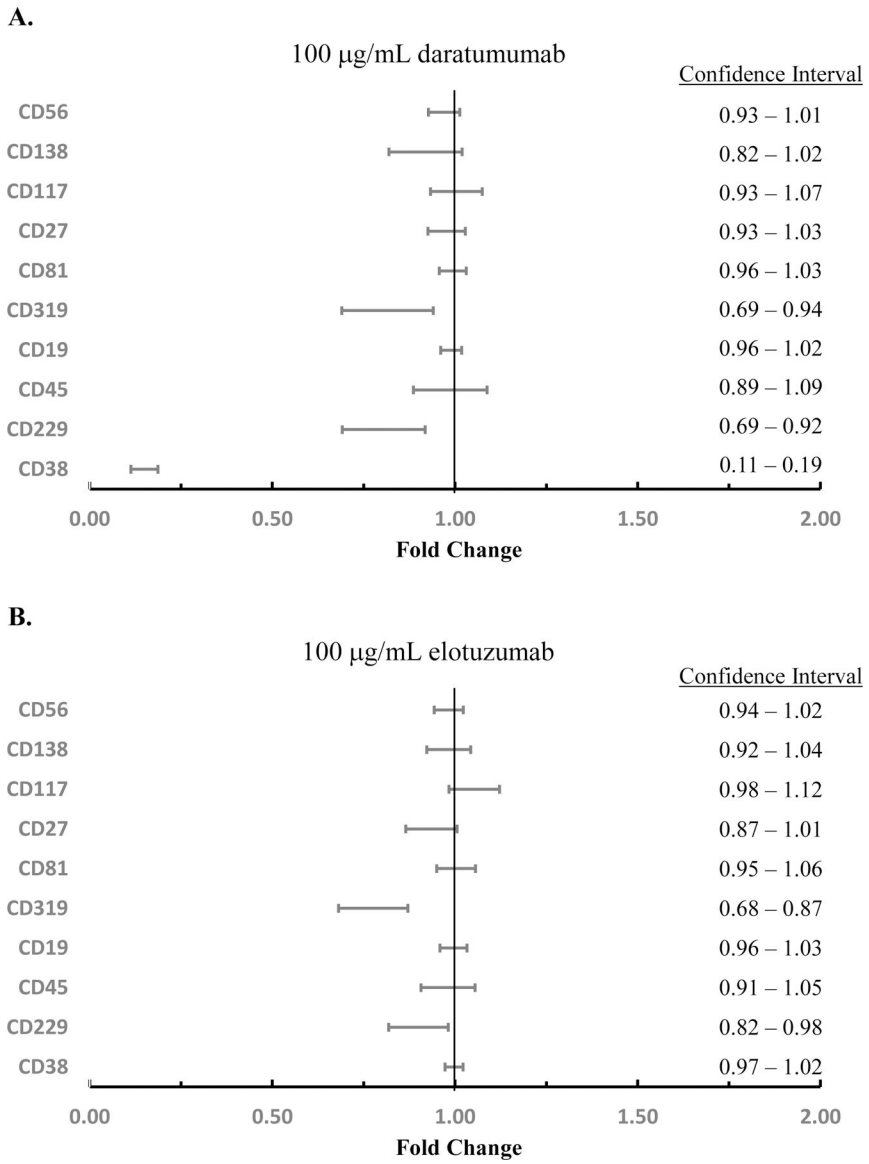

Figure 3. Impact of daratumumab and elotuzumab administration on the expression of different surface antigens.

A total of 5 bone marrow samples from patients with plasma cell neoplasms were tested. These samples were incubated with 10 μg/mL of daratumumab or 100 μg/ml of elotuzumab prior to surface staining. The resultant staining intensity of each marker was normalized to untreated samples. 95% confidence intervals are plotted.

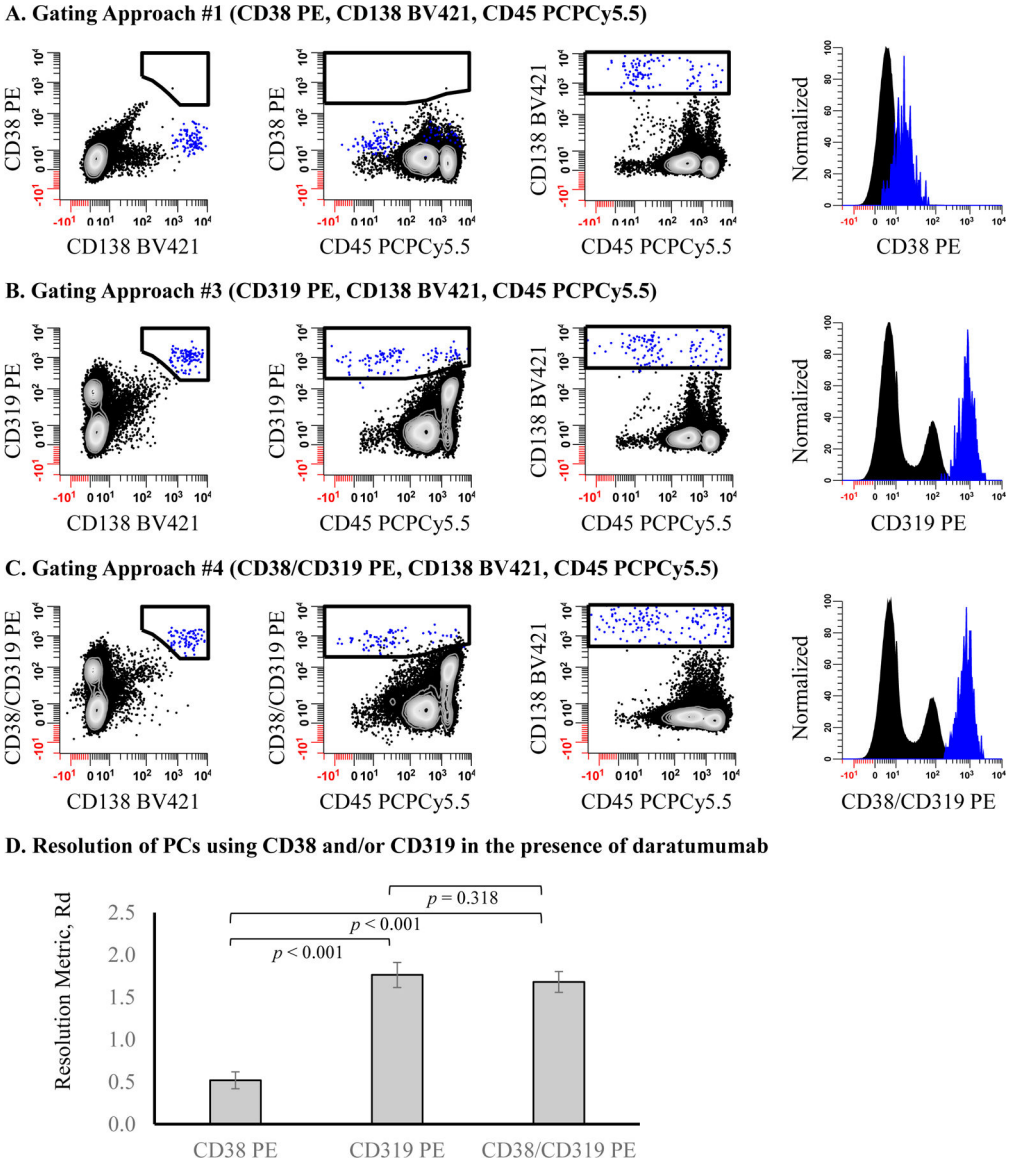

Figure 4. Detection of plasma cells in the presence of antibody-based therapy.

A total of 4 bone marrow aspirates from MM patients who received daratumumab as therapy were stained for flow cytometric acquisition. Different permutations of antibody combinations consisting CD38, CD138, CD319, and/or CD45 were tested to resolve plasma cells (blue dots) from the background hematopoietic cells based on the following gating strategies: (A) CD38+, CD138+, CD45+/−; (B) CD319+, CD138+, CD45+/−; and (C) CD38+/CD319+, CD138+, CD45+/−. Single parameter histograms show plasma cell (blue filled) with background cell populations (black filled) to visually compare their separation. In (D), the resolution metric was calculated to numerically assess the ability of CD38 PE, CD319 PE, and CD38/CD319 PE to resolve plasma cells from background cells; error bars represent the standard deviation of 4 bone marrow samples stained in triplicate.

Table 1:

Antibody panels used for flow cytometric analysis in this study

Panel	Blue laser (488 nm 50 mw)				Red laser (640 nm 40 mw)		Violet laser (405 nm 50 mw)			UV laser (355 nm 60 mw)	Purpose
	530/30	575/26	695/40	780/60	670/14	780/60	450/50	525/50	610/20	379/28	
1.	CD38 FITC	Test[a]	CD45 PCPCy5.5	CD19 PECy7	CD56 APC	CD4 APCH7	CD138 BV421	CD3 BV510	CD14 BV605	CD8 BUV395	Test expression of markers on various leukocyte subsets
2.	p63 FITC	CD56 PE	CD45 PCPCy5.5	CD19 PECy7	-	-	CD138 BV421	CD38 BV510	CD14 BV605	CD3 BUV395	Test expression of p63 in plasma cells
3.	CD38 FITC	CD229 PE	CD45 PCPCy5.5	-	CD319 APC	-	-	-	-	CD138 BUV395	Test stability of markers after storage
4.	CD38 FITC	CD229 PE	CD45 PCPCy5.5	CD19 PECy7	CD319 APC	CD81 APCH7	CD56 BV421	CD27 BV510	CD117 BV605	CD138 BUV395	Test ability of CD229 and CD319 to distinguish PCs in samples treated with daratumumab and elotuzumab
5.	CD56 BB515	Antibody cocktail[b]	CD45 PCPCy5.5	CD19 PECy7	CD117 APC	CD81 APCH7	CD138 BV421	CD27 BV510	-	-	Test CD38/ CD319 combination in a myeloma MRD panel

[a] A total of 15 surface antigens were tested in this study. All antibodies were conjugated to PE. These antigens were CD29, CD40, CD47, CD48, CD54, CD229, CD269, CD317, CD307, CD353, CD84, CD150, CD244, CD319, and CD352

[b] Containing CD38 PE and/or CD319 PE

Table 2:

Expression profiles of markers on plasma cells

Rank	Antigen	Frequency of antigen expression on plasma cells[a]		Sensitivity, %[b]
		Mean ± SD	Range	
1[c]	CD319	99.6 ± 0.5	98.5 – 100	100.0
2[c]	CD54	99.5 ± 0.6	97.6 – 100	100.0
3[c]	CD229	99.0 ± 1.4	95.7 – 100	100.0
4	CD317	94.8 ± 9.3	68.5 – 100	76.9
5	p63	93.3 ± 13.6	52.9 – 99.8	83.3
6	CD48	86.4 ± 13.7	48.3 – 99.9	46.2
7	CD29	89.4 ± 13	65.5 – 99.7	61.5
8	CD47	90.4 ± 9.4	74.5 – 98.4	53.8
9	CD269	89.1 ± 19.2	28.4 – 99.7	76.9
10	CD352	81.4 ± 31.1	15.9 – 100	69.2
11	CD150	72.6 ± 41.1	0.4 – 99.9	61.5
12	CD307	51.8 ± 40.2	5.6 – 98.9	30.8
13	CD84	37.2 ± 29.5	6.6 – 83.9	0.0
14	CD244	22.8 ± 31.5	0.4 – 77.8	0.0
15	CD353	7.2 ± 19.9	0.2 – 73.2	0.0
16	CD40	1.9 ± 2.3	0.1 – 8.3	0.0

[a] Plasma cells were defined as CD38br, CD138+, and CD45lo

[b] Percentage of patient cases with >90% plasma cells expressing the antigen

[c] Markers selected for further analysis

Sample size (n) = 13; except p63 (n) = 12

Table 3.

Ability of selected markers to resolve plasma cells from other leukocytes.

Marker	Resolution Metric, Rd (SD)							
	CD3+ T Cell	CD4+ T Cell	CD8+ T Cell	B Cell	NK Cell	Monocyte	Granulocyte	All Cells Devoid of PCs
CD319	1.67 (0.59)	2.08 (0.55)	1.42 (0.52)	2.10 (0.72)	1.47 (0.62)	1.87 (0.75)	2.08 (0.72)	2.04 (0.86)
CD229	0.70 (0.30)	0.76 (0.30)	0.62 (0.35)	1.24 (0.39)	1.03 (0.36)	1.51 (0.43)	1.57 (0.44)	1.47 (0.45)
CD54	1.29 (0.55)	1.38 (0.52)	1.16 (0.56)	1.16 (0.6)	1.08 (0.57)	1.05 (0.44)	1.24 (0.59)	1.22 (0.60)
CD150	1.05 (0.44)	0.92 (0.44)	1.20 (0.42)	1.16 (0.43)	1.26 (0.45)	1.19 (0.43)	1.24 (0.44)	1.23 (0.44)
p63	2.94 (1.57)	-	-	1.38 (0.77)	3.61 (2.35)	0.79 (0.35)	1.25 (0.57)	1.19 (0.54)
CD269	1.26 (0.35)	1.26 (0.35)	1.25 (0.36)	1.22 (0.36)	1.22 (0.35)	1.03 (0.34)	1.11 (0.35)	1.11 (0.35)
CD352	0.57 (0.44)	0.65 (0.39)	0.68 (0.57)	0.58 (0.57)	0.72 (0.67)	1.16 (0.61)	1.29 (0.60)	1.06 (0.57)
CD317	0.87 (0.42)	0.98 (0.42)	0.8 (0.41)	0.77 (0.38)	0.74 (0.31)	0.71 (0.56)	1.12 (0.40)	1.03 (0.41)
CD307	1.53 (1.09)	1.59 (1.29)	1.46 (0.9)	0.46 (0.35)	1.49 (1.04)	0.58 (0.38)	0.67 (0.37)	0.72 (0.38)
CD48	0.45 (0.20)	0.43 (0.25)	0.48 (0.14)	0.42 (0.16)	0.48 (0.26)	0.50 (0.26)	0.89 (0.30)	0.72 (0.34)
CD29	0.47 (0.26)	0.53 (0.30)	0.40 (0.26)	0.62 (0.37)	0.46 (0.29)	0.85 (0.53)	0.64 (0.4)	0.58 (0.37)
CD244	0.37 (0.17)	0.61 (0.33)	0.97 (0.36)	0.75 (0.47)	2.20 (0.95)	1.28 (0.55)	0.54 (0.37)	0.46 (0.37)
CD47	0.31 (0.14)	0.25 (0.18)	0.40 (0.13)	0.26 (0.16)	0.39 (0.17)	0.21 (0.09)	0.52 (0.23)	0.45 (0.22)
CD84	0.34 (0.24)	0.26 (0.14)	0.57 (0.33)	0.29 (0.13)	0.24 (0.15)	1.27 (0.48)	0.50 (0.30)	0.34 (0.25)
CD353	0.45 (0.34)	0.46 (0.34)	0.43 (0.32)	0.41 (0.31)	0.42 (0.28)	0.35 (0.21)	0.29 (0.16)	0.32 (0.19)
CD40	0.38 (0.23)	0.39 (0.23)	0.37 (0.23)	0.26 (0.15)	0.37 (0.21)	0.22 (0.12)	0.24 (0.16)	0.26 (0.11)

[a] Resolution metric, R_d is calculated by subtracting the median fluorescence intensity of a given marker on non-plasma cells from the median fluorescence intensity of the marker on plasma cells, divided by the total robust standard deviation of both populations. Confidence interval (CI) was calculated after taking sample size (n = 13), p-value ($\alpha = 0.05$), and standard deviation into consideration.

[b] p63 was not included because it was detected using FITC

Rd values are compared within column:

Low R_d — Mid R_d — High R_d

Table 4.

Stability of surface antigens on plasma cells

Markers	Fold change (SD)[a]		
	24 hours	48 hours	72 hours
A. 25°C			
CD38 FITC	0.93 (0.09)	0.86 (0.16)	0.84 (0.20)
p-value	NS	NS	NS
CD229 PE	1.01 (0.16)	1.06 (0.31)	1.02 (0.37)
p-value	NS	NS	NS
CD319 APC[b]	1.05 (0.08)	1.03 (0.04)	**1.12 (0.07)**
p-value	NS	NS	**0.041**
CD138 BUV395[b]	0.91 (0.14)	0.85 (0.30)	**0.69 (0.11)**
p-value	NS	NS	**0.008**
B. 4°C			
CD38 FITC	0.98 (0.05)	0.98 (0.07)	0.99 (0.12)
p-value	NS	NS	NS
CD229 PE	1.09 (0.16)	1.13 (0.14)	1.13 (0.23)
p-value	NS	NS	NS
CD319 APC	1.01 (0.11)	0.98 (0.05)	1.02 (0.06)
p-value	NS	NS	NS
CD138 BUV395[b]	0.62 (0.24)	**0.33 (0.26)**	**0.26 (0.22)**
p-value	0.054	0.0120	0.004

Bone marrow aspirates from patients with plasma cell neoplasms (n = 3) were stored in RPMI 1640 at 4°C and 25°C over a period of 72 hours. An aliquot was stained every 24 hours for flow cytometric assessment. The expression levels of CD38, CD229, CD319, and CD138 on plasma cells were assessed.

[a]Measured fold change was normalized to fresh bone marrow samples stained within 3 hours of collection.

[b]Bold: antigens with expression level altered significantly from baseline

NS: not significant.

Table 5.

Comparison of gating strategies used for detecting plasma cells after treatment with daratumumab and elotuzumab

Gating parameters[a]	Fold change (SD)[b]			
	Untreated	10 μg/mL daratumumab	100 μg/mL elotuzumab	10 μg/mL daratumumab & 100 μg/mL elotuzumab
A. Approach 1				
CD38, CD138, CD45	1.00 (0)[b]	0 (0)	1.06 (0.09)	0 (0)
p-value	-	< 0.001	NS	< 0.001
B. Approach 2				
CD319, CD138, CD45	1.00 (0.11)	1.01 (0.07)	1.05 (0.09)	0.75 (0.24)
p-value	NS	NS	NS	NS
C. Approach 3				
CD38/CD319, CD138, CD45	1.03 (0.12)	0.96 (0.03)	1.00 (0.04)	0.79 (0.33)
p-value	NS	0.086	NS	NS

[a]All cells were pre-gated using Time vs. FSC-A, FSC-A vs. FSC-H, and FSC-A vs. SSC-A to identify hematopoietic cells that are valid, singlet, and viable.

[b]Untreated plasma cells characterized by CD38br, CD138+, CD45lo was considered as the reference population; all analyses were normalized to this population.

NS: not significant.