The Mechanism of Action of the Anti-CD38 Monoclonal Antibody Isatuximab in Multiple Myeloma

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Abstract

Purpose: Knowledge about the mechanism of action (MoA) of monoclonal antibodies (mAb) is required to understand which patients with multiple myeloma (MM) benefit the most from a given mAb, alone or in combination therapy. Although there is considerable research about daratumumab, knowledge about other anti-CD38 mAbs remains scarce.

Experimental Design: We performed a comprehensive analysis of the MoA of isatuximab.

Results: Isatuximab induces internalization of CD38 but not its significant release from MM cell surface. In addition, we uncovered an association between levels of CD38 expression and different MoA: (i) Isatuximab was unable to induce direct apoptosis on MM cells with CD38 levels closer to those in patients with MM, (ii) isatuximab sensitized CD38hi MM cells to bortezomib plus dexamethasone in the presence of stroma, (iii) antibody-dependent cellular cytotoxicity (ADCC) was triggered by CD38in and CD38th tumor plasma cells (PC), (iv) antibody-dependent cellular phagocytosis (ADCP) was triggered only by CD38th MM cells, whereas (v) complement-dependent cytotoxicity could be triggered in less than half of the patient samples (those with elevated levels of CD38). Furthermore, we showed that isatuximab depletes CD38th B-lymphocyte precursors and natural killer (NK) lymphocytes ex vivo—the latter through activation followed by exhaustion and eventually phagocytosis.

Conclusions: This study provides a framework to understand response determinants in patients treated with isatuximab based on the number of MoA triggered by CD38 levels of expression, and for the design of effective combinations aimed at capitalizing disrupted tumor–stroma cell protection, augmenting NK lymphocyte-mediated ADCC, or facilitating ADCP in CD38th MM patients.

See related commentary by Malavasi and Faini, p. 2946

Introduction

Growing knowledge about tumor and immune cell biology led to continuous development of immunotherapies against each of the four major nodes of vulnerability in the cancer–immune relationship: direct targeting of surface antigens; boosting of numbers and functioning of immune effectors; activating tumor antigen-specific immunity; and; overcoming inhibitory immune suppression (1). Multiple myeloma (MM) is no exception to this; however, among the four major nodes of cancer immunotherapy, monoclonal antibodies (mAb) are at the forefront of recent clinical development with two new drugs approved in 2015 for the treatment of relapsed/refractory disease (2).

Characteristics that make antigens attractive as targets for mAb-based therapy include, amongst others, the density of expression of the target molecule by malignant and benign cells (3). Other desirable characteristics of target tumor antigens vary depending on the mAb construct and the mechanism of action (MoA) triggered by such constructs (3). Some mAbs that target antigens on the surface of malignant cells can induce apoptosis by direct transmembrane signaling (4). There is also evidence that mAbs kill target cells by complement-dependent cytotoxicity (CDC; ref. 5), or by inducing antibody-dependent cellular cytotoxicity (ADCC; ref. 6) and phagocytosis (ADCP; ref. 7). Thus, exquisite understanding about the MoA of novel mAbs is warranted, because precise identification of the (one or more) effector mechanisms triggered by these can have considerable impact on optimal selection of patients’ candidates to receive a given mAb based on tumor phenotypes, the design of effective treatment combinations, and correct immune monitoring to predict treatment failure (i.e., precision medicine).

CD38 is a type II transmembrane glycoprotein without an internal signaling domain that, although at variable levels (8),
Mechanism of Action of Isatuximab in Myeloma

Translational Relevance
Greater knowledge about the immune effector mechanisms of monoclonal antibodies is a prerequisite for better prediction of patients’ response and optimal monitoring of treatment effects. Here, we performed a comprehensive analysis about the mechanism of action of isatuximab in multiple myeloma. Our results underline similarities and differences between isatuximab and other anti-CD38 monoclonal antibodies and unveiled a direct association between the levels of CD38 expression and the mechanisms triggered by isatuximab; accordingly, antibody-dependent cellular cytotoxicity emerges as the most prevalent effector mechanism by which isatuximab eliminates tumor cells. They also provide information that could explain, at least in part, why patients with lower expression of CD38 may respond poorly to these drugs than patients with higher CD38 expression. We also provide new insight about the paradoxical depletion of natural killer (NK) lymphocytes following treatment with anti-CD38 monoclonal antibodies; further development of therapeutic strategies to overcome this phenomenon should become a priority.

Results
Isatuximab induces internalization of CD38 but not its significant release from MM cell surface
Recent evidence indicates that daratumumab induces rapid (i.e., less than 4 hours) redistribution of CD38 molecules and formation of polar aggregates leading to the release of CD38 in microvesicles (25). Thus, we sought to investigate whether isatuximab has a similar effect on CD38 density in the surface of MM cells. First, we started by selecting the H929, MM1S, OPM2, and RPMI-8226 cell lines to investigate MM cells with a well-defined range of specific isatuximab antibody-binding capacity (SABC) and CD38 antigen density. Thus, when compared with MM patients (n = 13; median SABC of 120.486), anti-CD38 SABC found in H929, MM1S and OPM2 cell lines was considerably lower (7.079, 10.421, and 21.886, respectively) and equivalent to anti-CD38 SABC values found in only one patient (Fig. 1A). By contrast, RPMI-8226 MM cells displayed SABC levels comparable to the median value of patients with MM (137.759 vs. 120.486, respectively). Therefore, H929, MM1S, and OPM2 cell lines are representative of patients whose tumor PCs have low CD38 expression (CD38lo), whereas RPMI-8226 cells are representative of patients whose tumor PCs have high CD38 levels (CD38hi). To measure CD38 occupancy and changes in CD38 surface expression upon treatment with isatuximab, we used monoclonal (targeting a single epitope that competes with isatuximab) and polyclonal (targeting multiple epitopes) anti-CD38–FITC-conjugated antibodies, to

Materials and Methods
A complete description of methods and techniques used in this study is available in the Supplementary Methods
Briefly, ex vivo studies were performed in bone marrow samples from 13 patients with MM (six newly diagnosed and seven with relapse/refractory disease). All samples were collected after informed consent was given by each patient, according to the local ethics committee and the Helsinki Declaration. We used the RPMI-8226, MM1S, OPM2, and H929 MM cell lines for studying internalization or release of CD38 after isatuximab binding. The effect of isatuximab on proliferation and apoptosis was measured using diphenyltetrazolium bromide (MTT) and flow cytometry assays, whereas the ability of isatuximab to trigger CDC, ADCP and ADCC was determined using various flow cytometry methods. In parallel, we evaluated the immune modulatory effects of isatuximab in the series of patients described above, and purified NK lymphocytes from healthy individuals to understand the mechanisms behind their activation and depletion after treatment with isatuximab, using fluorescence-activated cell sorting (FACS) and gene expression profiling (GEP). We also evaluated isatuximab MoA in NOD/scid/γ−/− (NSG) xenotransplant models, which were inoculated with freely luciferase-expressing MM1S cells in the presence or absence of immune effector cells (i.e., peripheral blood mononuclear cells [PBMC] obtained from healthy donors). We examined the potential of an anti-CD137 mAb, lenalidomide and bortezomib to enhance isatuximab-mediated activation of NK lymphocytes and prevent their exhaustion, using flow cytometry methods. The Wilcoxon signed rank test was used to evaluate the statistical significance of the differences observed between CD16 mean fluorescence intensity (MFI) levels of NK lymphocytes untreated versus treated with isatuximab, whereas the Mann–Whitney U and the Kruskal–Wallis tests were used to estimate the statistical significance of differences observed between two or more groups, respectively. Correlation studies were performed using the Pearson test. Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. The SPSS software (version 20.0; SPSS Inc.) was used for all statistical tests.

is expressed on malignant plasma cells (PC) from all patients with MM (9). CD38 mAbs currently available or being developed for the treatment of MM include daratumumab, isatuximab, MOR202, and Ab19. Except the latter, all other three mAbs are under clinical development but, so far, only daratumumab has been approved for the treatment of patients with MM. Coincidently, the MoA of isatuximab has been considerably investigated and several effector mechanisms were identified: tumor cell apoptosis upon FcγR cross-linking (10), CDC (11), ADCP (12), and ADCC (13). More recently, Krejci and colleagues (14, 15) have shown that daratumumab depletes CD38+ immune regulatory cells, which promoted the expansion of TCR-related T lymphocytes. Although these findings are surprising because they suggest that daratumumab is capable of depleting cells with considerable lower levels of CD38, they also indicate that drugs able to enhance the immune system may be preferred partners to combine with anti-CD38 mAbs (16). Accordingly, both preclinical (11, 13, 17, 18) and clinical data have confirmed the efficacy of daratumumab in combination with lenalidomide (19, 20) or pomalidomide (21). Isatuximab has also demonstrated single-agent and combination activity in heavily pretreated patients with relapsed/refractory MM (22); however, preclinical data to support the clinical development of isatuximab are scarce (23, 24).

Here, we aimed to gain an understanding of isatuximab MoA. Our results allowed us to identify thresholds of CD38 expression required for triggering different MoA, which can be used for optimal patient selection and to design effective treatment combinations. In addition, we unveil potential mechanisms by which natural killer (NK) lymphocytes decrease after treatment with anti-CD38 mAbs.

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Isatuximab induces internalization of CD38 but not its significant release from MM cell surface. A, SABC and CD38 antigen density in RPMI-8226, OPM2, MM1S, and H929 MM cell lines, as well as on primary tumor PCs (n = 13 patients). B and C, MM cell lines were cultured in triplicates and in the presence of isatuximab (10 μg/mL) for 24, 48, 72, and 96 hours (h). After 96 h, cells were collected for flow cytometry immunophenotyping; half were labeled with a mAb, whereas the other half were labeled with a polyclonal antibody to measure the MFI levels of the two CD38-FITC-conjugated antibodies. Baseline values of each cell line were normalized, and the variability of CD38 MFI levels is shown every 24 h. D, MM1S and RPMI-8226 MM cell lines were treated with A647-labeled isatuximab for 0.5, 1, 2, 4, 24, and 48 hours (h). After treatment, cells were washed with PBS, and flow cytometry analysis showed that, over time, there was an increase in MFI of the CD38/isatuximab-A647 signal (measured intracellularly and in the surface membrane) with respect to the control sample. E, Surface membrane CD38-isatuximab complexes were measured with a secondary antibody FITC-anti-human IgG Fab after treatment with unlabeled isatuximab for 0.5, 1, 2, 4, 24, and 48 hours (h). No significant differences on CD38-isatuximab surface levels were noted with respect to the Control-T0 [MM cells incubated with A647-labeled isatuximab or unlabeled isatuximab (10 μg/mL) at 4°C for 15 minutes]. The MFI levels measured in each time point were normalized with respect to the Control-T0. All experiments were performed in triplicate.
label the four MM cell lines after treatment with isatuximab for 24 up to 96 hours. Although the MFI of CD38 measured with the monoclonal antibody was significantly lower after the first 24 hours compared with baseline and remained low thereafter (median decrease of 73% in all cell lines, \( P = 0.04; \) Fig. 1B), there was no significant variation in the MFI of CD38 when using the polyclonal antibody (median variation of 12% in all cell lines, \( P = 0.36; \) Fig. 1C). Similarly, RPMI-8226 cells treated during 24 hours and cultured in RPMI medium with 10% normal human serum (NHS) or heat-inactivated NHS (HI-NHS), in presence or absence of PBMCs and stained with the polyclonal anti-CD38-FITC-conjugated antibody, displayed continuous intracellular and surface expression of CD38 despite the formation of CD38 polar aggregates (Supplementary Fig. S1).

To further investigate whether CD38 persisted in MM cells after binding to isatuximab, RPMI-8226 and MM1S MM cells were cultured up to 48 hours at 4°C or 37°C in the presence of unlabeled versus A647-labeled isatuximab. Flow cytometry analysis of MM cells cultured at 37°C in the presence of isatuximab-A647 revealed a significant increase in MFI levels after 24 and 48 hours (Fig. 1D). By contrast, MFI levels remained unaltered when MM cells were cultured with isatuximab-A647 at 4°C (Supplementary Fig. S1D). To confirm the presence of surface CD38-isatuximab complexes over time, we cultured RPMI-8226 and MM1S MM cells with unlabeled isatuximab and stained them with a secondary FITC goat anti-human IgG Fab antibody to measure the amount of isatuximab bound to CD38 in the surface of MM cells. No significant differences were observed in the MFI measured in the FITC channel during 24 hours (Fig. 1E). Thus, our results indicate that isatuximab effectively binds CD38 and may induce internalization but not significant release of CD38 from MM cells.

**Isatuximab sensitizes CD38hi MM cells to bortezomib plus dexamethasone but does not induce apoptosis by direct transmembrane signaling on MM cells with CD38 levels closer to those in MM patients**

After demonstrating that there was no significant release of CD38 after binding to isatuximab, we then investigated whether the drug had a direct antiproliferative or pro-apoptotic effect on MM cells. Upon treating CD38hi and CD38lo MM cells with increasing concentrations of isatuximab, we have not observed a significant (\( P > 0.05 \)) effect on viability, proliferation or survival of MM cells (Supplementary Fig. S2A–S2C). To understand whether, in the absence of direct apoptosis, isatuximab was modulating MM cells at the molecular level, we analyzed the transcriptome of CD38hi RPMI-8226 MM cells after treatment with isatuximab. No significant gene deregulation (using a B statistic cutoff B > 0 and log FC > 1) induced by treatment with isatuximab was observed when compared with untreated RPMI-8226 MM cells (Supplementary Fig. S2D).

Because CD38 may synergize with the CXCR4 pathway and cooperate in CXCL12-mediated homing (26), we investigated whether CD38 blocking by isatuximab could increase the efficacy of anti-myeloma drugs known to have reduced effect in the presence of stroma (e.g., bortezomib and dexamethasone; ref. 27). Interestingly, we observed that in the presence of stroma, isatuximab significantly increased the percentage of dying CD38hi RPMI-8226 cells after treatment with bortezomib plus dexamethasone (mean increment, 12%; \( P = 0.006; \) Supplementary Fig. S2E). However, adhesion of RPMI-8226 and MM1S MM cells to the H55 stromal cell line or patient-derived bone marrow stromal cells was not reduced by treatment with isatuximab (Supplementary Fig. S2F and S2G). Thus, our results suggest that isatuximab has no direct effect on MM cell lines with CD38hi and CD38lo levels but may sensitize CD38hi MM cells to combined bortezomib and dexamethasone through mechanisms other than disrupting tumor-stromal cell adhesion.

**Isatuximab activates complement in MM cells with elevated levels of CD38**

In the absence of a direct effect on MM cells with CD38 levels comparable to that of most MM patients, we then explored which immune effector mechanisms mediate the anti-myeloma surface expression of CD38 despite the formation of CD38 polar aggregates (Supplementary Fig. S1).

Isatuximab activates complement in MM cells with elevated levels of CD38

To determine whether isatuximab triggered activation of complement, we measured the deposition of C3-related fragments in the four MM cell lines as well as in primary tumor PCs (\( n = 9 \) patients). Although no deposition of C3-related fragments was noted in CD38lo and CD38hi MM cell lines (Fig. 2A), there was a non-significant increment of C3-related fragments deposition in primary tumor PCs (mean C3 MFI levels of 1.8 and 7.0 in the absence versus presence of isatuximab; \( P = 0.13 \)). More detailed analyses showed a significant correlation between anti-CD38 SABC and deposition of C3-related fragments in primary tumor PCs (\( r = 0.86; \) \( P = 0.003; \) Fig. 2B), as well as a trend for a correlation between tumor PC depletion and deposition of C3-related fragments (\( r = 0.61; \) \( P = 0.08; \) Fig. 2C). Thus, our results suggest that although isatuximab may induce CDC in patients with elevated levels of CD38, immune effector mechanisms other than CDC must mediate the activity of isatuximab against MM cells with lower levels of CD38 expression.

**Isatuximab induces ADCC in MM cells with a broad range of CD38 expression and selective ADCP in CD38hi MM cells**

To determine whether isatuximab triggered ADCC, we started by incubating CD38hi and CD38lo MM cells in absence or presence of the drug, with or without donor NK lymphocytes. When MM cells were incubated only with isatuximab, survival was nearly 100%, thus reproducing our previous findings that indicated lack of direct apoptosis. By contrast, significant cell death was observed when both isatuximab and NK lymphocytes were cultured with MM1S, OPM2 and RPMI-8226 but not with H929 MM cells (Fig. 3A), which had the lowest isatuximab anti-CD38 SABC (Fig. 1A). We also evaluated the potential of isatuximab to trigger ADCC against primary tumor PCs (\( n = 13 \) patients) and observed significant tumor PC depletion (median 51%; \( P = 0.003 \)); importantly, we noted a significant correlation between depletion of tumor PCs and their anti-CD38 SABC (\( r = 0.58; \) \( P = 0.04; \) Fig. 3B).

Because, in addition to NK lymphocytes, part of T lymphocytes also express FcRIIA (CD16A), we sought to determine the relative contribution of each subset to the ADCC triggered by isatuximab. Accordingly, we used FACS to culture CD38hi RPMI-8226 MM cells either with donor PBMCs, or with PBMCs without NK lymphocytes (W/o NK), or with PBMCs without CD16+ T lymphocytes (W/o T), in the presence or absence of isatuximab. Our results show that the percentage of tumor PCs remained stable in the absence of NK lymphocytes, whereas significant tumor depletion was noted in the absence of CD16+ T lymphocytes (Fig. 3C), thus indicating that NK lymphocytes are the critical mediators of ADCC triggered by isatuximab.

**Mechanism of Action of Isatuximab in Myeloma**

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3179

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Afterwards, we investigated if isatuximab also triggered ADCP by labeling MM cells with violet proliferation dye (VPD) prior culturing them with unlabeled M2-like macrophages. Thus, the intensity of VPD measured in CD11b$^+$ macrophages, after culture with VPD$^+$ MM cells and in the presence vs absence of isatuximab, would be a surrogate for ADCP. Our results show non-significant ADCP against CD38lo MM cells, but significant ADCP against CD38hi RPMI-8226 MM cells (median of 43%; $P = 0.005$; Fig. 3D).

To evaluate isatuximab MoA in vivo, we inoculated NOD/scid/γc$^-$ (NSG) mice (which are deficient in NK cells but retain phagocytic cells) with firefly luciferase-expressing MM1S cells and treated with isatuximab in the presence or absence of immune effector cells (i.e., PBMCs from healthy donors). Our results show that treatment with isatuximab significantly prolonged survival of mice in the absence and presence of immune effector cells, indicating that ADCP and ADCC were triggered in vivo by isatuximab (Fig. 3E and F).

**Effect of isatuximab on other immune cells**

Immune modulation triggered by daratumumab due to depletion of CD38$^+$ immune regulatory cells has been recently described (14, 15). Thus, we investigated whether isatuximab also depleted specific immune cell subsets by treating primary bone marrow samples from 13 patients with MM with isatuximab. As a control for the effect of isatuximab, we previously demonstrated that the drug significantly depleted tumor PCs (median of 51%; $P = 0.003$; Fig. 3B). Interestingly, we observed significant depletion of CD38hi B-lymphocyte precursors (median of 54%; $P = 0.009$), basophils (median of 26.5%; $P = 0.006$) and NK lymphocytes (median of 18%; $P = 0.002$) after treatment with isatuximab. By contrast, no differences were noted in the relative abundance of other immune cell subsets.

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**Figure 2.**

Isatuximab may induce complement activation in MM cells with elevated levels of CD38. A, Deposition of C3-derived fragments in CD38lo (H929, MM1S, and OPM2) and CD38hi (RPMI-8226) MM cells after complement activation in the presence of 10 μg/mL of isatuximab for 24 hours (treated) or saline (untreated). B, Deposition of C3-derived fragments in tumor PCs from primary bone marrow samples (n = 9 patients) untreated and treated with 10 μg/mL of isatuximab for 24 hours. Data are expressed as the ratio of the MFI between cells incubated with 10% serum and cells incubated with its respective complement inactivated serum. Bar graphs represent the median ± the 95% confidence intervals (from three independent experiments). The statistical significance was evaluated using the Mann-Whitney U test.
Figure 3.
Isatuximab induces ADCC in MM cells with a broad range of CD38 levels and selective ADCP in CD38^hi MM cells. A, ADCC was measured according to the percentage of Annexin V–positive CD38^lo (H929, MM1S, and OPM2) and CD38^hi (RPMI-8226) MM cells cocultured with donor NK lymphocytes, and after treatment with 10 μg/mL of isatuximab for 4 hours. B, Tumor PC depletion was determined in bone marrow samples from 13 patients with MM after treatment with 10 μg/mL of isatuximab for 24 hours, and correlated with the SABC and CD38 antigen density per tumor PC. C, To determine the relative contribution of NK and CD16^+ T lymphocytes to the ADCC triggered by isatuximab against CD38^hi RPMI-8226 MM cells, we used FACS to coculture CD38^hi RPMI-8226 MM cells either with donor PBMCs, with PBMCs W/o NK, or with PBMCs without CD16^+ W/o T, in the presence versus absence of 10 μg/mL of isatuximab for 24 hours. D, CD38^lo (H929, MM1S, and OPM2) and CD38^hi (RPMI-8226) MM cells were labeled with VPD and were preincubated with 10 μg/mL of isatuximab before adding monocyte-derived M2-like macrophages obtained from healthy donors in a 1:4 MM cell ratio. VPD was measured on CD11b^+ macrophages after 2 hours of coculture. All experiments were performed in triplicate. In A, C, and D, bars represent median values and vertical lines the upper bound of the 95% confidence intervals; the statistical significance was evaluated using the Kruskal–Wallis (A and C) and the Mann–Whitney U (D) tests. E, Mice (n = 7 per group) were inoculated with 5 x 10^6 MM1S-GFP-Luc cells on day 0, and either were left untreated or were treated with isatuximab (20 mg/kg) on days 7 and 14. F, In another two groups of mice (n = 7 per group), 10 x 10^6 PBMCs were administered on day 8. Survival was monitored twice weekly.
percentage of regulatory T cells (Treg), and T lymphocytes were not immune modulated after exposure to isatuximab (Supplementary Fig. S3). Other hematopoietic cells (including CD34⁺/CD19⁻ hematopoietic progenitors, neutrophils or monocytes) were not depleted by isatuximab (Supplementary Fig. S3E and S3F).

**Activation and depletion of NK lymphocytes upon treatment with isatuximab**

Because NK-mediated ADCC was the only MoA developed by isatuximab to kill CD38lo and CD38hi MM cells, but at the same time isatuximab was depleting (CD38hi) NK lymphocytes, we sought to gain further insight into the mechanism behind the direct effect of isatuximab on NK-lymphocyte depletion. Thus, we compared the transcriptome of NK lymphocytes FACSorted after a 24-hour culture with CD38hi RPMI-8226 MM cells, in the presence or absence of isatuximab. Interestingly, NK lymphocytes showed deregulated expression of 70 genes in the presence of isatuximab (Fig. 4A), for which Ingenuity analysis attributed biological processes such as cell chemotaxis, cytolysis and defense response. One such gene was TNFRSF9 (i.e., 4-1BB or CD137), whose upregulation was also observed at the protein (antigen) level (Fig. 4D). These results suggest that isatuximab induces NK-lymphocyte activation beyond tumor–NK cell cross-talk. Because in previous experiments with primary patient samples \((n = 13)\), we observed significant downregulation of CD16 MFI levels on NK lymphocytes after treatment with isatuximab (62% down-regulation, \(P < 0.001\); Fig. 4B), we thus investigated whether isatuximab was activating NK lymphocytes through Fc binding, or if activation was also being triggered after binding to CD38 on CD38hi NK lymphocytes. We observed that in the presence of increasing concentrations of an Fc blocker containing specialized human IgG, NK lymphocytes cultured with RPMI-8226 showed increased expression of the activation markers CD69 and CD137 in a dose-dependent manner. Of note, the extent of activation induced by the Fc blocker was consistently lower than the extent of activation observed in the presence of isatuximab alone. Thus, the combination of Fc blocker with isatuximab induced a significant increment in the expression of the activation markers CD69 and CD137 compared with NK lymphocytes treated with Fc blocker in the absence of isatuximab (Fig. 4C and D). Altogether, these

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**Figure 4.** Activation of NK lymphocytes upon treatment with isatuximab. **A,** Heat map of genes with significantly different expression (using a B statistic cutoff B > 0 and log FC > 1) from NK lymphocytes cocultured with CD38hi RPMI-8226 MM cells, left untreated versus treated with 10 \(\mu\)g/mL of isatuximab for 24 hours. **B,** CD16 MFI levels were measured on NK lymphocytes from bone marrow samples of 13 patients with MM treated with 10 \(\mu\)g/mL of isatuximab for 24 hours. **C and D,** The percentage of CD69 (C) and CD137 (D) expression on NK lymphocytes cocultured with RPMI-8226 MM cells, left untreated or treated with increasing concentrations of an Fc blocker (0.5, 1, and 2.5 \(\mu\)g/mL), in the absence versus presence of 10 \(\mu\)g/mL of isatuximab. Experiments in A, C, and D were performed in triplicate. In C and D, bars represent median values and vertical lines the upper bound of the 95% confidence intervals; the statistical significance was evaluated using the Wilcoxon (B) and Kruskal-Wallis (C and D) tests.
results suggest that in the presence of target cells, isatuximab activates NK lymphocytes by Fc binding and CD38 transmembrane signaling. Afterwards, we investigated whether the depletion of CD38hi NK lymphocytes occurred due to coating and activation by isatuximab (i.e., fratricide). Accordingly, VPD-labeled NK lymphocytes (VPD-NK) preincubated with isatuximab were cultured with VPD-unlabeled NK lymphocytes (NK) from the same subject. Our results show that in the absence of isatuximab, there was no NK-lymphocyte activation or depletion; however, exposure to isatuximab induced significant activation (mean 17% increment in CD69 expression, \( P = 0.001 \)) and death (mean 27% increment, \( P = 0.027 \)), without any differences in activation and death rates when VPD-NK lymphocytes were cultured with VPD-unlabeled NK lymphocytes (Fig. 5A). These results suggest that rather than fratricide, activation followed by exhaustion would be responsible, at least in part, for depletion of NK lymphocytes after treatment with isatuximab.

Driven by the upregulation of CD137, we subsequently evaluated if an anti-CD137 agonist mAb could prolong isatuximab-mediated activation of NK lymphocytes and prevent their exhaustion. However, no significant differences were noted (Supplementary Fig. S5A and S5B). As expected, immune suppression of NK lymphocytes induced by proteasome inhibition after pretreatment with bortezomib resulted in lower NK cell viability (Supplementary Fig. S5C).

Because recent findings suggesting that SLAMF7 could be critical for phagocytosis of hematopoietic cells (7) and, coincidentally, some NK lymphocytes co-express SLAMF7 and CD38 with levels above that of RPMI-8226 MM cells (Fig. 6), we investigated whether phagocytosis could also contribute to the depletion of some NK lymphocytes. Accordingly, we labeled increasing numbers of NK lymphocytes with VPD and cultured them with M2-like macrophages, in the presence or absence of isatuximab; the percentage of VPD⁺ macrophages would thus indicate the extent compared with isatuximab alone (Fig. 5B); accordingly, no differences in tumor cell depletion were noted by adding the anti-CD137 mAb (Supplementary Fig. S4A). Equivalent results were found using similar experimental conditions in primary patient bone marrow samples (Supplementary Fig. S4B). We also investigated whether lenalidomide could prolong isatuximab-mediated activation of NK lymphocytes and prevent their exhaustion, by pretreating PBMCs with increasing doses of lenalidomide before culture with RPMI-8226, MM1S and OPM2 MM cells, in the presence or absence of isatuximab. However, no significant differences were noted (Supplementary Fig. S5A and S5B).
of NK-lymphocyte phagocytosis. Interestingly, our results showed a non-significant increment of NK-lymphocyte phagocytosis with isatuximab (P = 0.16; Fig. 5C), suggesting that some NK lymphocytes (possibly those with co-expression of SLAMF7 and CD38hi) could be phagocytosed by M2-like macrophages in the presence of isatuximab.

Discussion

Several anti-CD38 mAbs have been developed for the treatment of MM (28). Thus, in-depth understanding of their MoA is of utmost importance to select patients who, according to their tumor and immune phenotypes, are potential candidates to benefit the most from a given mAb, alone or within rational treatment combinations. Although there is considerable research about the MoA of daratumumab (10, 11, 12–15, 17, 25, 29, 30, 31), preclinical data about other anti-CD38 mAbs in clinical development remain scarce (23, 24). Here, we performed a comprehensive analysis on the MoA of isatuximab in MM, and uncovered a direct association between the levels of CD38 expression and the mechanisms triggered by isatuximab; accordingly, ADCC emerges as the most prevalent effector mechanism by which isatuximab eliminates tumor PCs.

An antigen that can be effectively targeted by a cancer-specific mAb needs to be found on the surface of tumor cells in numbers high enough to trigger one or more effector mechanisms. Here, we showed that continuous exposure to effective concentrations of isatuximab does not result in a decrease of CD38 numbers on the surface of MM cells, which contrasts with daratumumab-induced polar aggregates and release of CD38 in microvesicles almost immediately after antigen–antibody binding in vitro (25). These findings suggest that sequential analyses of antigen saturation and turnover could be valuable to design cost-effective anti-CD38 treatment schedules.

Some mAbs can induce tumor cell death in the absence of immune effector mechanisms; the strength of this effect varies considerably depending on the mAb, the target antigen and the target cell (32, 33). Direct killing by daratumumab (upon cross-linking) and isatuximab (without cross-linking) has been previously reported, which is in contrast with the data shown here. However, such results were obtained in Burkitt’s lymphoma or CD38-overexpressing MM cell lines (i.e., following lentiviral transduction; refs. 10, 23, 24), and it should be noted that, as compared with normal PCs, CD38 density is downregulated in clonal PCs from nearly half of patients with MM (8), to levels that are similar to that of parental (non-transduced) MM cell lines.

Figure 6.
Schematic representation of the different mechanisms of action possibly triggered by isatuximab according to the levels of CD38 expression in normal and tumor cells. We measured, in normal bone marrow samples from healthy individuals (n = 3), the levels of CD38 expression in T-, NK- and B-lymphocyte subsets, normal PCs, distinct myeloid cells and nucleated red blood cells. Using the InfiCyte software (Cytognos; Salamanca, Spain), we merged the FCS files from normal bone marrow samples with those from H929, MM1S, OPM2, and RPMI-8226 cell lines, as well as from bone marrow samples from 13 patients with MM. The levels of CD38 expression (measured with the clone HB7 conjugated with APC-H7; BD Biosciences) are represented by population-band histograms and ordered from the lowest to highest expression detected among normal bone marrow cells, MM cell lines, and primary tumor PCs. Of note, patient MM-01 was studied at relapse after being treated with daratumumab. Levels of CD38 expression required to trigger different MoA induced by isatuximab are represented by dashed lines as a schematic representation in accordance with the results reported in this study.
(Fig. 6). Here, we showed that isatuximab had no effect on viability, proliferation and survival of four different MM cell lines with variable levels of CD38 expression. It was also surprising to see that isatuximab does not induce gene expression changes in MM cells with normal CD38 expression. Functions ascribed to CD38 include receptor-mediated adhesion, signaling events, or bifunctional ectoenzymatic activities that contribute to intracellular calcium mobilization (34), although none of these functions have been actually demonstrated on MM cells. Furthermore, given the end-stage long-lived and quiescent phenotype of bone marrow PCs, it could be hypothesized that rather than signaling, CD38 acts as a marker of adhesion to the bone marrow stroma (35, 36). Interestingly, we demonstrated that tumor cells became more sensitive to the combined administration of bortezomib and dexamethasone when CD38 was blocked by isatuximab; however, those findings appear to be unrelated to a disruption of tumor-stroma cell adhesion. Thus, other functions ascribed to CD38 such as its ectoenzymatic activity should be considered. CD38 together with CD203a (PC-1) and CD73 may flank the CD39/CD73 canonical pathway in the production of adenosine (ADO), which could favor tumor survival (37, 38). Accordingly, Morandi and colleagues (39) unveiled that MM PCs express high levels of ectoenzymes contributing to higher catabolism of ADO, and production of ADO has been demonstrated in cultures of MM and stromal cell lines (25). Notwithstanding, our results suggest that direct apoptosis triggered by isatuximab may only occur in selected patients (i.e., those in which CD38 is expressed at elevated levels).

The ability of a given mAb to fix complement and to induce ADCC is partly dependent on antigen concentration, the orientation of the antigen in the membrane, and if the antigen is present in the surface as a monomer or polymer. CDC can also depend on the mAb isotype and the characteristics of the target cell, including whether the malignant cell expresses complement-regulatory proteins (3). Here, we showed that isatuximab triggered the deposition of C3-related fragments (i.e., a pre-requisite for CDC) in less than half of the MM patient samples included in this study. Although CDC has been previously ascribed as a killing mechanism to daratumumab and isatuximab, this has been demonstrated in Burkitt’s lymphoma cell lines (11, 23) and in primary patient samples without depletion of NK lymphocytes (23, 29, 30); thus, the contribution of ADCC to the killing of MM cells in the later experiments cannot be ruled out, as deposition of C3 was not measured. Furthermore, surface expression of complement inhibitory proteins such as CD46, CD55, and CD59 was not associated with clinical response to daratumumab in the GEN501 and SIRIUS studies (29). Although the results obtained with MM cell lines should be interpreted with caution, our findings suggest that CDC may occur only in selected patients (i.e., those in which CD38 is expressed at elevated levels).

mAbs can induce cytotoxicity by binding to FcRs, which are expressed by a variety of immune effector cells, including NK lymphocytes as well as macrophages (i.e., ADC and ADCP, respectively; ref. 3). ADCP triggered by daratumumab and isatuximab has also only been demonstrated against Burkitt’s lymphoma cell lines (12, 23) and in primary patient samples without depletion of NK lymphocytes (12). Here, we showed that isatuximab only induced significant ADCP in CD38+ MM cells. By contrast, the extent of ADCC was significant in all but H929 cells (the one with the lowest anti-CD38 SABC), and detectable against tumor PCs in most patient samples (11 out of 13, Fig. 4B). Interestingly, the extent of ADCC triggered by isatuximab against primary tumor PCs (median of 51%) was similar to that reported for daratumumab in other preclinical studies (11, 13, 17, 30), suggesting that in the event of different efficacy between both anti-CD38 mAbs, such difference would be related to mechanisms other than ADCC.

It has been reported that daratumumab depletes CD38-ve immune regulatory cells which have substantially lower MFI levels of CD38 as compared with normal and tumor PCs as well as MM cell lines (Fig. 6; ref. 14). These observations are surprising because daratumumab (refs. 40, 41; or isatuximab; ref. 22) has not shown significant hematologic toxicity, suggesting that most mechanisms triggered by these drugs require high CD38 density and spare all other cells with considerable lower MFI levels of CD38 (14). Here, we demonstrated that, although isatuximab significantly killed primary tumor PCs, it also depleted B-lymphocyte precursors and basophils. The density of CD38 in B-lymphocyte precursors and basophils are the second and third highest (after PCs) among all bone marrow cells (Fig. 6), and overlaps with that found in immature/transitional B lymphocytes (42, 43), which are also depleted by daratumumab (14). Altogether, these data suggest that anti-CD38 mAbs can target B lymphocytes with elevated levels of CD38, but although this may be valuable if these cells are immune suppressive (14), it could also delay the regeneration of mature B lymphocytes (including normal PCs and normal immunoglobulin production; ref. 44). By contrast, and as expected on the basis of the observation that isatuximab had no effect on H929 MM cells (Fig. 6), other hematopoietic cells with CD38 MFI levels below the former were not depleted by the drug. Treg cells were depleted by isatuximab in our experimental conditions which supports the findings of Krejcik and colleagues (14) that only rare CD38-ve and not CD38-ve Tregs were eliminated during treatment with daratumumab. Similarly, there were no significant differences in the percentage of neutrophils and monocytes after exposure to isatuximab, consistent with the low levels of CD38 expression found in these cell types (Fig. 6). Because daratumumab induces release of CD38 from the surface of MM and other CD38-ve hematopoietic cells (15, 25, 29), it is uncertain if the drug depletes CD38-ve immune regulatory cells, or if these cells become undetectable after release of CD38 from their surface (in this case, monitoring of CD38 in CD38-ve cells such as a positive control may be useful). Indeed, the EuroFlow uses a multiparameter anti-CD38 antibody and simultaneous surface plus intracellular staining to measure CD38 expression during MRD assessment (9). That notwithstanding, it cannot be excluded that other mechanisms beyond cell depletion induced by anti-CD38 mAbs can lead to decreased levels of immune suppressive cells as well as enhanced adaptive immunity (e.g., immune modulation after robust lymphocyte activation). Altogether, these findings urge for in-depth and longitudinal monitoring of patients with MM (i.e., from baseline to MRD and eventually disease progression) to fully understand both tumor and immune cell mechanisms behind the response and resistance to anti-CD38 mAbs.

Another interesting finding of this study was that, whereas NK-lymphocytes emerged as the most relevant immune subset for the efficacy of isatuximab, anti-CD38 mAbs may induce their depletion through exhaustion and, eventually, CD38/SLAMF7-mediated phagocytosis (7). These results, if applied more broadly, could help to explain the rapid decrease in absolute counts of NK lymphocytes after the first infusions of daratumumab in patients enrolled into the GEN501, GEN503 and SIRIUS...
studies (14, 15). Accordingly, drugs able to enhance or prolong the functionality of NK lymphocytes (e.g., lenalidomide; refs. 20, 22; or pomalidomide; ref. 19) emerge as attractive partners to combine with anti-CD38 mAbs. On the basis of the upregulation of CD137 expression on NK lymphocytes following CD16 ligation to isatuximab, we explored whether the combination of anti-CD38 plus anti-CD137 mAbs could increase activation and prolong the lifespan of NK lymphocytes; however, our results were not comparable to those reported in lymphoma with CD137 stimulation and anti-CD20 mAbs (45). Similarly, we found that pretreatment with lenalidomide did not increase isatuximab-mediated activation of NK lymphocytes or prevented their exhaustion and death. Thus, further investigations are warranted to assess if immunotherapies other than immunomodulatory drugs can potentiate ADCC, as well as to define if there is a correlation between the absolute counts of NK lymphocytes and patients’ response to anti-CD38 mAb therapy.

Greater knowledge about the immune effector mechanisms of anti-CD38 mAbs is a prerequisite for better prediction of patients’ response and optimal monitoring of treatment effects. Here, we performed a comprehensive analysis about the MoA of isatuximab in MM; our results underline similarities and differences between isatuximab and other anti-CD38 mAbs. They also provide information that could explain, at least in part, why patients with lower expression of CD38 may respond poorly to these drugs compared with patients with higher CD38 expression. We also provide new insight about the paradoxical depletion of NK lymphocytes following treatment with anti-CD38 mAbs; further development of therapeutic strategies to overcome this phenomenon should become a priority.

Disclosure of Potential Conflicts of Interest

P. Rodriguez-Otero reports receiving speakers bureau honoraria from Celgene, Janssen, and Bristol-Myers Squibb, and is a consultant/advisory board member for Celgene, Janssen, and Takeda. C. Panizo reports receiving speakers bureau honoraria from Roche Pharma, Janssen, and Bristol-Myers Squibb, and is a consultant/advisory board member for Takeda and Janssen. J.F. San-Miguel is a consultant/advisory board member for Takeda, Janssen, Sanofi, Bristol-Myers Squibb, Amgen, Novartis, and Celgene. B. Paiva reports receiving speakers bureau honoraria from Celgene, is a consultant/advisory board member for Amgen, Celgene, Janssen, Karyopharm, Takeda, and Sanofi, and reports receiving commercial research support from Sanofi, Celgene, and Takeda. No potential conflicts of interest were disclosed by the other authors.

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The Mechanism of Action of the Anti-CD38 Monoclonal Antibody Isatuximab in Multiple Myeloma

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