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# CD200 immune checkpoint expression is associated with inferior outcome in multiple myeloma patients treated with anti-CD38 monoclonal antibodies

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**ABSTRACT**

In multiple myeloma (MM), the anti-CD38 monoclonal antibody Daratumumab has become essential in the therapeutic arsenal, although very few predictive factors of response to Daratumumab have been identified in clinical studies. We have prospectively collected biological data from 97 patients treated with Daratumumab in first line or at relapse in our center between 2016 and 2020. These data included multiparameter flow cytometry phenotype (CD200, CD117, CD56, CD38, CD45, and CD27), cytogenetic, and transcriptomic gene expression profiling (GEP) of tumor plasma cells before treatment with Daratumumab. We first looked for predictive factors of response to Daratumumab. We found that high CD56 expression and CD45 expression were significantly associated with better progression free survival (PFS) whereas high CD200 expression was significantly associated with poorer PFS. Then, we showed that the CD200-CD200R immune synapse is responsible for a decrease in Daratumumab response through the alteration of NK cells' activity. Finally, we demonstrated that inhibition of CD200 increase response to Daratumumab in MM patient samples, highlighting its potential as a predictive biomarker for Daratumumab response and as a possible therapeutic target in combination with Daratumumab. This study is the first to identify phenotypic and molecular factors' predictor of response to Daratumumab.

**KEY POINTS**

(1) CD200 expression on tumor plasma cell is associated with poorer progression free survival.
(2) CD200-CD200R axis is responsible for a decrease in anti-CD38 response through the alteration of NK cell activity.

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## Introduction

Multiple myeloma (MM) is the second most common hematological malignancies after non-Hodgkin lymphoma with more than 100 000 case per year worldwide,[1] defined by abnormal plasma cells' accumulation in the bone marrow (BM). MM is characterized by high molecular and clinical heterogeneity, making it difficult to treat and incurable. The overall survival (OS) of MM patients has been significantly improved during the last decade with the introduction of new therapies, especially immunotherapies. Daratumumab is a human monoclonal immunoglobulin G1 Kappa (IgG1) that targets CD38. It was the first monoclonal

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antibody approved for the treatment of relapsed/refractory MM, leading to a major improvement in outcome and opening the way to the development of immunotherapy in this pathology.[2,3] CD38 is a transmembrane glycoprotein that can act as an ectoenzyme but also as a receptor.[4] CD38 is ubiquitously expressed in the human body, particularly in mature lymphoid cells (T, B, and natural killer (NK)) and myeloid cells.[5] The role of CD38 in immune cells ranges from modulating cell differentiation to effector functions during inflammation, where CD38 may regulate cell recruitment, cytokine release, and NAD availability.[4]

In MM, Daratumumab mechanisms of action are mainly triggered by its Fc region: induction of complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP) against MM cells, but it also triggers tumor plasma cells apoptosis induction via cross-linking.[6] In addition, Daratumumab has been described to have immunomodulatory effects related to the elimination of CD38-positive immune suppressor cells, such as regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells.[7–9] Daratumumab was first approved in MM as monotherapy in 2016, for patients with relapsed or refractory disease.[10] It was then approved in several combinations for relapsed and refractory patients.[11,12] It finally became available in first line of treatment.[11,12] Daratumumab shows highly variable inter-individual activity and treated patients eventually relapse with several mechanisms of resistance that have been identified. First, through loss of CD38 expression by MM cells during treatment. Second, via immunomodulatory effects such as the CD47 overexpression by MM cells that will inhibit the ADCP in a mouse model,[13,14] the high CD55 and CD59 expression that protects MM cells against CDC,[15] or the depletion of NK cells following Daratumumab infusion, through fratricide ADCC activation.[15] Finally, it is noticed that the BM microenvironment is also involved in Daratumumab resistance due to the interaction between MM cells and BM stem cells. The latter will induce overexpression of the anti-apoptotic protein surviving in MM cells, allowing them to escape Daratumumab-induced apoptosis.[16] In this context, the identification of Daratumumab efficiency predictive markers is of first interest. Still, to date, predictive markers such as low expression of CD38[15,17] or FcRIIIa and FcRIIb polymorphisms[18] need confirmation.

Our objective was to identify predictive response factors to Daratumumab through clinical, phenotypic, karyotypic, and gene expression profiling (GEP) parameters and validate them functionally. To this end, we studied a biologically documented cohort of MM patients treated with Daratumumab.

## Patients & methods

### *Patients*

Patients provided informed consent for the study, which was in accordance with the principles of the Declaration of Helsinki. This study is monocentric, with prospective biological data collection and retrospective clinical data collection. It included MM patients (as defined by International Myeloma Working Group (IMWG) criteria)[19] from Montpellier University Hospital who had received at least one cycle of Daratumumab in first line or at relapse and who had BM or blood multiparametric flow cytometry analyses of tumor plasma cells prior to the initiation of Daratumumab. Details on patients included can be found in the supplementary methods.

### *Flow cytometry*

To discriminate between abnormal and normal plasma cells, we used conventional surface markers CD138 (PE), CD38(-PerCP-Cy5.5), CD45(–BUV421), CD56(–BUV 395), CD19(PE-CF594), cytoplasmic (–CF594) and (–FITC) immunoglobulin light chain, and CD20(APC-Cy7), CD27(–BV786), CD117(-PerCy-7) and CD200(–APC) surface markers were added to the flow panel. We used a single multiparametric panel with 11 fluorescence channels. For this study, we collected CD38, CD200, CD56, CD45, CD27, and CD117 expression on tumor plasma cells at myeloma diagnosis when it was available and closest to diagnosis when it was not available, always before Daratumumab infusion. When available, we also collected plasma cell phenotypes at relapse just before initiation of Daratumumab. Details on flow cytometry data can be found in the supplementary methods.

### Study endpoint and statistical analysis in the cohort

The primary endpoint was to identify predictive factors of response to Daratumumab in MM by examining clinical, phenotypic, karyotypic, and GEP parameters. The supplementary methods provide details on statistical analyses.

### Research of factors predicting response to Daratumumab

We looked for factors predicting response to Daratumumab on clinical, therapeutic, and biological qualitative and quantitative variables, including phenotypic markers (details can be found in the supplementary data). The two groups of "responders" and "non-responders" were compared for the level of expression of Affymetrix U133 plus 2.0 probe-sets using Gene Set Expression Analysis (GSEA). The genes that had significantly different expression between the two groups (either differentially enriched or repressed) were picked up. Details on determining factors predicting response to Daratumumab can be found in the supplementary methods.

### Human myeloma cell lines (HMCLs) and ADCC assay

LP1 cell line was purchased from DSMZ (Braunsweig, Germany). XG-1 was generated in the laboratory as previously described.[20] XG-1-CD200+ and LP-1-CD200+ are two HMCLs engineered in the lab to overexpress CD200. HMCLs were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Eurobio, Les Ulis, France) and interleukin 6 (IL6, Peprotech, Rocky Hill, New Jersey, USA) for XG cell lines, and maintained at 37°C with 5% CO2. The ADCC was assessed after 24 hours using flow cytometry as described previously.[21] Details on human myeloma cell lines and primary MM samples used in this study can be found in the Supplementary Methods.

### Availability of data

Additional data are available from the corresponding author upon reasonable request.

## Results

### Patient characteristics and outcomes

A total of 97 patients suffering from MM were included. The diagnosis was made between 1995 and 2019, and Daratumumab was started between 2016 and 2020. The median age at diagnosis was 63 years (range 40–82 years). Only 3% of patients had plasma cell leukemia at diagnosis. Demographics and baseline disease characteristics are presented in Table 1. Most patients received Daratumumab in relapse (94%). The median number of previous lines was 3 (range 0–7). Overall, 81% patients had already received an immunomodulator (IMiD) and a proteasome inhibitor (IP). Overall, 57% patients had received an autologous stem cell transplant (ASCT) before treatment with Daratumumab. Daratumumab was given in combination with lenalidomide in 24% of patients, with bortezomib in 15%, with pomalidomide in 5%, and with both in 3% of cases, 48% of patients were treated in monotherapy. The median number of cycles administered was 7 (range 1–7).

The response status is missing for one of the 97 patients. The analyses regarding response subgroups were conducted on 96 patients. The overall response rate (ORR) for the whole cohort was 60.4%: 21.8% of patients obtained partial response (PR), 31.3% very good PR, and 7.3% complete response (CR). Overall, 39.5% patients were considered non-responders (stable disease or progressive disease). Clinical and biological characteristics of responders and non-responders are presented in Table 2. After a median follow-up of 26.4 months (95% CI 16.8–39.3), the median PFS of the whole cohort was 8 months (95% CI 5.4–13.5) (Figure 1a). Median PFS was 3.0 months (95% CI 2.5–5.3) for non-responders and 16.1 months (95% CI 11.8–43.2) for responders (Figure 1b). The median OS of the whole cohort was 38.1 months (95% CI 22.1-NR) (Supplementary Figure S1A). Median OS was 9.7 months (95% CI 6.71–22.3) for non-responders

**Table 1.** Baseline demographics and clinical characteristics.

| Characteristics | *N* = 97(%) |
|---|---|
| Age at diagnosis, yr (%) | |
| median, yr | 63 years |
| <65 yr (*n*,%) | 56 (58) |
| Age at Dara start, yr median | 68 years |
| Sexe, n (%) | |
| Male | 59 (61) |
| ISS disease stage at diagnosis, *n* (%) | |
| I | 38 (39) |
| II | 27 (28) |
| III | 27 (28) |
| Plasma cell leukemia at diagnosis, *n* (%) | 3 (3) |
| Renal failure according to IMWG (creatinine ≥177 µmol/L), n (%) | 19 (20) |
| Bone lesion at diagnosis | 67 (69) |
| Anemia at diagnosis (Hb < 10 g/dl) | 26 (27) |
| Hypercalcemia at diagnosis (calcemia ≥2.6 mmol/L) | 26 (27) |
| Type of multiple myeloma, *n* (%) | |
| Ig G | 56 (58) |
| Ig A | 20 (21) |
| Light chain myeloma | 19 (20) |
| Cytogenetic profile n/total *n* (%) | |
| t(4;14) | 12/75 (16) |
| del(17p) | 8/38 (21) |
| del(1p) | 6/22 (27) |
| gain(1q) | 5/20 (25) |
| Number of previous lines of therapy | |
| median | 3 |
| Previous therapy, *n* (%) | |
| Autologous stem cell transplantation (ASCT) | 55 (57) |
| Hematopoietic stem cell transplantation (HSCT) | 17 (18) |
| Bortezomib | 89 (92) |
| Carfilzomib | 2 (2) |
| Ixazomib | 10 (10) |
| Lenalidomide | 81 (84) |
| Pomalidomide | 52 (54) |
| At least one proteasome inhibitor and immunomodulatory drug | 79 (81) |
| Use of Daratumumab in combination, *n* (%) | |
| lenalidomide | 23 (24) |
| bortezomib | 15 (15) |
| pomalidomide | 5 (5) |
| both (proteasome inhibitor and immunomodulatory drug) | 3 (3) |
| Use of Daratumumab in monotherapy, *n* (%) | 47 (48) |
| Cycles of Daratumumab | |
| median | 7 |

and not reached for responders (Supplementary Figure S1B). As expected, response to Daratumumab was significantly associated with prolonged PFS ($p < 0.0001$, Figure 1b) and OS ($p < 0.0001$, Supplementary Figure S1B). PFS and OS according to the level of response to Daratumumab are presented, respectively, in Figure 1c and Supplementary Figure S1C.

### *Daratumumab outcomes were significantly associated with CD200, CD45, and CD56 expression*

We first performed a MaxStat analysis of phenotypic markers (CD200, CD45, CD117, CD27, CD56, and CD38) to identify their expression cutoff correlated with PFS. We thus found that high CD56 expression (≥95%) and high CD45 expression (≥50%) were significantly associated with better PFS (Figure 2a) ($p = 0.029$ and $p = 0.0159$, respectively). In contrast, CD200 high expression (≥95%) was significantly associated with lower PFS ($p = 0.0238$). CD27 expression, CD117 expression, and CD38 MFI were not significantly associated with PFS ($p = 0.17$, $p = 0.52$, and $p = 0.13$, respectively) (data not shown). COX regression model performed on these samples for CD45, CD56, and CD200 finally confirmed that CD200 high (≥95%), CD56 high (≥95%), and CD45 (≥50%) were independent prognostic markers in univariate ($p = 0.02$, $p = 0.04$, and $p = 0.01$, respectively) and multivariate analysis ($p = 0.001$, $p = 0.02$, and $p = 0.02$, respectively) (Supplementary Table S1). We then created a phenotypic-based survival score based on CD200, CD45, and CD56 expression that could classify patients into three distinct survival groups according to their expression level of CD200, CD45, and CD56 (Figure 2a). Better outcomes were demonstrated in patients with low

**Table 2.** Clinical and biological characteristics of responders and non-responders.

| Characteristics | Responders $N = 58$(%) | Non-responders $N = (38\%)$ | $p$-value (uunivariate logistic regression) |
|---|---|---|---|
| Male | 33 (57) | 25 (66) | 0.38 |
| Median age at diagnosis | 63 | 63 | |
| Median number of previous lines | 2 | 3 | |
| Isotype | | | |
| IgG myeloma | 30 (52) | 25 (66) | *ref* |
| IgA myeloma | 15 (26) | 5 (13) | *0.11* |
| Light chain myeloma | 12 (21) | 7 (18) | *0.51* |
| Bone lesions at diagnosis | 41 (71) | 25 (66) | 0.80 |
| Anemia at diagnosis (Hb < 10 g/dl) | 15 (26) | 11 (29) | 0.67 |
| Renal failure at diagnosis (creatinine ≥177 µmol/L) | 13 (22) | 6 (16) | 0.46 |
| Hypercalcemia at diagnosis (calcemia ≥2,6 mmol/L) | 17 (29) | 9 (24) | 0.52 |
| ISS score at diagnosis | | | |
| 1 | 22 (41) | 16 (43) | *ref* |
| 2 | 17 (31) | 10 (27) | 0.68 |
| 3 | 15 (28) | 11 (30) | 0.99 |
| Prior autologous stem cell transplantation | 30 (52) | 25 (66) | 0.18 |
| Prior treatment with Bortezomib | 50 (86) | 38 (100) | 0.99 |
| Prior treatment with Lenalidomide | 43 (74) | 37 (97) | 0.02 |
| Use of dara in first or 2$^{nd}$ line | 26 (45) | 1 (3) | <0.01 |
| Use of dara in combination | 38 (66) | 12 (32) | <0.01 |
| Cytogenetics data | | | |
| del(17p) | 2/24 (8) | 6/14 (43) | 0.02 |
| t(4;14) | 7/48 (15) | 4/26 (15) | 0.93 |
| Molecular classification | | | |
| CD1 | 3/43 (7) | 1/23 (4) | *ref* |
| CD2 | 6/43 (14) | 4/23 (17) | 0.60 |
| HY | 10/43 (23) | 3/23 (13) | 0.94 |
| LB | 10/43 (23) | 3/23 (13) | 0.94 |
| MF | 2/43 (5) | 2/23 (9) | 0.47 |
| MS (bad prognosis) | 6/43 (14) | 3/23 (13) | 0.77 |
| PR (bad prognosis) | 6/43 (14) | 7/23 (30) | 0.33 |
| PCLI ≥1% | 18 (31) | 10 (30) | 0.94 |

CD200 and high CD45 and CD56 expression. In contrast, high CD200 and low CD45 and CD56 expression were associated with a bad prognosis in this cohort of patients treated with Daratumumab (Figure 2b).

Then, we tested clinical, biological, and genetic markers that could predict response to Daratumumab. In univariate analysis, four variables were significantly associated with a better response to Daratumumab with a p-value <0.05: prior treatment with lenalidomide ($p = 0.02$), use of daratumumab in the first or second line ($p < 0.01$), use of daratumumab in combination ($p < 0.01$) and del17p ($p = 0.02$) (Table 2). In multivariable logistic regression, the following parameters were significantly associated with better response to Daratumumab: use of Daratumumab in the first or second line ($p = 0.018$) and expression of CD56 on tumor cells ≥95% ($p = 0.003$) (Supplementary figure S2). The expression of CD200 was not included in this multivariate analysis because, although it was associated with PFS, it was not significantly linked to response in the univariate comparison.

Finally, we evaluated if the GEP could give an insight into the response to Daratumumab, and we performed a GSEA analysis on 36 patients with material, divided into 24 "responders" and 12 "non-responders." Interestingly, inflammatory response genes, genes encoding for extracellular matrix, polycomb repressive complex 2 (PRC2) polycomb target genes, and H3K27me3 target genes were significantly enriched in "responders" versus "non-responders" (Figure 2c).

## *CD200 expression on tumor MM cells affects NK cell population distribution*

Based on our previous observation, we aimed to get a better insight into the functional role of CD200 association with poor prognosis when treated with Daratumumab. The ligation of CD200 to its receptor CD200R induces an immunosuppressive signal, inhibiting T cell immune response[22] and NK cytotoxicity.[23] In addition, CD200 has been described as a prognostic factor associated with a bad prognosis in MM.[24] First, we analyzed the NK cells' sub-populations in MM patients with CD200$^{low}$ ($n = 5$) and CD200$^{high}$ ($n = 25$) phenotypes at diagnosis. The five populations of NK cells were distinguished based on their CD56 and

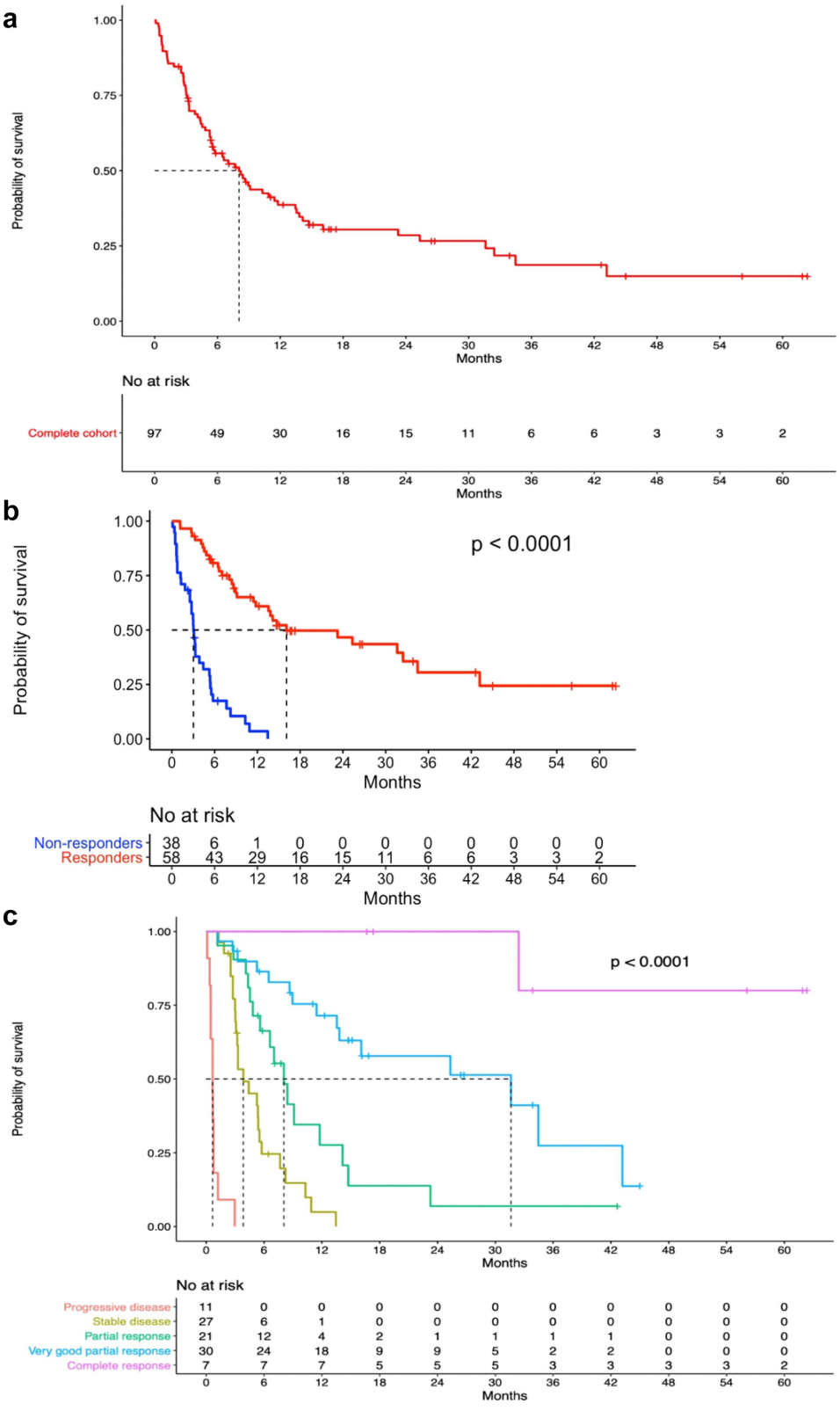


**Figure 1.** Progression free survival (a) PFS of the whole cohort. *the median follow-up was 26.7 months (95% CI 16.6–45.0) and), median PFS of the whole cohort was 8.1 months (95% CI 5.4–13.5)*. (b) PFS of responders and non-responders to Daratumumab. *median PFS was 3.0 months (95% CI 2.5–5.3) for "non-responders" and 16.1 months (95%CI 11.8–43.2) for "responders."* (c) PFS according to the level of response to Daratumumab. *median PFS was 0.7 months (95% CI 0.461-NR) for progressive disease, 3.0 months (95% CI:3.1–5.8 months) for stable disease, 8.1 months (95% CI 5.6–14.7) for partial response, and 31.6 months (95% CI 13.8-NR) for very good partial response. The median PFS was not reached for stringent complete response..*

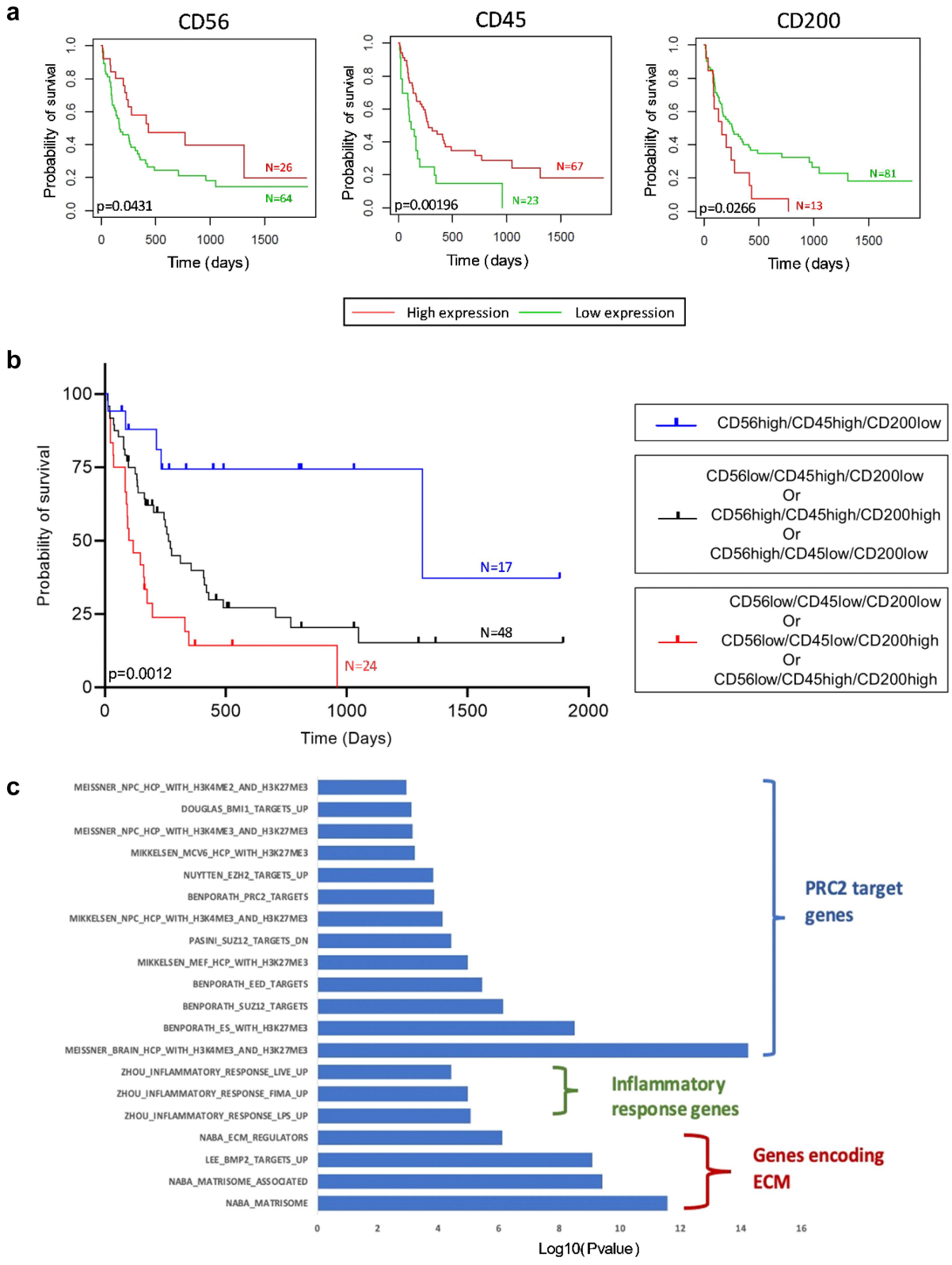


**Figure 2.** Analysis of phenotypic markers expression and their relation with PFS (a) Prognosis value of CD56 (left panel), CD45 (middle panel) and CD200 (right panel). (b) PFS according to phenotyping score. Score 1, blue line: CD56 high (≥95%), CD45 high (≥50%) and CD200 low ( <95%). Score 2, black line: CD56low ( <95%)/CD45high/CD200low or CD56high/CD45high/CD200high (≥95%) or CD56high/CD45low ( <50%)/CD200low. Score 3, red line: CD56low/CD45low/CD200low or CD56low/CD45low/CD200high or CD56low/CD45high/CD200high. (c) GSEA analysis performed on 36 patients (24 responders and 12 non-responders). Only the significant signaling pathways are represented (FRD q ≤ 0,05) as log10 (p value).

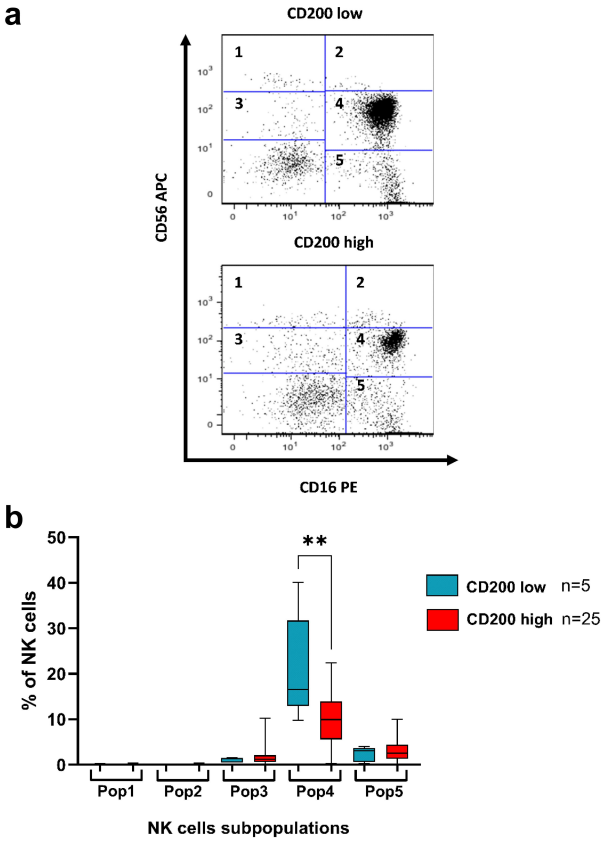


**Figure 3.** CD200 phenotype on MM primary cells influence NK cells subsets frequency on MM patients' samples (a) NK cells populations assessed by flow cytometry using anti-CD56-APC and anti-CD16-PE antibodies. (b) Comparison of NK cells populations percentages between patients with CD200low and CD200high phenotype and highlight a decrease in NK cells pop4% in CD200high group of patients. CD200 high expression was determined if more than 15% of the tumor plasma cells expressed CD200 on their cell surface.

CD16 expression that determined the activity of the NK cells, with $CD56^{dim}$ $CD16^{high}$ population (pop 4) representing the population of activated NK cells[23] (Figure 3a).

Interestingly, we observed a significantly lower percentage of activated NK cells (10.1%) in the $CD200^{high}$ MM patients compared to $CD200^{low}$ MM patients (21.2%, $p = 0.003$) (Figure 3b), suggesting a link between the CD200 expression on tumor cells and the effector NK cells frequency within the BM. Furthermore, using RNA-seq data of the non-tumor BM fraction from 112 patients, we explored the correlation between *CD200* expression and the abundance of immune cell subpopulations within these samples. Using the CIBERSORTx suite,[25] we estimated the immune cell type abundance in bulk RNA-seq from MM BM samples (Supplementary Figure S3). Of interest, we identified a significantly lower abundance of monocytes and macrophages in the BM of $CD200^{high}$ compared to $CD200^{low}$ MM patients. Altogether, these data underlined a significant deregulation of the immune microenvironment in $CD200^{high}$ MM patients.

In an effort to initiate mechanistic investigations into the downstream signaling pathways of CD200-CD200R interaction in MM cells, we compared gene expression profiling of purified malignant plasma cells with high and low CD200 expression from a cohort of 208 MM patients. Notably, GSEA analysis revealed significant overexpression of genes that were enriched in polycomb PRC2 target genes, the PI3K pathway, and MAPK pathway signaling (Supplementary Figure S4).

## *High CD200 expression significantly reduces anti-CD38 activity* in vitro *and* ex vivo

To better understand the role of CD200 in response to Daratumumab treatment, we used two parental and two CD200 overexpressing MM cell lines (XG-1/XG-1CD200+, LP-1/LP-1 CD200+) that showed similar CD200 ectopic expression than observed in tumor plasma cells from MM patient (Supplementary Figure

S5A). While CD200 overexpression was different between the cell lines (measured with the Antibody Binding Capacity (ABC) technique), overexpressing cell lines (XG-1CD200+, LP-1CD200+) and parental cell lines (XG-1, LP-1) showed similar CD38 cell surface expression (Supplementary Figure S5B). These HMCLs were treated with Daratumumab (1 µg/ml) or Isatuximab (1 µg/ml) in the presence of NK cells or PBMC purified from healthy donors as effector cells. Interestingly, high CD200 expression significantly decreases the ability of the two anti-CD38 therapeutic antibodies to induce ADCC on the HMCLs in presence of NK effector cells that were shown to express CD200R (data not shown). XG-1 and LP-1 cell lines were more sensitive to anti-CD38 (mean of cell lysis of 39.5% and 41.3%, respectively) than XG-1 CD200+ and LP-1 CD200+ cell lines (mean of cell lysis of 14.7% and 18%, respectively) ($p < 0.05$) (Figures 4a,b).

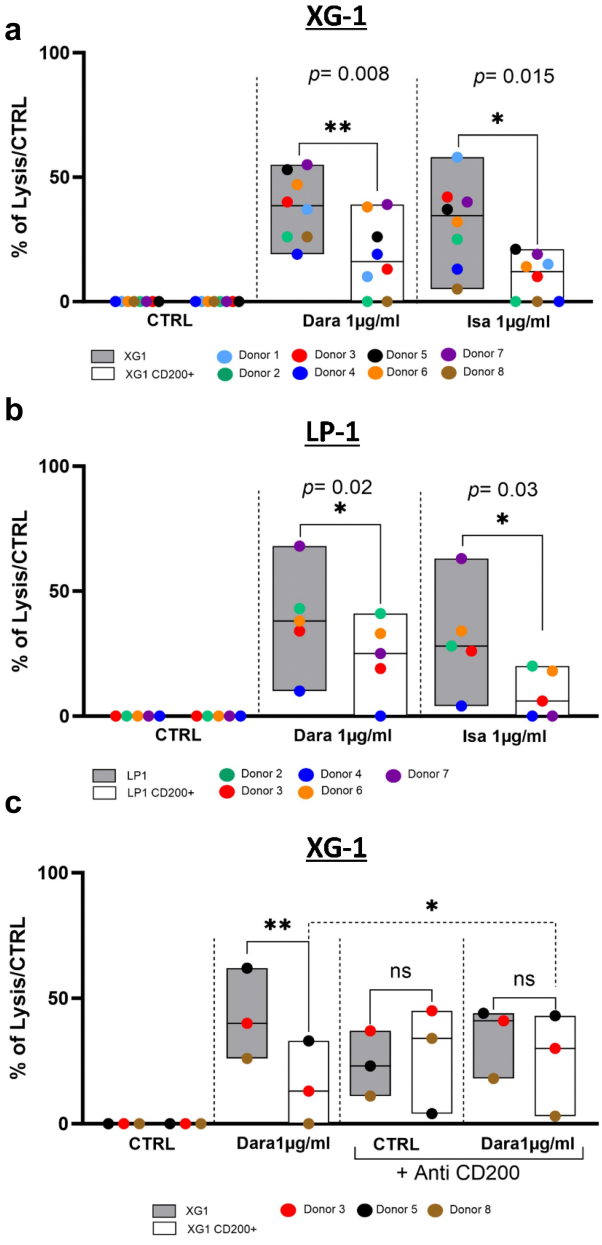


**Figure 4.** CD200 overexpression reduce HMCL sensitivity to anti-CD38 monoclonal antibodies in presence of NK effector cells (a) and (b) representation of HMCL (LP-1/LP-1 CD200+ and XG-1/XG-1 CD200+) lysis induced by 1 µg/ml of anti-CD38 monoclonal antibodies (Daratumumab or isatuximab) in vitro in the presence of natural killer from healthy donors as effector cells. (c) The blocking of CD200-CD200R axis rescue the activity of Daratumumab on MM cell lines with CD200high phenotype. The comparison of XG-1 and XG-1 CD200+ cell lysis co-cultured with NK cells, preincubated with 0.125 µg/ml of anti-CD200 antibody and treated with 1 µg/ml of Daratumumab.

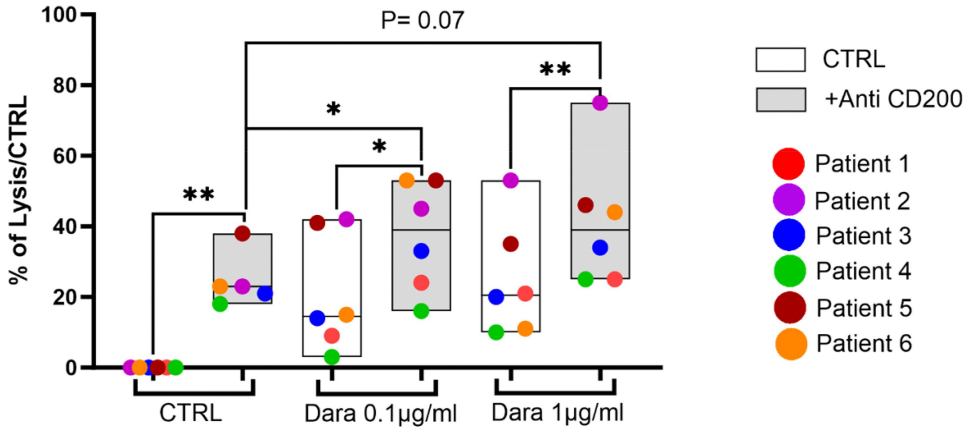


**Figure 5.** The inhibition of CD200 improves CD200high MM patient samples response to Daratumumab treatment. 48 h of daratumumab treatment (0.1 µg/ml and 1 µg/ml) with anti-CD200 antibody demonstrate a better cell lysis than daratumumab alone in 6 MM patient samples ($p < 0.01$).

Similar significant results were observed in the presence of PBMC effector cells (Supplementary Figure S6A and B). Altogether, these results suggest that CD200 expression on target cells negatively impacts their ADCC lysis.

To further confirm the role of CD200, we next performed an ADCC experiment on the XG1 and XG1-CD200+ models in the presence of Daratumumab 1 µg/ml and a first anti-CD200 antibody (BD; Biosciences) to block the CD200-CD200R immune checkpoint axis. Remarkably, while in the presence of Daratumumab only, we assessed an expected decrease in the lysis of CD200 overexpressing cells, the addition of anti-CD200 led to similar ADCC lysis in both XG1 and XG1-CD200+ cells, demonstrating that blockade of the CD200-CD200R checkpoint can rescue the ADCC lysis induced by Daratumumab in the XG-1 CD200+ cell line (Figure 4c). Finally, we tested the role of CD200 on MM cell response to anti-CD38 using CD200+ primary MM samples isolated from patients ($n = 6$) (Figure 5). Primary MM tumor cells were cultured in the presence of their microenvironment and treated with two concentrations of Daratumumab (0.1 µg/ml and 1 µg/ml) for 48 h. In parallel, duplicates of the same conditions were supplemented with 0.5 µg/ml of anti-CD200 antibody to blockade the CD200-CD200R axis. After 48 h of Daratumumab treatment, we observed a dose-dependent tumor cell lysis. Interestingly, despite a lysis effect of the anti-CD200 alone on tumor cells, the addition of anti-CD200 antibody to Daratumumab treatment significantly increased and improved the tumor cells' lysis mediated by Daratumumab, therefore confirming our previous observation.

Finally, we included analyses of T cell exhaustion and macrophage polarization markers. We conducted additional experiments in which five primary myeloma cells were cultured within their microenvironment for 48 hours with or without Daratumumab (1 µg/ml) and Samalizumab (0.2 µg/ml), another anti-CD200 antibody (detailed Methods in Supplemental Material). These experiments demonstrated a statistically significant improvement in lysis induced by Daratumumab at 1 µg/ml in co-treatment with anti-CD200 (Samalizumab 0.2 µg/ml), further confirming our previous results (Supplementary Figure S7A). However, we did not observe any effect of anti-CD200 treatment on macrophage polarization; we found the same quantity of M1 macrophages (CD86+ cells) and M2 macrophages (CD206+ cells) with or without Samalizumab treatment (Supplementary Figure S7B and C). We also analyzed the effects of Daratumumab and anti-CD200 antibodies on the T cell subpopulations. The only significant difference identified revealed a higher percentage of CD8 T cells PD1+ in the combo or anti-CD200 treated cells compared to the control (Supplementary Figure S7D).

## Discussion

Here, we describe a new series of 97 highly documented patients, both at the clinical and biological level, who received Daratumumab in their treatment history. We identified CD200 as a marker that could predict

the response to Daratumumab, validated the role of CD200 in ADCC induced by Daratumumab, and demonstrated that high expression of CD200 reduces the efficacy of Daratumumab.

Other studies described cohort of Daratumumab treatment but usually to assess the efficiency of this treatment used in different combination therapies[26,27] or on various classes of patients[28] rather than to identify new prognosis factors. We used cytometry data with transcriptomic and treatment history to create an original Daratumumab cohort. The multiparameter phenotyping of minimal residual disease by flow cytometry in MM is known to give prognostic value on PFS and OS.[29–31] However, there is a high degree of heterogeneity within tumor plasma cell phenotypes. The correlation between the amount of CD38 surface levels on myeloma cells and response to Daratumumab treatment remains controversial.[15,17] Our results suggest that even a low expression of CD38 is sufficient for Daratumumab activity on MM plasma cells. Since the MFI of CD38 expression in our cohort was not significantly associated with response to Daratumumab.

CD200 has been shown to be a negative prognosis marker in MM using transcriptomic data, as patients with CD200 expression showed a significantly lower event-free survival (EFS),[24] and its upregulation in MM progression is associated with regulatory T cell expansion.[32] In the present study, we demonstrated using flow cytometry data that high surface expression of CD200 (95% of tumor plasma cells) is associated with poorer PFS, also in patients treated with Daratumumab. CD200 is a type 1 glycoprotein expressed in B, T, endothelial, neuronal, and ovarian cells. The receptor of CD200 is found on the surface of a subset of T cells, and myeloid/monocyte lineage cells, and interaction between CD200 and CD200R induces suppression of T cell-mediated immune responses,[33] thus acting like an immune checkpoint. In AML and CLL, CD200-CD200R interaction has been described as a regulatory axis targeting the number and the activity of NK and T-cells,[23,34] impacting anti-tumor response. Moreover, a recent study demonstrated that monocytes from MDS patients highly express CD200, and the interaction between CD200 (expressed on monocytes) and CD200R (expressed on NK cells) inhibits NK cells' antitumoral function.[35] This function could explain the CD200 prognosis capacity in our Daratumumab cohort. Our observation on the more active NK phenotype in CD200 low patients and our *in vitro* results on HMCL ADCC-mediated lysis favor an immune checkpoint role in MM and further highlight CD200 as a predictive biomarker for Daratumumab treatment outcome. Interestingly, we also observed a potential additional effect of anti-CD200 and Daratumumab in primary patient samples. A CD200 humanized monoclonal antibody, Samalizumab,[33] is currently in development. It mainly acts by preventing the binding of CD200 to its receptor, thus indirectly activating the lymphocyte-mediated immune response. We also analyzed the effects of Daratumumab and two anti-CD200 antibodies (one from BD, and Samalizumab) on the T cell subpopulations and macrophage polarization. The only significant difference identified revealed a higher percentage of CD8 T cells PD1+ in the combo or anti-CD200 treated cells compared to the control. Inhibitory properties of PD-1 receptor engagement on activated T cells are well established in physiologic and pathological contexts. Nonetheless, it is also established that PD-1 expression on antigen-specific T cells reflects the functional avidity and anti-tumor reactivity of these T cells.[36] Since no significant differences in TIM3 or LAG3 positive T cells were identified, the upregulation of PD1+ CD8+ T cells could be related to the fact that the level of PD-1 expression is also associated with the strength of TCR signaling, and thus to the functional avidity of specific T cells.[37] Finally, it has recently been shown that the CD200-CD200R axis can be exploited to enhance CAR-T cells' efficacy.[38]

In our study, we also showed that a high expression of CD56 (95%) on tumor cells was associated with better PFS and a better response to Daratumumab. This is in accordance with previous studies that showed that CD56-negative patients displayed a more aggressive disease, with higher levels of β2-microglobulin and a higher incidence of extramedullary disease[39] and plasma cell leukemia.[40] Interestingly, in the context of breast cancer, CD56 promotes the formation of a cytotoxic immunological synapse via cell–cell adhesion by homophilic interaction.[41] In myeloma, similar observations have been made with the description of a subtype of NK $CD94^{low}$ $CD56^{dim}$ that is very effective in destroying myeloma cells, mainly when patients express $CD56^{high}$ myeloma cells.[42] Recently, Robinette et al. showed that CD56 expression is a novel positive predictive factor of response to Daratumumab and that CD56 expression is altered by Daratumumab treatment.[43]

Remarkably, we assessed a significant enrichment of PRC2 target genes in the patients with the best response to Daratumumab treatment. PRC2 is a multi-subunit epigenetic protein complex that represses

gene transcription through the tri-methylation of lysine 27 of histone 3 (H3K27me3) with its catalytic subunit enhancer of zeste homolog 2 (EZH2). In myeloma cells, PRC2 complex is overexpressed in association with cell cycle deregulation,[44,45] and targeting of PRC2 is a potential strategy in this pathology.[46] More recently, a link between EZH2 and CD38 expression, with a rescue of Daratumumab efficiency in samples treated with EZH2 inhibitors, has also been shown.[21] Altogether, our observation could thus suggest that EZH2 is downregulated in the responders, rescuing CD38 expression and paving the way to better response to Daratumumab.

The main limitation of this study is the non-uniform timing of plasma cell phenotyping (most often done at diagnosis). It would have been interesting to systematically determine tumor plasma cell phenotype before Daratumumab treatment, since phenotypic markers may change over time and according to treatment pressure. Indeed, phenotype changes have already been described in the literature, notably concerning CD19, CD20, CD45, CD56, CD117, and CD200, with a rate of change variable depending on studies (between 40% and 80%). [47–49] Here, we also observed a change in the phenotype of tumor plasma cells at relapse. However, our observations are robust as we used multiple parameters that confirmed our results on the cohort, and we further validated *in vitro* the role of CD200 in Daratumumab response.

Finally, this study is the first to identify phenotypic and molecular prognostic factors in the context of Daratumumab treatment. Altogether, our results underlined the role of CD200 immune checkpoint in MM that may provide new therapeutic avenues to investigate the efficacy of CD200 inhibition in overcoming resistance.

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## Author contributions

D.C., C.P. and C.H. contributed to the overall design and performed research; D.C., H.D.B., J.M., L.G., and C. H. analyzed the data and performed the statistical analyses; D.C., A.B., A.M., and H.D.B. designed and performed *in vitro* studies, G.R., N.R., C.P., L.G., L.F., C.V., G.C., L.V., and C.H. provided clinical care and collected data; D.C., C. P., V.J., H.D.B., J.M., and C.H. wrote, reviewed, and edited the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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