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NK Cell Phenotype Is Associated With Response and Resistance to Daratumumab in Relapsed/Refractory Multiple Myeloma

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ABSTRACT

The CD38-targeting antibody daratumumab has marked activity in multiple myeloma (MM). Natural killer (NK) cells play an important role during daratumumab therapy by mediating antibody-dependent cellular cytotoxicity via their FcγRIII receptor (CD16), but they are also rapidly decreased following initiation of daratumumab treatment. We characterized the NK cell phenotype at baseline and during daratumumab monotherapy by flow cytometry and cytometry by time of flight to assess its impact on response and development of resistance (DARA-ATRA study; NCT02751255). At baseline, nonresponding patients had a significantly lower proportion of CD16$^+$ and granzyme B$^+$ NK cells, and higher frequency of TIM-3$^+$ and HLA-DR$^+$ NK cells, consistent with a more activated/exhausted phenotype. These NK cell characteristics were also predictive of inferior progression-free survival and overall survival. Upon initiation of daratumumab treatment, NK cells were rapidly depleted. Persisting NK cells exhibited an activated and exhausted phenotype with reduced expression of CD16 and granzyme B, and increased expression of TIM-3 and HLA-DR. We observed that addition of healthy donor-derived purified NK cells to BM samples from patients with either primary or acquired daratumumab-resistance improved daratumumab-mediated MM cell killing. In conclusion, NK cell dysfunction plays a role in primary and acquired daratumumab resistance. This study supports the clinical evaluation of daratumumab combined with adoptive transfer of NK cells.

INTRODUCTION

The CD38-targeting antibody daratumumab induces several classic Fc-dependent immune effector functions, such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity and antibody-dependent cellular phagocytosis, as well as modest inhibition of CD38 enzymatic activity.[1–3] Furthermore, daratumumab has immunomodulatory effects by depleting CD38$^+$ immune suppressor cells such as regulatory T cells, resulting in T-cell expansion and enhanced cytotoxic capacity.[4–6] Daratumumab has single-agent activity[7–9] and can be successfully combined with immunomodulatory agents (IMiDs) and/or proteasome inhibitors (PIs).[10,11] However, multiple myeloma (MM) remains an incurable disease, and treatment options for patients who become refractory to IMiDs, PIs, and CD38-targeting antibodies are limited.

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Indeed, triple-class refractory MM carries a poor prognosis.[12] A better understanding of determinants that affect response and/or resistance to daratumumab may further improve treatment strategies.

We and others have previously shown that tumor-related features play a role in primary and acquired resistance to daratumumab. First, the efficacy of daratumumab has been shown to be partially dependent on baseline CD38 expression on MM cells.[13] However, the role of CD38 in acquired resistance remains unclear, because upon initiation of daratumumab treatment, there is rapid and sustained CD38 downregulation on MM cells in both responding and nonresponding patients.[13–16] We recently showed that all-trans retinoic acid (ATRA) can induce a transient increase in CD38 expression at the time of daratumumab resistance, but clinical activity of the combination of daratumumab and ATRA in daratumumab-refractory patients was limited.[17] In addition, increased expression of complement inhibitory proteins on the tumor cell surface contributes to daratumumab resistance.[13] Daratumumab is also less effective in patients with high-risk cytogenetic abnormalities or with extramedullary disease.[8,18]

Natural killer (NK) cells are a critical component of the innate immune system, as they provide protection against viral infections and play an important role in the immune surveillance of cancer. Activation of NK cells depends on the integration of activating and inhibitory signals from cell-surface receptors, upon contact with ligands expressed on target cells. FcγRIII receptor (CD16) is the most potent NK cell activating receptor, and is essential for ADCC by NK cells via its interaction with the Fc portion of the therapeutic antibody.[19] Importantly, NK cells from MM patients are characterized by qualitative and quantitative abnormalities, with substantial heterogeneity between patients, which may relate to differences in age, tumor burden, pathogen exposure, and extent of treatment with immunosuppressive anti-MM therapies.[20–23]

Although preclinical studies have demonstrated the importance of NK cells in daratumumab-mediated antitumor activity,[24,25] there was no association between peripheral blood (PB) NK cell frequency and response to daratumumab monotherapy.[26] In addition, FcγR polymorphisms, with different affinity for certain IgG subclasses, had no or minimal impact on clinical outcomes in MM patients treated with daratumumab.[27] However, the impact of NK cell fitness before and during daratumumab treatment on efficacy and development of resistance remains largely unknown. Therefore, we evaluated the frequency and characteristics of NK cells in serial bone marrow (BM) and PB samples obtained from heavily pretreated relapsed/refractory (RR) MM patients, who received daratumumab monotherapy in part A of the DARA-ATRA study (NCT02751255).

MATERIALS AND METHODS

Study population

The study population consisted of patients enrolled in part A of the DARA-ATRA study, which was described previously.[17] Briefly, the DARA-ATRA study was a prospective, investigator-initiated, nonrandomized, multicenter, open-label phase 1 dose finding trial, followed by a phase 2 expansion trial at the recommended phase 2 dose of ATRA, to evaluate safety, tolerability, and efficacy of daratumumab combined with ATRA in daratumumab-refractory RRMM patients. Patients were eligible if they had relapsed from or were refractory to at least 2 prior lines of treatment. Patients were first treated in part A of the trial, which consisted of daratumumab monotherapy. Patients were subsequently enrolled in part B of the study (daratumumab combined with ATRA) in case of insufficient response to daratumumab monotherapy, defined as disease progression during the first cycle of treatment in part A, less than minor response (MR) after the second treatment cycle, or less than partial response (PR) after the third treatment cycle, or in case of disease progression after an initial response (at least PR). Daratumumab and ATRA treatment was continued until disease progression. The study was approved by the institutional medical ethical committee in each participating center and was performed in accordance with the declaration of Helsinki. All patients provided written informed consent. The trial was registered at www.ClinicalTrials.gov as NCT02751255. In this analysis, all 63 patients enrolled in part A of the study were included (Table 1).

Drug administration

In part A, daratumumab was administered intravenously at a dose of 16 mg/kg according to the approved schedule, which consists of 8 weekly infusions, then 8 biweekly infusions, and monthly infusions thereafter.[7,8]

Efficacy assessments

Treatment response was assessed at the end of each cycle according to the International Myeloma Working Group Uniform Response Criteria.[28]

Analysis of BM and blood samples from patients treated with daratumumab monotherapy using flow cytometry and cytometry by time of flight

Flow cytometry was used to assess the frequency and phenotype of NK cells in blood and BM samples, obtained at baseline, during treatment, and at the time of daratumumab resistance. In a subset of BM samples, cytometry by time of flight (CyTOF) was performed for additional analysis of shifts in NK cell composition.

BM samples were also obtained from age-matched patients, who underwent cardiothoracic surgery, and used as healthy controls.

Details are presented in the Supplemental Data, available on the *HemaSphere* website.

NK cell isolation

NK cells were isolated from Buffy Coats obtained from healthy donors using the EasySep Human FITC Positive Selection Kit II (Stemcell Technologies) to deplete $CD3^+$, $CD14^+$, and $CD19^+$ cells in the presence of anti-human CD32 (Fc gamma RII) Blocker (Stemcell Technologies). Subsequently, the remaining cells were stained with CD3 FITC (Beckton Dickinson), CD56 PE-Cy7, CD45 Krome Orange (both Beckman Coulter), and LIVE/DEAD Fixable Near-IR Dead Cell fluorescent dye (Invitrogen). Lineage negative and $CD56^+$ NK cells were sorted on the FACSaria IIu (Beckton Dickinson), and the purity of NK cells (>95%) was checked during the procedure.

Flow cytometry-based ex vivo cytotoxicity assays with BM-MNCs

Cryopreserved BM mononuclear cells (MNCs) derived from a total of 8 MM patients with primary or acquired daratumumab resistance, containing 9%–55% $CD138^+$ tumor cells, as well as all immune cells, including effector NK cells and potential immune suppressive cells, were used in flow cytometry-based cytotoxicity assays. Sample viability at start of the assays, assessed by Trypan Blue staining, was >75%. BM-MNCs were incubated in RPMI + 10% fetal bovine serum with or without 10 µg/mL daratumumab, and with or without purified NK cells (>95% pure) from healthy donors, in 96-well U-bottom plates for 48 hours at 37°C. Cells were stained with HuMax-003 FITC (Genmab/Janssen), CD138 APC (Beckman Coulter), and LIVE/DEAD Fixable Near-IR Dead Cell fluorescent dye (Invitrogen). The survival of $CD138^+$ MM cells was determined by flow cytometry as previously described.[13,16] Briefly, surviving MM cells were enumerated by single platform flow cytometric analysis of $CD138^+$ cells in the presence of Flow-Count

Table 1

Baseline Characteristics

Characteristic	Part A (n = 63)	
Median age, y (range)	69 (38–80)	
<65	20 (31.7)	
65 to <75	30 (47.6)	
≥75	13 (20.6)	
Sex, n (%)		
Female	24 (38.1)	
Male	39 (61.9)	
WHO performance score, n (%)		
0	29 (46.0)	
1	25 (39.7)	
2	7 (11.1)	
Unknown	2 (3.2)	
Extramedullary plasmacytomas, n (%)		
No	53 (84.1)	
Yes	8 (12.7)	
Type of monoclonal heavy chain, n (%)		
IgG	36 (57.1)	
IgA	7 (11.1)	
Light chain only	13 (20.6)	
Bence-Jones	7 (11.1)	
Type of light chain, n (%)		
Kappa	40 (63.5)	
Lambda	23 (36.5)	
Time since start of first treatment, median years (range)	6.8 (1.5–16.0)	
Prior lines of treatment, median (range)	4 (2–11)	
>3 prior lines, n (%)	41 (65.1)	
Autologous SCT, n (%)	38 (60.3)	
Allogeneic SCT, n (%)	8 (12.7)	
Double-class refractory, n (%)[a]	42 (66.7)	
Triple-class refractory, n (%)[b]	2 (3.2)	
Prior IMID, n (%)	Exposed	Refractory[c]
Thalidomide	32 (50.8)	11 (17.5)
Lenalidomide	63 (100)	51 (81.0)
Pomalidomide	23 (36.5)	22 (34.9)
Prior PI, n (%)	Exposed	Refractory[c]
Bortezomib	61 (96.8)	37 (58.7)
Carfilzomib	7 (11.1)	7 (11.1)
Ixazomib	2 (3.2)	2 (3.2)
Prior monoclonal antibody, n (%)	Exposed	Refractory[c]
Daratumumab	3 (4.8)[d]	2 (3.2)
Elotuzumab	2 (3.2)	2 (3.2)
Durvalumab	6 (9.5)	6 (9.5)
Cytogenetic risk profile, n (%)[e]		
High-risk	36 (57.1)	
Standard-risk	14 (22.2)	
Not available	13 (20.6)	
International Staging System at enrollment, n (%)		
ISS I	19 (30.2)	
ISS II	25 (39.7)	
ISS III	13 (20.6)	
Unknown	6 (9.5)	
Revised International Staging System at enrollment, n (%)		
R-ISS I	10 (15.9)	
R- ISS II	26 (41.3)	
R- ISS III	7 (11.1)	
Unknown	20 (31.7)	
Laboratory values at baseline, median (range)		
Absolute neutrophil count, $\times 10^9$/L	2.92 (0.60–8.87)	
Hemoglobin level, mmol/L	7.0 (4.8–8.9)	
Platelet count, v10^9/L	168 (34–479)	
Creatinin, µmol/L	88 (53–228)	
Calcium, mmol/L[f]	2.41 (2.05–2.95)	
LDH, U/L	204 (119–1356)	
Beta-2 microglobulin, mg/L	3.4 (0.6–20.6)	
Bone marrow plasma cell percentage in biopsy (median, range)[g]	50 (0–100)	

[a]Double-class refractory is defined as both IMiD and PI refractory disease.
[b]Triple-class refractory is defined as IMID, PI, and CD38-targeting antibody refractory disease.
[c]Refractory disease is defined as progressive disease during therapy, no response (less than PR), or progressive disease within 60 d of stopping treatment, according to the International Uniform Response Criteria for Multiple Myeloma.
[d]Three patients had previously been treated with daratumumab 16, 42, and 48 mo before the registration;
[e]As determined by FISH or SNP array on purified MM cells before start of daratumumab treatment; according to the criteria proposed by Sonneveld et al. Blood 2016: high-risk disease is defined by the presence of t(4;14), t(14;16), t(14;20), del(17/17p), and/or gain(1q).
[f]Calcium corrected for serum albumin using formula: corrected calcium = calcium measured + ([40 − albumin] × 0.02).
[g]Based on the data from 39 patients (62%).
IMID = immunomodulatory drug; FISH = fluorescence in situ hybridization; LDH = lactate dehydrogenase; MM = multiple myeloma; PI = proteasome inhibitor; PR = partial response; SCT = stem cell transplantation; SNP = single-nucleotide polymorphism; WHO = World Health Organization.

Fluorospheres (Beckman Coulter). The percentage of lysis induced by daratumumab was calculated using the following formula: % lysis MM cells = 1 – (absolute number of surviving MM cells in treated wells)/(absolute number of surviving MM cells in untreated wells) × 100, as described previously.[16] In these short-term cytotoxicity assays, daratumumab kills MM cells predominantly via NK cell-dependent ADCC.[24,29]

Impact of proportion of CD16$^+$ NK cells on daratumumab-mediated lysis in ex vivo cytotoxicity assays

BM-MNCs were obtained from 47 daratumumab-naïve MM patients (17 newly diagnosed MM and 30 RRMM patients). These patients were not treated in the DARA-ATRA study. Samples contained 2.0%–67% CD138$^+$ tumor cells, as well as autologous effector cells and immune suppressive cells. The frequencies of MM cells, NK cells, and CD16$^+$ NK cells in these samples at the start of the assay were determined by staining 1.0×10^6 cells with HuMax-003 FITC (Genmab/Janssen), CD138 PE, CD45 Krome Orange, CD56 PC7 (all Beckman Coulter), CD3 V450, and CD16 APC (both BD Biosciences). Sample viability at start of the assays, assessed using 7-AAD (BD Biosciences) was >95%. BM-MNCs were incubated with daratumumab 10 µg/mL in duplicate for 48 hours, after which MM cell-specific lysis was assessed by flow cytometric analysis, as described above.

MM cell line and culture

The luciferase (LUC)-transduced CD38-positive MM cell line UM9[4] was cultured in RPMI-1640 (Invitrogen, Carlsbad, CA), supplemented with 10% HyClone FetalClone I serum (GE Healthcare Life Sciences) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). UM9 was obtained after prolonged in vitro culture of the BM aspirate of a MM patient. Monthly mycoplasma testing was performed using real-time PCR (Microbiome). The cell line was authenticated

by short-tandem repeat profiling carried out maximal 6 months before the most recent experiment. The cell line was used for a time period no longer than 4 months.

Impact of daratumumab-induced NK cell activation on expression of NK cell-surface markers

Cryopreserved PB-MNCs, obtained from healthy donors, were thawed and cultured overnight in RPMI-1640 containing 20% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Next, PB-MNCs were cocultured with UM9 target cells in a 50:1 ratio, in the presence of CD107a-BV421 (Beckton Dickinson), with or without daratumumab 10 µg/mL for 4 or 24 hours. Subsequently, cells were stained with CD45 KO, CD56 PC7, CD138 PE (Beckman Coulter), HLA-DR APC-H7, TIM-3 BV605, CD3 BUV395, and CD16 BUV737 (Beckton Dickinson). LIVE/DEAD Fixable Near-IR Dead Cell fluorescent dye (Invitrogen) was used to determine viability. Degranulation (CD107a positivity) and surface markers were assessed in T cells and NK cell subsets using flow cytometry (LSRFORTESSA instrument, Becton Dickinson).

Statistics

Overall response rate was defined as PR or better. Progression-free survival (PFS) for daratumumab monotherapy, followed by daratumumab plus ATRA (PFS-AB), was defined as time from registration until disease progression during part B or death, whichever came first. Patients still on treatment at last follow-up were censored. For patients not enrolled in part B (not meeting eligibility criteria or withdrawal of consent), progression during part A was defined as event. PFS for part A alone could not be calculated, since a large proportion of patients was treated in part B because of suboptimal response to daratumumab monotherapy (according to the protocol criteria). These patients are primary refractory to daratumumab, but do not fulfill criteria for progression. Overall survival (OS) was defined as time from registration until death from any cause. Patients still alive at last follow-up were censored. PFS and OS were estimated using the Kaplan-Meier method. Predictive factors for response were determined using the Fisher exact test for categorical variables, and Mann-Whitney *U* test for continuous variables. The impact of continuous and categorical variables on PFS and OS was evaluated using univariate Cox regression analysis. Only factors that had a significance of $P < 0.05$ in the univariate analyses were included in the multivariate analysis (Cox regression, forward selection based on the Wald statistic) to assess independent determinants of response.

Comparisons between continuous variables were performed using Mann-Whitney test, (un)paired *t* test, Wilcoxon matched-pairs signed rank test, Kruskall Wallis test (with Dunn multiple comparisons test), RM 1-way ANOVA (with Holm-Šídák's multiple comparisons test) or Friedman test (with Dunn multiple comparisons test), dependent on whether data followed a normal distribution.

P-values below 0.05 were considered significant. Statistical analyses were performed using SPSS software (version 26), GraphPad prism (version 9) or R (version 4.0.3). Clinical data were monitored by the Clinical Research Bureau (Amsterdam University Medical Center).

RESULTS

Patient characteristics

Sixty-three RRMM patients with a median age of 69 years (range, 38–80), and with a median of 4 prior lines of treatment (range, 2–11), were treated with daratumumab monotherapy in part A of the DARA-ATRA study (Table 1). All patients were previously exposed to lenalidomide and a PI; 89% were IMiD-refractory, 71% PI-refractory, and 67% double-refractory to both an IMiD and a PI. Three patients were previously treated with daratumumab monotherapy in the GEN501 study,[7] 16, 42, and 48 months before the study enrollment, with as best response MR in 1 and very good partial response (VGPR) in 2 patients. At data cutoff (June 17th, 2021), 26 patients (41.3%) achieved a PR or better during daratumumab monotherapy, including VGPR in 14.3% and (stringent) complete remission in 4.8% (Figure 1A).

Fifty of these 63 patients were subsequently treated with daratumumab and ATRA in part B: 14 patients in phase 1 and 36 patients in phase 2. Thirteen patients were not enrolled in part B: 3 were still on treatment in part A, 8 did not meet the eligibility criteria for part B, and 2 withdrew consent. With a median follow-up of 50.2 months in surviving patients, median PFS from the start of daratumumab monotherapy followed by the ATRA combination (PFS-AB) was 7.2 months (95% confidence interval [CI], 4.6-9.8 mo) and median OS was 24.7 months (95% CI, 12.0-37.4 mo).

Characterization of NK cells in the BM microenvironment of MM patients

We first evaluated the immune composition of the BM microenvironment of these IMiD/PI-exposed patients before initiation of daratumumab (available BM samples obtained at baseline n = 51), and compared these profiles with those of healthy age-matched controls (n = 10; median age, 66 years) by using flow cytometry (Figure 1B and Suppl. Figure S1). The BM of these MM patients contained a significantly lower proportion of B cells, similar proportion of T cells, but a higher proportion of NK cells (median 7.9 versus 4.0%; $P = 0.011$), compared with healthy controls. NK cells were also evaluated for several surface proteins: CD16, CD38, inhibitory receptors (TIM-3, PD-1, and LAG3), and the activation marker HLA-DR.[22,30] NK cells from MM patients had significantly higher HLA-DR (median 12.9 versus 2.7%; $P < 0.001$) and PD-1 cell-surface expression (median 6.1 versus 2.4%; $P < 0.001$), but similar expression of other tested cell-surface markers (CD16, CD38, TIM-3, and LAG3), compared with healthy controls. $CD16^+$ NK cells had significantly higher expression of CD38, compared with $CD16^{neg}$ NK cells in BM samples from patients ($P < 0.001$; Suppl. Figure S2). NK cell frequency and phenotype were not affected by the type of treatment administered directly before the study enrollment (38 patients [74.5%] received IMiD-based therapy, 6 [11.8%] PI-based therapy, 6 [11.8%] PI and IMiD-based therapy, and 1 [2.0%] another treatment [melphalan-prednisone]; Suppl. Figure S3).

Primary resistance to daratumumab is associated with a higher percentage of $TIM\text{-}3^+$ and $HLA\text{-}DR^+$ NK cells, and lower proportion of $CD16^+$ NK cells in the BM

Next, we evaluated the impact of the baseline frequency and phenotype of NK cells in the BM on clinical outcomes with daratumumab monotherapy. To this end, we compared BM samples obtained from 21 responding patients (≥PR) with those from 30 nonresponding (primary refractory) patients by using flow cytometry (Figure 2A). The proportion of NK cells was similar between both the groups. Interestingly, nonresponding patients had higher proportions of $HLA\text{-}DR^+$ (15.3 versus 9.3%, $P = 0.043$) and $TIM\text{-}3^+$ NK cells (median 9.0 versus 5.6%; $P = 0.005$). In addition, nonresponders also had a lower proportion of $CD16^+$ NK cells (73.3 versus 85.8%; $P = 0.017$). There were no differences in the frequency of $CD38^+$, $LAG3^+$, and $PD\text{-}1^+$ NK cells between both the groups. To assess whether $HLA\text{-}DR^+$, $TIM3^+$, and $CD16^{neg}$ cells were comprising a single NK cell subset, we performed additional computational analyses. We found that virtually all $TIM\text{-}3^+$ cells were $HLA\text{-}DR^+$ and $CD16^{neg}$, consistent with an activated/exhausted phenotype (metacluster 1 in Suppl. Figure S4). HLA-DR expression was also observed

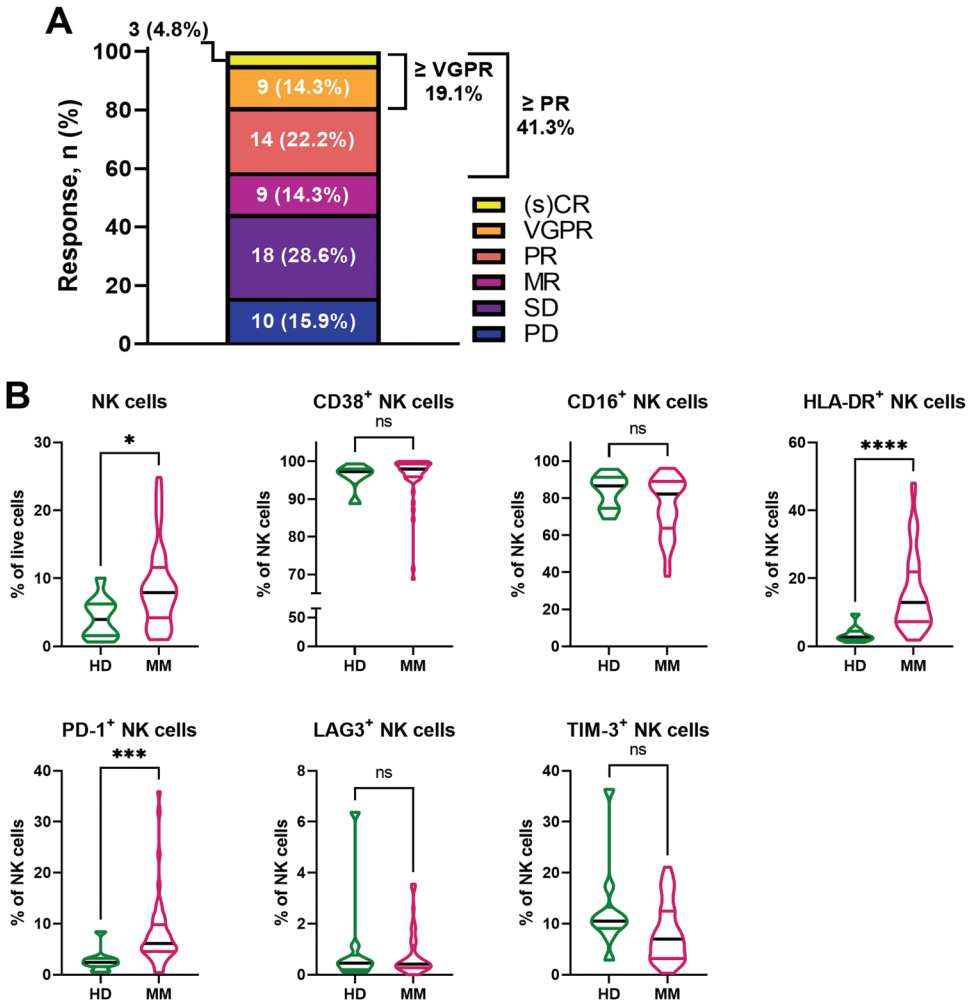

Figure 1. Response to daratumumab monotherapy in DARA-ATRA study and differences in BM-resident NK cells between healthy donors and MM patients. (A) Response rates of 63 patients enrolled in part A of the DARA-ATRA study, treated with daratumumab monotherapy. (B) Frequency of NK cells and NK cell phenotype were assessed by flow cytometry in BM samples obtained from relapsed/refractory MM patients (n = 51) and from HD of comparable age (n = 10). Data are depicted as violin plots, indicating the distribution, including median and interquartile range. Groups were compared using Mann-Whitney test. *P < 0.05; ***P < 0.001; ****P < 0.0001. BM = bone marrow; HD =healthy donors; PD = progressive disease; PR = partial response; MM = multiple myeloma; MR = minimal response; NK = natural killer; ns = not significant; (s)CR = (stringent) complete remission; SD = stable disease; VGPR = very good partial response.

in 2 small TIM-3^{neg} NK cell subsets: 1 CD16^{+} and 1 CD16^{neg} population (0.98% and 1.6% of total NK cells). Because the majority of CD16^{neg} NK cells (79.2%) did not express TIM-3 or HLA-DR, in further analyses, we continued to separately determine the impact of CD16, TIM-3, and HLA-DR expression on clinical outcome parameters. Importantly, a high frequency of TIM-3^{+} or HLA-DR^{+} NK cells was associated with significantly reduced PFS and OS, while a high proportion of CD16^{+} NK cells was associated with improved PFS and OS (Figure 2B, C).

In line with these findings, we observed a positive correlation between the potency of daratumumab to lyse MM cells and the ratio of CD16^{+} NK cells to MM cells, when we performed ex vivo, fully autologous, ADCC assays with a different set of BM samples from 47 daratumumab-naïve MM patients (r = 0.500, P < 0.001; Figure 2D). In agreement with our previous findings,[13] there was also a positive correlation between MM cell lysis and CD38 expression on tumor cells (r = 0.378, P = 0.008). TIM-3 and HLA-DR expression were not assessed in these BM samples.

Clinical determinants associated with response, PFS, and OS following daratumumab monotherapy

Patients with World Health Organization (WHO) performance status of 1 or 2 had a significantly lower response rate and inferior PFS, compared with patients with WHO performance status 0. In addition, elevated levels of lactate dehydrogenase and beta-2 microglobulin, as well as the presence of extramedullary plasmacytomas and ISS stage 3 disease, were associated with inferior PFS and OS. Revised-ISS stage 3 was associated with impaired OS (Suppl. Table S1).

To determine independent predictive factors for PFS and OS, we performed a multivariate analysis by including the clinical features and NK cell characteristics that showed a significance of P < 0.05 in the univariate analyses (Suppl. Tables S1 and S2). The presence of extramedullary disease (hazard ratio [HR], 6.08; 95% CI, 2.01-18.3) and frequency of HLA-DR^{+} NK cells (HR, 1.06; 95% CI, 1.03-1.09) were independent predictors for PFS; and frequency of HLA-DR^{+} NK cells (HR, 1.12; 95% CI, 1.07-1.17) and TIM-3^{+} NK cells (HR, 1.10; 95% CI, 1.02-1.19) were predictive for OS.

Daratumumab resistance is associated with increased proportion of NK cells expressing inhibitory receptors and decreased proportions of CD16^{+} NK cells

Next, we assessed the alterations in the phenotype and frequency of NK cells in the BM, when patients developed daratumumab-resistance by comparing BM samples obtained at

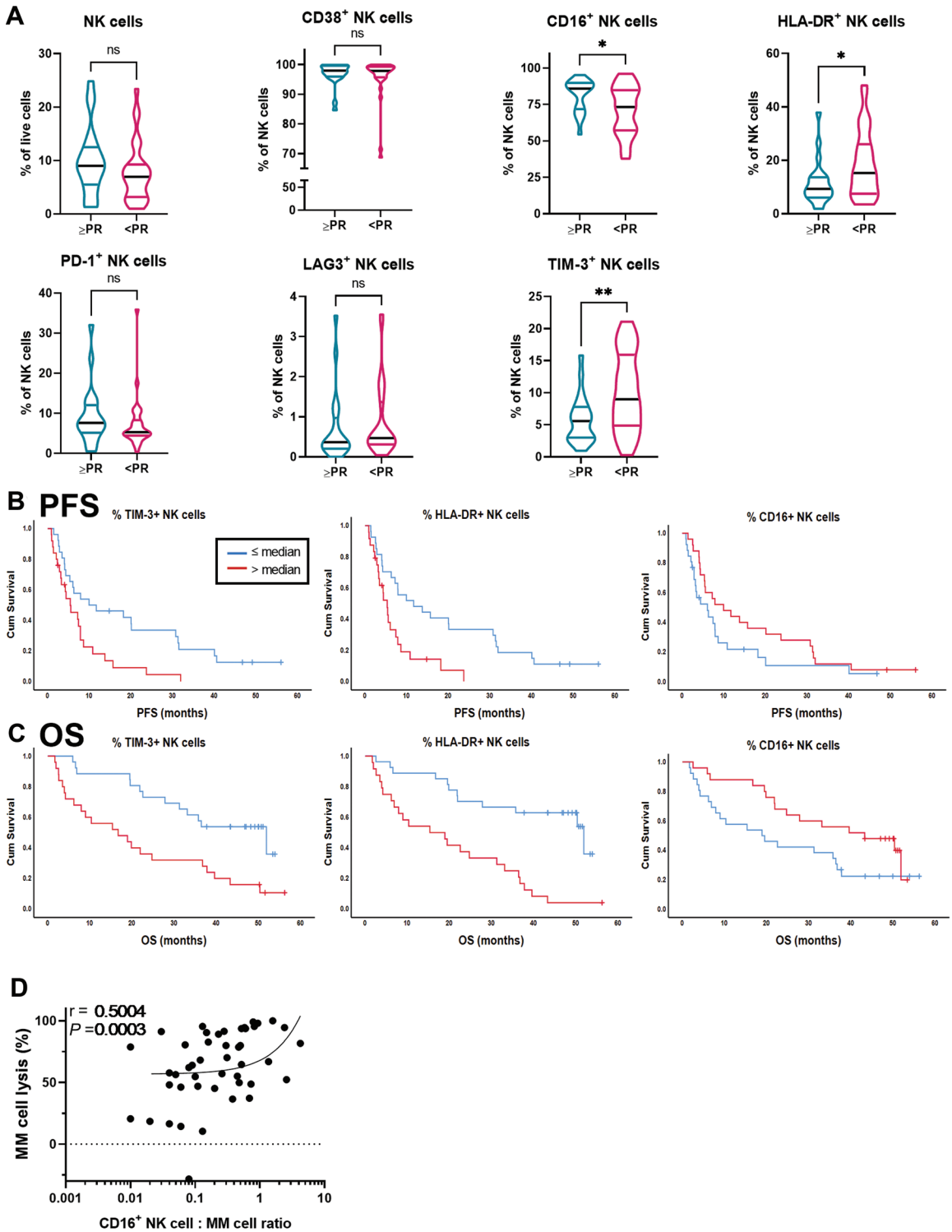

Figure 2. High proportions of HLA-DR+ NK cells and TIM-3+ NK cells, as well as a low proportion of CD16+ NK cells in the BM microenvironment were associated with inferior response and inferior survival following initiation of daratumumab monotherapy. (A) Both the frequency of NK cells and NK cell phenotype at baseline were assessed by flow cytometry in BM samples obtained from patients who achieved a PR or better (responder; n = 21) following initiation of daratumumab treatment, and from patients with less than PR (nonresponder; n = 30). Data are depicted as violin plots, indicating the distribution, including median and interquartile range. Groups were compared using Mann-Whitney test. (B) Kaplan-Meier curves representing PFS from start part A to progression, death, or last follow-up in part B (daratumumab + ATRA), according to the median proportion of TIM-3+, HLA-DR+, or CD16+ NK cells. (C) Kaplan-Meier curves representing OS from start part A to death or last follow-up, according to the median proportion of TIM-3+, HLA-DR+, or CD16+ NK cells. Red curve represents the patients with proportion of specific subset of NK cells above the median, and the blue curve represents patients with a proportion of NK cells equal to or below the median. (D) BM-MNCs obtained from 47 daratumumab naive-MM patients were incubated with 10 μg/mL daratumumab in duplicate for 48 h, after which MM cell-specific lysis was assessed by flow cytometry. The correlation between daratumumab-mediated MM cell lysis and the baseline ratio between CD16+ NK cells and MM cells was calculated using Spearman's correlation coefficient (r). Dots represent individual experiments. ns = not significant; *$P < 0.05$; **$P < 0.01$. PFS = progression-free survival; OS = overall survival; BM = bone marrow; NK = natural killer; MM = multiple myeloma; PR = partial response; ATRA = all-trans retinoic acid; MNC = mononuclear cells.

baseline (n = 51) with those obtained at the time of confirmed daratumumab resistance, defined as either disease progression after prior response (ie, acquired resistance, n = 17) or as insufficient response to daratumumab monotherapy (ie, primary resistance, n = 30) (Figure 3A, and Suppl. Figure S5 for gating strategy of NK cell phenotype).

Similar to our previous studies,[29] daratumumab treatment resulted in a marked decrease of NK cells in the BM, as well as reduced CD38 expression levels on nondepleted NK cells. More importantly, when compared with baseline, the BM samples obtained at the time of daratumumab resistance (end of treatment part A [EOT-A]) appeared to contain significantly higher proportions of HLA-DR$^+$ (12.9 versus 32.7%, $P < 0.001$), LAG3$^+$ (0.4 versus 1.3%, $P < 0.001$), and TIM-3$^+$ NK cells (median 7.0 versus 11.7%, $P = 0.003$), and a significantly lower proportion of CD16$^+$ NK cells (82.2 versus 46.6%; $P < 0.001$), compatible with an activated/exhausted phenotype. The percentage of PD-1$^+$ NK cells remained unchanged. These phenotypic changes were observed in patients with primary as well as with acquired daratumumab-resistance, except for the increase in TIM-3$^+$ NK cells, which was only observed in patients with acquired resistance (Figure 3B).

Supervised analysis of CyTOF data demonstrates a decreased proportion of granzyme B$^+$ NK cells at the time of daratumumab resistance

Because our flow cytometry panel did not have intracellular markers, and contained only a maximum of 15 surface parameters, we used CyTOF for deeper analysis with a different antibody panel, including a granzyme B antibody (Suppl. Table S3), in a subset of 37 patients (29 baseline and 23 EOT-A samples). Here, we first confirmed that at baseline CD16$^+$ NK cells were granzyme B$^+$ and CD56^{dim}, and therefore most likely cytotoxic NK cells (Suppl. Figure S6).

Next, NK cells from different conditions were visualized in a plot using FreeViz algorithm (Figure 4A), highlighting differences at baseline between responders and nonresponders, as well as between baseline and EOT-A, with a shift from granzyme B$^+$ to HLA-DR$^+$ NK cells upon progression. We then clustered the projected cells and used a linear mixed effects model to estimate the significance of these shifts (Figure 4B). Consistent with our flow cytometry results, daratumumab significantly reduced the number of NK cells (Figure 4B). Moreover, CyTOF confirmed enrichment of granzyme B$^+$ CD56^{dim} cytotoxic NK cells in responders compared with nonresponders at baseline (Figure 4C, F, lower panel). These cells were different from those depleted upon treatment in responders (Figure 4D), or compared with nonresponders upon treatment (Figure 4E). Additionally, it appeared that nonresponders were enriched for CD38$^+$ NK cells, with varying levels of HLA-DR expression (Figure 4F, upper panel). At the time of daratumumab resistance, the BM contained a significantly reduced frequency of granzyme B$^+$ CD56^{dim} NK cells, which was more pronounced in patients with acquired resistance (previous response), than in patients with primary daratumumab resistance (nonresponders; Figure 4G; boxplots for granzyme B$^+$ NK cells are shown in Figure 4H). Because it was recently demonstrated that TIM-3 signaling reduces the expression of granzyme B in Vγ9Vδ2 T cells,[31,32] we also assessed whether there was an association between changes in TIM-3 and granzyme B expression in NK cells at the time of primary or acquired daratumumab resistance. Two-dimensional bin analysis demonstrated that the decrease in granzyme B levels was independent of changes in TIM-3 expression, both for patients with primary or acquired daratumumab resistance (Suppl. Figure S7). In conclusion, consistent with the flow cytometry results, the CyTOF data suggest that impaired NK cell fitness contributed to differences in response and development of resistance.

Rapid induction of phenotypic changes in NK cells by daratumumab treatment

We next questioned whether the daratumumab-mediated quantitative and phenotypic changes of NK cells as observed in BM were also present in PB. Because PB samples were also collected after 1 cycle of daratumumab therapy (cycle 2 day 1 [C2D1]), we could also address the dynamics of NK cell alterations in the circulation.

We first assessed the impact of daratumumab on absolute NK cell counts in PB from 61 patients by using flow cytometry with a limited antibody panel, including antibodies against CD3, CD45, CD56, and CD16 (Figure 5A). Similar to what we observed in the BM samples, the baseline frequency of NK cells in PB was comparable between responding and nonresponding patients. Rapidly after initiation of daratumumab therapy (at C2D1), the frequency of NK cells and CD16$^+$ NK cells in PB decreased, irrespective of the patient's response.

To assess the effect of daratumumab on circulating NK cells in more detail, we next analyzed PB samples from 20 patients by using an extended flow cytometry panel, which included antibodies detecting additional NK cell markers, including activating receptors (CD16, NKG2C, NKG2D, NKp30, NKp46, CD160, and DNAM-1), inhibitory receptors (NKG2A, KLRG1, PD-1, TIM-3, and TIGIT), the activation marker HLA-DR, and the terminal differentiation marker CD57 (2 new NK cell panels were used; gating strategy is shown in Suppl. Figure S8).[22,30] After the initiation of daratumumab therapy, there was a progressive decrease in NK cells expressing CD16 (median proportion of CD16$^+$ NK cells: 80.0% at baseline, 61.5% at C2D1, and 49.4% at EOT-A; Figure 5B). In contrast, the proportions of HLA-DR$^+$ and TIM-3$^+$ NK cells increased. There was also an increase in the proportion of NK cells expressing the inhibitory receptors KLRG1 and TIGIT, while the proportion of CD57$^+$ NK cells decreased. Furthermore, a modest increase in the activating receptors NKG2C, CD160, and NKp30 was observed, while DNAM-1 showed a modest decrease. These changes in NK cell phenotype occurred rapidly within 1 treatment cycle, with only limited differences between C2D1, and at the time of confirmed daratumumab resistance. Changes in NK cell phenotype were comparable between responding and nonresponding patients (Suppl. Figure S9).

Next, computational analysis was used to assess whether these cell-surface markers were expressed by the same NK cell-subpopulations. Comparable to the BM analysis, our PB analysis strongly suggests that NK cells expressing the highest levels of TIM-3 and HLA-DR belong to the same subset of NK cells, which is negative for CD16 (Suppl. Figure S10A-E). These HLA-DR$^+$, TIM3$^+$, and CD16^{neg} NK cells were enriched during daratumumab therapy (Suppl. Figure S10A, B, right panels). Interestingly, this NK cell subset did not (highly) express any of the other markers that increased during daratumumab therapy, as assessed by manual analysis (ie, KLRG-1, TIGIT, NKG2C, and NKp30), which indicates that the increase of these markers can be attributed to other NK cell subsets.

Daratumumab-induced NK cell activation results in downregulation of CD16 and upregulation of TIM-3 and HLA-DR

Nondepleted NK cells in PB and in BM display similar phenotypic changes with increased proportions of TIM-3$^+$ and HLA-DR$^+$ NK cells, and decreased frequency of CD16$^+$ cells. We, therefore, evaluated whether these phenotypic changes were caused by daratumumab-mediated NK cell activation. To this end, we treated the MM cell line UM9 with daratumumab or solvent control in the presence of PB-MNCs for 4 or 24 hours. Indeed, daratumumab rapidly upregulated TIM-3 and HLA-DR expression on nondepleted NK cells, followed by down-modulation of CD16 (Figure 6A, B). In these short-term assays, no significant upregulation of LAG3 on NK cells was observed (Suppl. Figure S11). Consistent with a NK cell-dependent mode

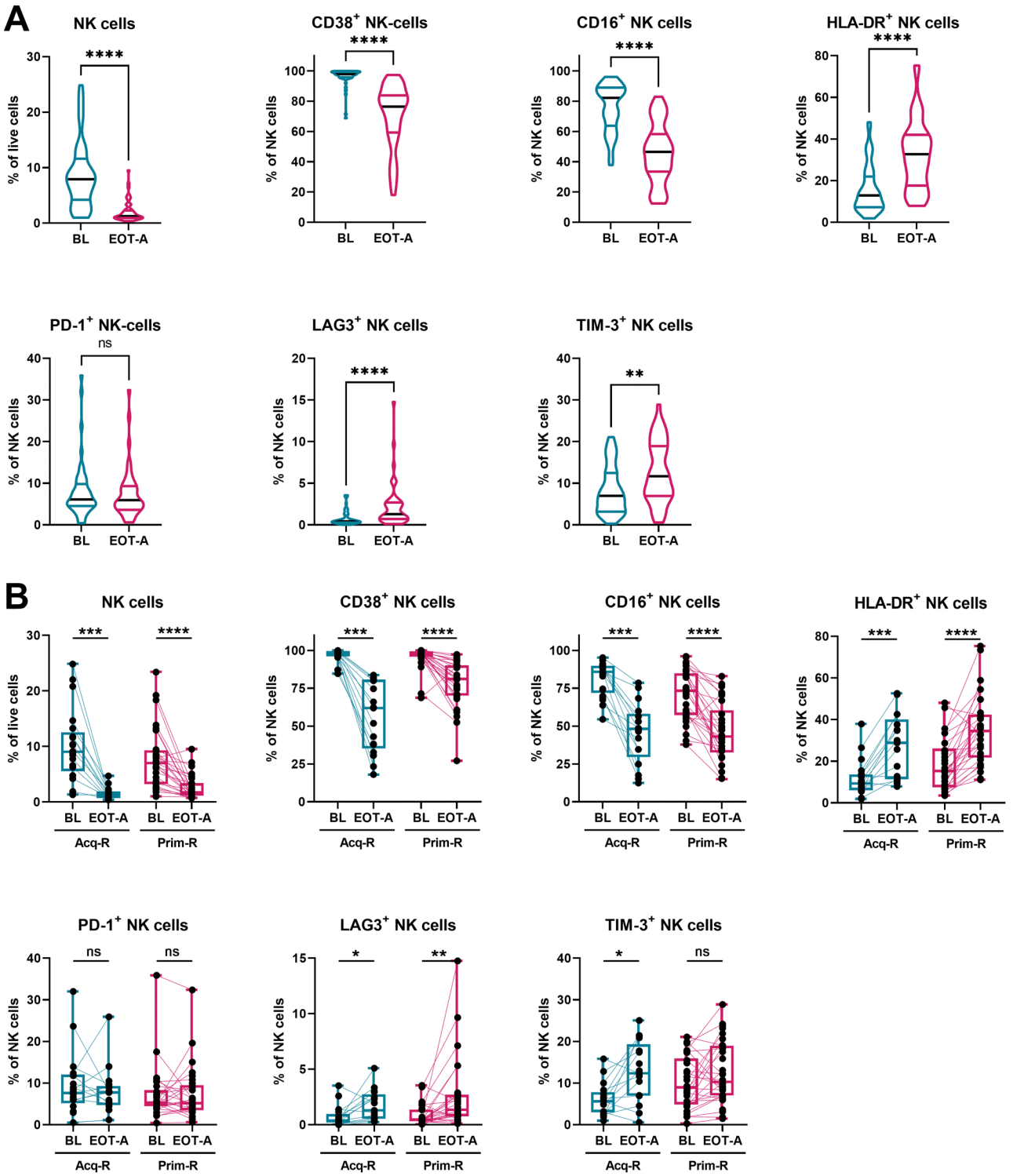

Figure 3. Development of daratumumab resistance is associated with an increased proportion of BM-resident NK cells expressing inhibitory receptors and a decreased proportion of CD16+ NK cells. (A) Proportion of NK cells and NK cell phenotype in BM samples at BL (n = 51) and at EOT-A (disease progression during the first cycle of treatment or after initial response in part A, <MR after the second treatment cycle, or <PR after the third treatment cycle; n = 47) were assessed by flow cytometry and depicted as violin plots, indicating the distribution, including median and interquartile range. (B) Both the proportion of NK cells and NK cell phenotype in BM samples at baseline and at EOT-A were also analyzed separately for patients who developed acquired daratumumab-resistance (Acq-R; patients who achieved a partial response or better before progression) and patients with Prim-R. Dots represent individual samples, with box and whiskers, representing median values, 25th to 75th percentile, and range. Groups were compared using Wilcoxon Matched-pairs signed rank test. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; ****$P < 0.0001$. ns = not significant; MR = minor response; Prim-R = primary resistance; BL = baseline; PFS = progression-free survival; BM = bone marrow; NK = natural killer; PR = partial response; EOT-A, end of treatment part A.

of action in these short-term killing assays, daratumumab treatment led to degranulation of NK cells, as evidenced by the increased cell-surface expression of CD107a on NK cells, but not on T cells (Figure 6C). NK cells characterized by either CD16 loss, high expression of TIM-3, or high levels of HLA-DR after the 4-hour or 24-hour incubation exhibited a higher extent of

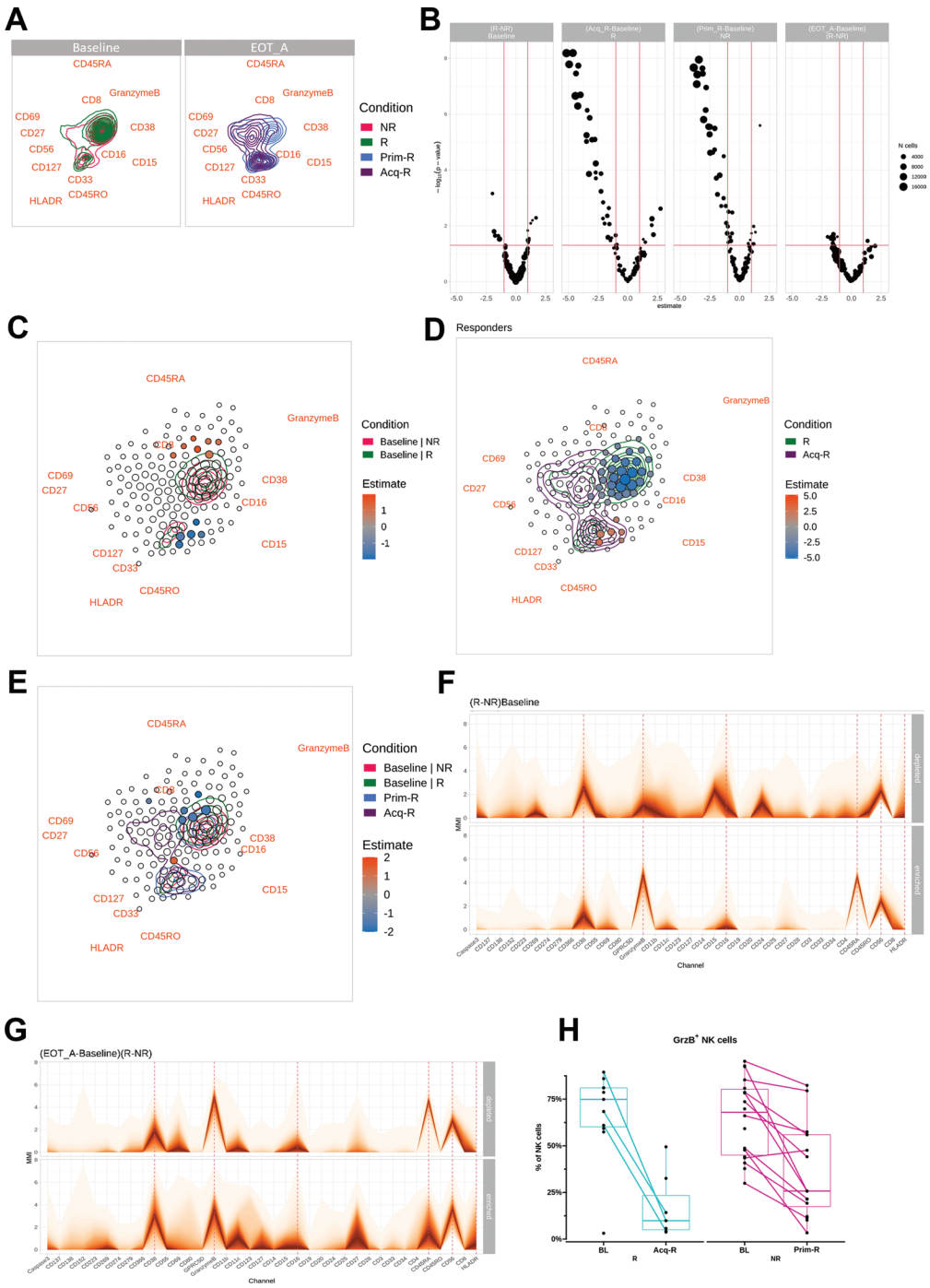

Figure 4. Differential marker expression in NK cells between responders vs nonresponders, and baseline vs time of daratumumab resistance (CyTOF analysis – bone marrow), using a supervised learning approach. CyTOF analysis was performed to explore shifts in NK cell composition in a data-driven way, in a subset of BM samples obtained from 37 patients (baseline, n = 29; EOT-A, n = 23). (A) FreeViz projection of NK cells at baseline from both R (n = 15) and NR patients (n = 22), and at the time of daratumumab resistance (primary or acquired resistance). The position of the different markers was optimized to maximize the difference between conditions of interest; the distance to the center of the projection corresponds to the weight of the markers (the longer, the more important). After projection, density lines were used to visualize the distribution of cells; markers with low weights are filtered out to increase readability. (B) Polyfunctionality analysis of FreeViz projection; volcano plots show a number of significant bins, representing different NK cell states, between all 4 contrasts of interest. (C) At baseline, bins enriched (orange) in responding patients, compared with nonresponding patients, are compatible with granzyme B^{+} CD56^{dim} NK cells. (D) In responding patients, bins depleted (blue) at the time of acquired daratumumab resistance, compared with baseline, are compatible with granzyme B^{+} CD56^{dim} NK cells. (E) When comparing baseline samples to those taken at the time of daratumumab resistance, bins compatible with granzyme B^{+} CD56^{dim} NK cells are depleted (blue), which was more pronounced in patients with acquired resistance compared with primary resistance. (F) At baseline, fan plots confirm that NK cells, corresponding to enriched bins in responders, are granzyme B^{+} CD56^{dim} (bottom panel). At baseline, CD38^{+} NK cells with varying levels of HLA-DR expression are enriched in nonresponders (top panel). (G) At the time of daratumumab resistance, granzyme B^{+} CD56^{dim} NK cells are depleted in patients with acquired and primary resistance, but to a significantly larger extent in patients with acquired resistance, compared with patients with primary resistance (top panel). HLA-DR^{+} CD56^{high} NK cells are significantly enriched in patients with acquired resistance, compared with patients with primary resistance to daratumumab (bottom panel). (H) Boxplot of the proportion of granzyme B^{+} NK cells over time in responders (blue) and nonresponders (purple) shows a more pronounced decrease of granzyme B^{+} NK cells in patients with acquired resistance. MMI = median marker intensity; NK = natural killer; NR = nonresponding; R = responding; CyTOF = cytometry by time of flight; Acq-R = patients with acquired resistance; Prim-R = patients with primary resistance; BL = baseline; EOT-A = disease progression during the first cycle of treatment or after initial response in part A, <MR after the second treatment cycle, or <PR after the third treatment cycle.

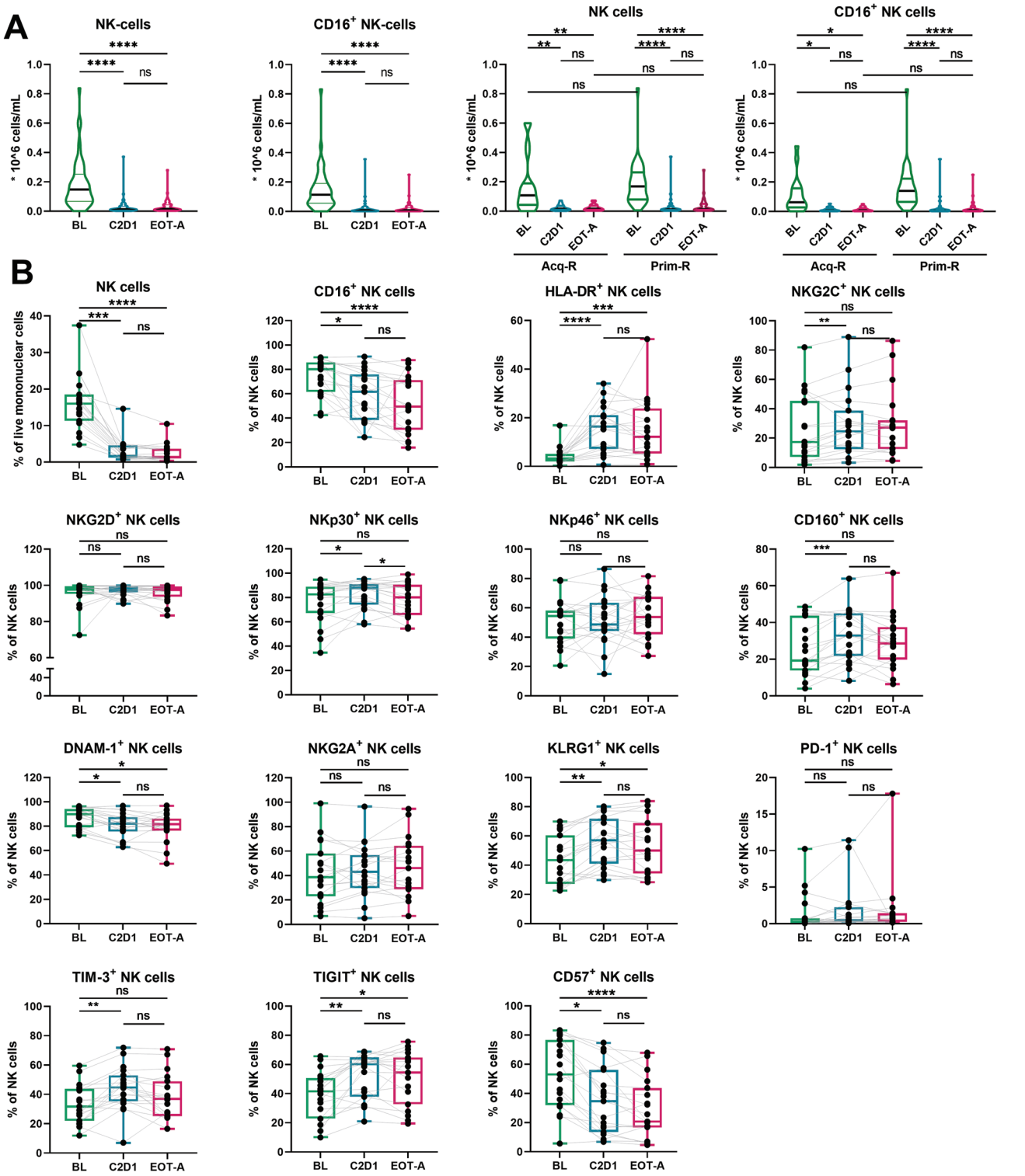

Figure 5. Immunophenotypic analyses of longitudinal blood samples shows that changes in NK cell phenotype occur rapidly after initiation of daratumumab. (A) Absolute NK cell counts and absolute CD16+ NK cell counts in peripheral blood samples obtained from 61 patients at BL, C2D1, and at EOT-A are depicted as violin plots, indicating the distribution, including median and interquartile range. Left 2 panels for all patients; and right 2 panels for patients who developed Acq-R and those with Prim-R separately. Groups were compared using mixed-effect analysis, with Tukey multiple comparisons posttest. (B) Peripheral blood samples from 20 patients (10 responders and 10 nonresponders) were subjected to in-depth immune profiling using flow cytometry. Dots represent individual samples, with box and whiskers, representing median, 25th–75th percentile, and range. Groups were compared using Friedman test with Dunn multiple comparison posttest (when data did not follow a normal distribution), or with RM 1-way ANOVA with Holm-Šídák multiple comparison posttest (when data followed a normal distribution). Data were analyzed using mixed-effect analysis with Holm-Šídák multiple comparison posttest. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; ****$P < 0.0001$. BL = baseline; NK = natural killer; ns = not significant; Prim-R = primary resistance; EOT-A = end of treatment part A; C2D1 = cycle 2 day 1; Acc-R = acquired resistance.

degranulation, as compared with NK cells with high CD16, low TIM-3, or low HLA-DR expression (Figure 6D). This indicates that NK cell phenotypic changes in the daratumumab-treated patients were caused, at least in part, by daratumumab-mediated NK cell activation.

Addition of healthy donor-derived NK cells partly reverses daratumumab resistance

Because our analyses demonstrated that an increased proportion of NK cells with an activated/exhausted phenotype was associated with primary and acquired daratumumab-resistance, we hypothesized that the MM cells of daratumumab-resistant patients could still be sensitive to daratumumab if these exhausted NK cells will be repleted with healthy donor-NK cells. Hence, we performed 48-hour ex vivo daratumumab-mediated killing assays with a number of daratumumab-resistant BM samples (n = 8), with or without the addition of purified healthy donor blood-derived NK cells. These NK cells from healthy donors had significantly lower TIM-3 expression, compared with levels on circulating NK cells from MM patients, both before initiation of daratumumab treatment ($P = 0.002$) or at the time of daratumumab-refractory disease ($P < 0.001$; Figure 7A). In addition, NK cells from healthy donors had lower HLA-DR expression ($P < 0.001$), and higher CD16 levels ($P = 0.008$), compared with daratumumab-refractory MM patients (Figure 7A).

We first tested baseline BM samples, obtained from patients who were primary refractory to daratumumab (n = 3; Figure 7B). As expected, daratumumab-mediated MM cell lysis was modest (mean lysis, 14%), but MM cell elimination significantly improved in the presence of healthy donor-NK cells (mean lysis 44%; $P = 0.003$). Next, we tested samples obtained at the time of progression after prior response (n = 5, of which 3 patients had obtained a VGPR and 2 patients a PR as best response; Figure 7C). In line with the clinical status, daratumumab was not effective in the ex vivo assays (mean lysis, –0.2%). However, adding healthy donor-derived NK cells restored sensitivity to daratumumab (mean lysis, 28%; $P = 0.042$). Baseline BM samples were available from 3 of these 5 patients. By analyzing MM cell lysis in these baseline samples, we observed that addition of purified NK cells did not completely restore daratumumab-mediated lysis to baseline levels in 2 of these 3 samples, while in 1 sample NK cell repletion increased killing capacity to the baseline value (Figure 7D).

DISCUSSION

MM is characterized by multiple immune defects, which contribute to disease progression and immunotherapy failure.[34] Our extensive assessment of quantitative and qualitative NK cell characteristics in patients treated with single-agent daratumumab reveals that NK cells are expanded in the BM of relapsed/refractory MM patients, which is similar to what has been described in newly diagnosed MM.[35–38] Our analysis further demonstrates that the NK cell frequency in BM and PB is similar between daratumumab-responding and nonresponding patients, which is also in line with prior studies.[26] However, we show for the first time that in the BM microenvironment, the expression of several NK cell-surface antigens is different between patients who are responsive or nonresponsive to daratumumab monotherapy. Patients with primary refractory disease had an increased frequency of NK cells in the BM with an exhausted and activated phenotype at baseline, with reduced CD16 and granzyme B expression, and increased TIM-3 and HLA-DR expression, compared with responding patients. An exhausted or activated NK cell phenotype was also associated with significantly impaired PFS and OS.

We also analyzed quantitative and phenotypic changes of BM-resident and circulating NK cells, when patients developed daratumumab-resistance. Similar to previous studies and irrespective of response, daratumumab treatment resulted in the rapid elimination of $CD38^+$ NK cells by NK cell fratricide.[19,26] Interestingly, the remaining, nondepleted NK cells were characterized by a decrease in CD16 expression and lower expression of the cytolytic molecule granzyme B, which may both limit the potency to induce ADCC. Remaining NK cells also had an activated phenotype ($HLA\text{-}DR^+$), and increased expression of several inhibitory receptors, such as TIM-3, TIGIT, KLRG-1, and LAG3, consistent with NK cell exhaustion.[22,39] Evaluation of serially obtained PB samples showed that these NK cell alterations occur early after initiation of the daratumumab treatment.

Altogether, these findings suggest that NK cell dysfunction and NK cell depletion contribute to both primary and acquired resistance to daratumumab. We, therefore, hypothesized that NK cell repletion could restore daratumumab sensitivity in patients with resistant disease. Indeed, in ex vivo killing assays with BM samples from primary refractory patients, daratumumab-mediated elimination of MM cells was significantly improved by the addition of healthy donor-derived NK cells. Furthermore, healthy donor-derived NK cells also partially restored daratumumab sensitivity in BM samples from patients, who had developed progression during daratumumab therapy after prior response.

CD16 is the activating NK cell receptor required for triggering ADCC. The daratumumab-mediated CD16 reduction can be explained by continuous NK cell activation by this antibody. Supporting this idea, our in vitro studies show that cell-surface expression of CD16 is rapidly decreased upon daratumumab-mediated NK cell activation, which was accompanied by increased TIM-3 and HLA-DR expression. Prior studies have shown that CD16 reduction following NK cell activation is caused by internalization as well as proteolytic cleavage of its extracellular portion by metalloproteinases.[40,41] CD16 downregulation prevents excessive immune responses, but also limits the efficacy of therapeutic antibodies, which work, at least in part, through ADCC.[19,40–43] Differences in CD38 expression between $CD16^+$ and $CD16^{neg}$ NK cells subsets may also contribute to the relative increase in $CD16^{neg}$ NK cells.

The strength of our study is that we analyzed, using 2 methodologies (flow cytometry and CyTOF), NK cells in sequential BM and blood samples obtained from patients, who were uniformly treated with daratumumab monotherapy, which allowed us to specifically dissect the impact of daratumumab on this immune cell subset. Currently most patients are treated with daratumumab combined with other agents, which complicates the evaluation of daratumumab-specific mechanisms of action and resistance. Although the results obtained by CyTOF are supportive and mirror the results generated by flow cytometric analysis, minor differences were observed. These differences most likely arise from the fact that the CyTOF analysis was performed in a subset of patients, compared with the patient group analyzed by flow cytometry. A limitation of our study is that patients received ATRA in case of progression or in case of insufficient response. Therefore, PFS for daratumumab monotherapy alone could not be calculated, and we used PFS for daratumumab followed by daratumumab and ATRA (PFS-AB) instead. Because the impact of ATRA on clinical outcomes was very limited, with a response rate of only 5%,[17] PFS-AB forms a good surrogate for PFS assessment. In addition, we were not able to assess the pattern of NK cell reconstitution after stopping daratumumab treatment, because PB and BM were not collected after the last daratumumab administration. We have previously shown that NK cell recovery occurs 3–6 months after the last daratumumab administration,[26] but it remains unknown how the frequency of different NK cell subsets changes over time during the NK cell reconstitution. Finally, due to the scarcity of BM and PB material, we were not able to

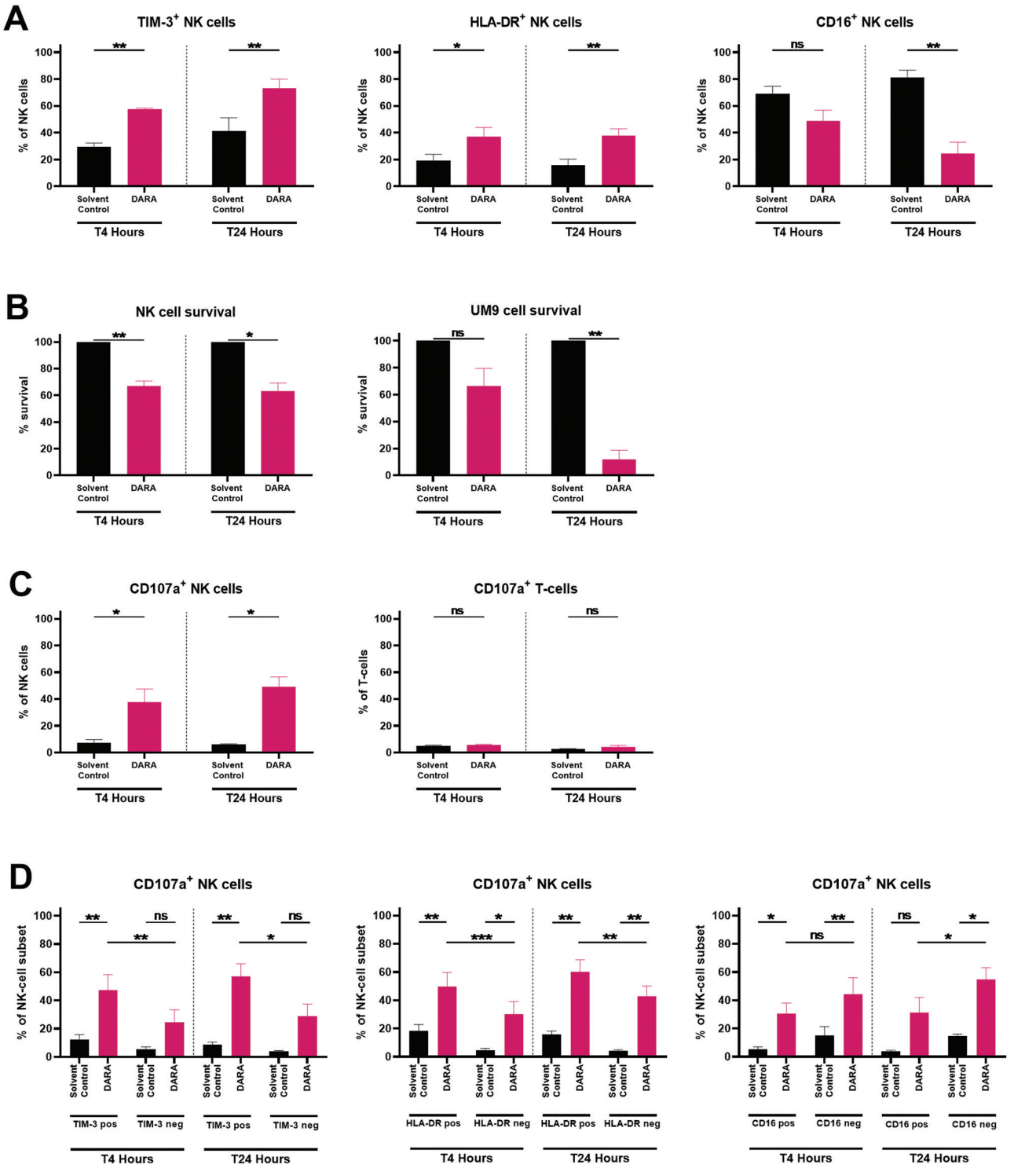

Figure 6. Daratumumab-induced NK cell activation results in downregulation of CD16 and upregulation of TIM-3 and HLA-DR. UM9 cells were incubated with solvent control or 10 µg/mL daratumumab in the presence of PB-MNCs (ratio of 50:1) for 4 or 24 h. (A) The proportion of TIM-3+, HLA-DR+, and CD16+ NK cells was determined by flow cytometric analysis. (B) Survival of NK cells and UM9 cells was determined and calculated as described in the Materials and Methods. Of note, we have previously shown that UM9 cells are not susceptible to daratumumab-dependent phagocytosis, and therefore MM cell elimination in these experiments is mainly by NK cells.[33] (C) Daratumumab-mediated degranulation of NK cells and T cells was assessed by flow cytometric analysis of CD107a cell-surface expression. (D) Frequency of CD107a-positive cells based on the presence or absence of TIM-3, HLA-DR, or CD16 expression on NK cells after the 4-h or 24-h incubation with daratumumab or solvent control. Data represent mean and SEM of 4 (at 4 h) or 3 (at 24 h) independent experiments, performed in duplicate. Paired Student *t* test was used to evaluate significance between both groups. **P* < 0.05; ***P* < 0.01, ****P* < 0.001. PB-MNCs = peripheral blood mononuclear cells; NK = natural killer; ns = not significant; SEM = standard error of mean.

purify NK cells from patients to study the relative contribution of NK cell exhaustion versus depletion to the development of daratumumab resistance.

An increased understanding of determinants of response and mechanisms of primary and acquired resistance may contribute to novel, rationally designed treatment strategies. Our

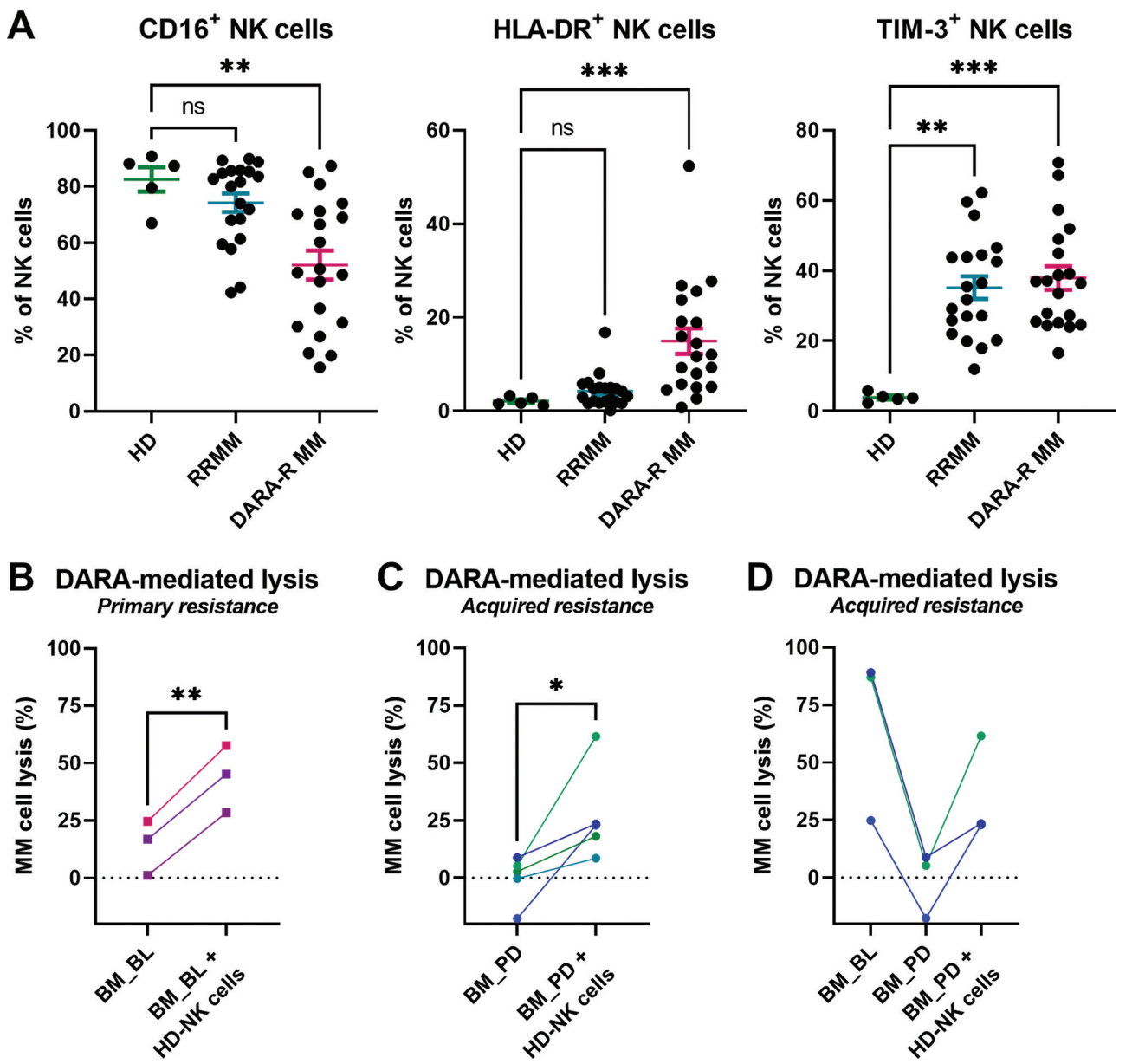

Figure 7. Healthy donor-derived NK cell repletion partially restores sensitivity to daratumumab in ex vivo experiments. (A) Expression of CD16, HLA-DR, and TIM-3 on NK cells in peripheral blood samples of 5 HD was compared with expression levels on NK cells in blood samples from daratumumab-naïve (n = 10) and daratumumab-refractory MM patients (n = 10), using Kruskal Wallis test. Dots represent individual samples, with bars indicating mean ± SEM. (B) In ex vivo cytotoxicity assays, 100,000 thawed cryopreserved BM mononuclear cells from nonresponding patients obtained at BL, before daratumumab treatment, were incubated for 48 h with solvent control or daratumumab (DARA; 10 µg/µL), with or without 20,000 healthy donor-derived NK cells. Data represent MM cell lysis of 3 samples, performed in triplicate. The percentage of MM cells ranged from 18% to 55% in these BM samples. Groups were compared using paired *t* test. (C) Similar experiments were performed with 5 BM samples obtained from previously responding patients at the time of PD (acquired daratumumab resistance). The percentage of MM cells ranged from 7% to 38%. (D) From 3 of the 5 responding patients, ex vivo daratumumab-mediated lysis could also be assessed at BL. The percentage of lysis induced by daratumumab was calculated using the following formula % lysis MM cells = 1 – (absolute number of surviving MM cells in daratumumab-treated wells)/(absolute number of surviving MM cells in solvent control-treated wells) × 100. Negative lysis values indicate that cell numbers are higher when compared with solvent control. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$. ns = not significant; NK = natural killer; BL = baseline; HD = healthy donors; MM = multiple myeloma; PD = progressive disease; BM = bone marrow; SEM = standard error of mean.

data support the clinical evaluation of the combination of a CD38-targeting antibody and adoptive transfer of expanded NK cells to improve depth and duration of response. However, addition of NK cells did not completely reverse acquired daratumumab resistance. Novel NK cell therapies hold promise to further improve efficacy of CD38-targeting antibodies.[19,44–47] This includes the use of NK cells engineered with a noncleavable version of CD16, resulting in improved ADCC activity,[47] expansion of specific NK cell subsets with superior ADCC activity,[44,48] or generation of NK cells with CD38 deletion to eliminate daratumumab-induced fratricide.[46] However, it is currently unknown whether these novel NK cell therapies can reverse primary or acquired resistance to a CD38-targeting antibody.

An alternative strategy to improve the efficacy of daratumumab is to enhance the effector function of endogenous NK cells. We observed an increased proportion of TIM-3+ NK cells in nonresponding patients at baseline, and an enhanced proportion of this NK cell subset at the time of acquired resistance. There is increasing evidence that TIM-3 impairs NK cell function,[49–51] while blocking TIM-3 has been shown to improve NK cell-mediated killing of cancer cells, including MM cells.[50,51]

Therefore, the ability of TIM-3 inhibiting antibodies to reverse daratumumab resistance should be explored in future studies.

Our data is important for the optimal sequencing of available and novel immunotherapies. The SLAMF7-targeting antibody elotuzumab mediates its anti-MM activity in part via CD16+ NK cells.[52] Decreased NK cell numbers and reduced CD16 and granzyme B expression may impair the efficacy of elotuzumab after daratumumab treatment. Indeed, in 2 retrospective studies, prior therapy with daratumumab had a negative impact on response to elotuzumab in subsequent lines of therapy.[53,54] In addition, daratumumab-induced NK cell depletion and NK cell alterations may also decrease the efficacy of novel NK cell engagers.[55,56] A CD38-targeting antibody-free interval of approximately 3–6 months, which allows for NK cell recovery, may be beneficial for subsequent NK cell-dependent therapies.[26] However, it is currently unknown to what extent the expression of CD16 and inhibitory receptors is restored to baseline levels after discontinuing daratumumab.

In conclusion, primary daratumumab resistance can be explained in part by an exhausted NK cell phenotype. Furthermore, next to NK cell depletion, NK cell exhaustion may also contribute to acquired daratumumab resistance. Our data support ongoing studies, which are evaluating the value of adoptive NK cell therapy in combination with a CD38-targeting antibody.

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AUTHOR CONTRIBUTIONS

CPMV, KAF, TM, and NWCJvdD designed the study and interpreted the results. MCM, MDL, AB, GMJB, MJK, SKK, PS, SZ, and NWCJvdD provided patient material. CPMV, KAF, MECB, CD, CAO, WB, PMHW, MMS, MK, TC, YA, RIV, TS, GV, DCS, LvS, ER, and SZ analyzed data and interpreted the results. KAF, CPMV, and NWCJvdD wrote the first draft of the article. All authors helped critically review the article and checked the final version of it.

DISCLOSURES

MCM has a consultancy or advisory role for Gilead Sciences, BMS, Alnylam, Janssen Cilag, Takeda, and Servier; all paid to employer, hospitality from Celgene. MDL serves in advisory boards for Roche, Janssen and Abbvia. AB receives honoraria from Celgene, Janssen, Amgen, Takeda, and Sanofi. MJK has received research support from Kite/Gilead, and honoraria from Kite/Gilead, Novartis, BMS/Celgene, Takeda, Roche, and Miltenyi Biotec (all paid to institution). MK, TC, YA, RIV, TS, GV, DCS, LvS, and ER are employed by Janssen. PS has received honoraria from Amgen, BMS, Celgene, Janssen, Karyopharm, Takeda, and receives research funding from Amgen, Celgene, Janssen, Karyopharm, SkylineDx, Takeda. SZ has received research funding from Celgene, Takeda, Janssen, and serves in advisory boards for Janssen, Takeda, BMS, Oncopeptides and Sanofi, all paid to institution. TM has received research support from Janssen Pharmaceuticals, Takeda, Genmab, Novartis, and ONK Therapeutics. NWCJvdD has received research support from Janssen Pharmaceuticals, AMGEN, Celgene, Novartis, Cellectis, and BMS, and serves in advisory boards for Janssen Pharmaceuticals, AMGEN, Celgene, BMS, Takeda, Roche, Novartis, Bayer, Adaptive, and Servier. All the other authors have no conflicts of interest to disclose.

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