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Adaptive Natural Killer Cells Facilitate Effector Functions of Daratumumab in Multiple Myeloma

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ABSTRACT

Purpose: To investigate the different roles of heterogeneous natural killer (NK)-cell subpopulations in multiple myeloma and to identify NK-cell subsets that support the robust anti-myeloma activity of daratumumab via antibody-dependent cellular cytotoxicity (ADCC).

Experimental Design: We performed single-cell RNA sequencing of NK cells from patients with newly diagnosed multiple myeloma (NDMM) and delineated adaptive NK cells in their bone marrow (BM). We further characterized the distinct immunophenotypic features and functions of adaptive NK cells by multicolor flow cytometry in 157 patients with NDMM.

Results: Adaptive NK cells exhibit a significantly lower level of CD38 expression compared with conventional NK cells, suggesting that they may evade daratumumab-induced fratricide. Moreover, adaptive NK cells exert robust daratumumab-mediated effector functions *ex vivo*, including cytokine production and degranulation, compared with conventional NK cells. The composition of adaptive NK cells in BM determines the daratumumab-mediated *ex vivo* functional activity of BM NK cells in patients with NDMM. Unlike conventional NK cells, sorted adaptive NK cells from the BM of patients with NDMM exert substantial cytotoxic activity against myeloma cells in the presence of daratumumab.

Conclusions: Our findings indicate that adaptive NK cells are an important mediator of ADCC in multiple myeloma and support direct future efforts to better predict and improve the treatment outcome of daratumumab by selectively employing adaptive NK cells.

Introduction

Multiple myeloma is a malignant plasma cell disorder that occurs in the bone marrow (BM), leading to organ damage associated with an accumulation of monoclonal protein in the blood or urine (1, 2). Despite recent therapeutic advances with the availability of proteasome inhibitors (PI) and immunomodulatory agents (IMiD), multiple myeloma remains largely incurable with few treatment options, especially for relapsed/refractory (R/R) patients (3, 4). Identification of high expression of surface antigens, CD38 and CS1, on malignant plasma cells has led to the development of anti-CD38 monoclonal antibodies (mAb), such as daratumumab and isatuximab, and the CS1-targeting mAb elotuzumab for the treatment of multiple myeloma (5–13).

These therapeutic antibodies are under preclinical and clinical development to prove their potential activity against multiple myeloma (5–14). The most advanced is daratumumab, which has shown efficacy in R/R patients as both monotherapy and triple combination with PIs and IMiDs (15–17). Encouraging results have led to the approval of daratumumab as part of the front-line triple combination therapy in patients with newly diagnosed multiple myeloma (NDMM; ref. 18). However, some patients are still refractory to daratumumab treatment for unclear reasons and these patients have extremely poor prognosis (19). Moreover, the benefit of mAbs in high-risk patients with NDMM is still uncertain (20–22). Therefore, further efforts are required to predict and improve the responses to therapeutic antibodies in multiple myeloma.

Antibodies directed against tumor cells rely on antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), and immunomodulatory effects for antitumor activity (23). Notably, natural killer (NK) cells are the primary mediator of the ADCC by which therapeutic antibodies exert antitumor effector functions (24). In particular, daratumumab and elotuzumab largely depend on ADCC mediated by NK cells to confer activity against multiple myeloma (25–29).

NK cells have been regarded as the primary effectors of innate immunity, but emerging evidence suggests that a subset of NK cells has adaptive immune features, including memory-like properties, such as long-term persistence, robust preferential expansion in response to viral infection, and enhanced antibody-dependent effector functions (30–38). This subset of NK cells is characterized by a lack of *FCER1G* (FcεRIγ) expression or high expression of *KLRC2* (NKG2C), which is used as a marker for adaptive NK cells (30–38). Recent studies with single-cell RNA sequencing of peripheral blood (PB) or BM NK cells from healthy individuals have also acknowledged this population (39, 40). Unlike conventional

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Translational Relevance

Efforts are required to predict and improve the clinical efficacy of therapeutic antibodies in patients with multiple myeloma. By single-cell RNA sequencing of natural killer (NK) cells from bone marrow (BM) of patients with newly diagnosed multiple myeloma, we identified adaptive NK cells with distinct immunophenotypic and functional features. Adaptive NK cells exert robust daratumumab-mediated effector functions and their composition in BM determines the daratumumab-mediated *ex vivo* functional activity of BM NK cells in patients with multiple myeloma. Our results highlight the adaptive NK cells as a potential candidate for predicting immunotherapy outcome in multiple myeloma and support future works to predict and promote the efficacy of mAb treatment in virtually any malignancy for which there are approved therapeutic antibodies.

NK cells, adaptive NK cells robustly induce ADCC in viral infection (30–35, 37, 38, 41). However, whether adaptive NK cells contribute to therapeutic antibody-mediated elimination of multiple myeloma cells remains unclear (42).

Here, we investigated the heterogeneity of NK cells in the BM of patients with NDMM and identified adaptive NK cells with distinct immunophenotypic and functional features compared with conventional NK cells. Adaptive NK cells had lower CD38 expression with robust effector functions upon *ex vivo* daratumumab treatment. In addition, adaptive NK cells sorted from the BM of patients with NDMM efficiently killed multiple myeloma cells in the presence of daratumumab, unlike conventional NK cells, suggesting their superior role in ADCC.

Materials and Methods

Patient samples

This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University of Medicine (Seoul, Republic of Korea; IRB 4-2014-0236), and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. We obtained paired PB mononuclear cells (PBMC) and BM mononuclear cells (BMMC) at the time of multiple myeloma diagnosis from 157 treatment-naïve patients between December 2014 and December 2019. BMMCs from healthy donors were purchased from ATCC or acquired from diagnostic BM aspirates of healthy individuals. PBMCs and BMMCs were isolated by Ficoll (GE Healthcare) density-gradient centrifugation and cryopreserved until use. The baseline characteristics of patients with multiple myeloma in this study are described in Supplementary Table S1.

Measurement of anti-human cytomegalovirus IgG antibody

BM plasma samples were subjected to the Architect CMV IgG testing (Abbott), an automated method using Architect i4000 (Abbott) based on chemiluminescent microparticle immunoassay used for the qualitative detection and semiquantitative determination of IgG antibodies to a viral lysate (AD169) of cytomegalovirus (CMV). Testing was performed by SCL Healthcare according to the manufacturer's specifications. The results were reported in arbitrary units/mL (AU/mL) as negative (< 6.0 AU/mL) or positive (≥ 6.0 AU/mL) without equivocal range.

NK-cell sorting

NK cells were isolated among thawed BMMCs from patients with NDMM using a human NK Cell Isolation Kit (Miltenyi Biotec). After magnetic bead separation according to the manufacturer's protocol, enriched cells were labeled with a mixture of APC–H7–CD45, PerCP–CD3/7-AAD, PE–TR–CD14/CD19, FITC–CD56, and APC–NKG2C antibodies. $CD3^-CD14^-CD19^-CD56^+$ NK cells were sorted for single-cell RNA sequencing, whereas $CD3^-CD14^-CD19^-CD56^+NKG2C^-$ (conventional NK) or $CD3^-CD14^-CD19^-CD56^+NKG2C^+$ (adaptive) NK subsets were sorted to >98% purity for cytotoxicity assays using a FACS Aria Fusion (BD Biosciences). Sorted NK cells were immediately processed for single-cell RNA sequencing or cytotoxicity assay. The gating strategy for NK-cell sorting is provided in Supplementary Fig. S1.

Droplet-based single-cell RNA sequencing

Sorted single cells were processed using the 10X Chromium Single cell 3′ Reagent Kit v3 (10X genomics) following the manufacturer's protocol as described previously (43). Briefly, sorted NK cells were suspended in PBS with 0.5% BSA and mixed with RT reagent mix and RT primer, which were then added to each channel in 10X chips targeting 5,000 cells. Cells were separated into gel beads in emulsion, where RNA transcripts from single cells were barcoded and reverse transcribed. After cDNA library construction and amplification, cDNAs were enzymatically fragmented, end-repaired, and A-tailed. After selecting the appropriate size of processed cDNA molecules through double-sized size selection using SPRI beads (Beckman Coulter), they were ligated with an adaptor and sample-index PCR performed. After another double-sized size selection using SPRI beads, the final library constructs were diluted 10-fold and run on the Agilent Bioanalyzer High Sensitivity Chip for quality control. Single-cell libraries were then sequenced using the Illumina HiSeq-X platform.

Preprocessing of single-cell RNA data

Raw sequencing data were demultiplexed and mapped to the human reference genome (GRCh38; 10× cell ranger reference GRCh38 v3.0.0) using the Cell Ranger 3.0.2 toolkit from 10X Genomics (http://10xgenomics.com). Using R package "Seurat" (version 3.0.0), raw expression matrices were built using the Read10X function. Initial quality control revealed high NK-cell purity, optimal library assembly, and sequencing. The majority of the sequenced cells had more than 3,000 median unique molecular identifiers (UMI) and a minimum of 1,000 genes associated with the cell barcodes. Cells with fewer than 1,000 detected genes or more than 6,000 detected genes (considered as potential doublets) were excluded. Most of the cells had < 7% of the total gene expression transcribed from mitochondrial genes, indicating robust cell viability. Cells with high mitochondrial gene-associated UMI counts (≥ 7% of total) were considered dead cells and excluded. Genes expressed by fewer than three cells were removed. In addition, a small number of contaminating immune cells, including B cells, plasma cells, T cells, and myeloid cells, were excluded after the initial round of clustering. After removing unwanted cells and genes, UMI counts for each gene in a cell were divided by the total expression of a given cell and multiplied by 10,000 and log transformed. The genes were then scaled and centered by regressing out variables, such as mitochondrial gene percentage and number of UMIs.

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Identifying variable genes and dimensionality reduction

R package "Seurat" was used for the clustering analysis as described previously (43). First, highly variable genes across datasets were identified using the FindVariableFeatures function in Seurat with option selection.method = "vst." The 2,000 genes with the highest variability were selected for downstream analysis. Using the identified variable genes, initial dimensionality reductions were performed using principal component analysis (PCA). JackStraw function was used to determine the significance of PCA scores. The most significant 30 principal components were used as input for the uniform manifold approximation and projection (UMAP) to reduce the data to a two-dimensional space.

Cluster analysis

We performed unsupervised clustering of the top 2,000 highly variable genes for all samples to cluster the cells by their transcriptional profiles as described previously (43). We built a shared nearest neighbors graph on the principal component space using the FindNeighbors function. Next, we applied the Louvain algorithm for modularity optimization-based clustering using the FindClusters function. Clustering resolution parameters were set at the point where clusters exhibited highly distinct transcriptional profiles. Cluster-specific markers were differentially expressed genes for each cluster identified through the FindMarkers function in Seurat with the following settings: min.pct = 0.3, logfc.threshold = 0.25, min.diff.pct = 0.15, and test.use = "MAST." Identified markers with an adjusted $P < 0.05$ were used for further analysis. To remove unwanted sources of variation, such as gender, cells were split by the presence of female-specific transcript *XIST* and integrated. To integrate different datasets, we log normalized each raw expression matrix and identified the top 2,000 highly variable genes for each dataset. Using the FindIntegrationAnchors function in Seurat, we identified anchors between datasets. Next, the datasets were integrated on the basis of the identified anchors via the IntegrateData function in Seurat. Integrated datasets were then scaled and the dimensions reduced for further cluster analysis. Clusters in the integrated datasets were annotated by the expression profiles of genes in the identified maker lists.

Gene ontology enrichment analysis

Gene ontology (GO) enrichment analysis of the cluster markers was performed using R package "goseq" (version 1.34.0) as described previously (43). Depending on the gene length, weightings for each gene were obtained and the Wallenius approximation used to identify associated GO terms. GO terms with an adjusted $P < 0.05$ and biological process categories were selected.

Multicolor flow cytometry

Cryopreserved PBMCs and BMMCs were thawed and stained using the Live/Dead fixable cell stain kit (Invitrogen) to exclude dead cells from the analysis. After washing with FACS staining buffer, these cells were stained with the indicated fluorochrome-conjugated antibodies for 20 minutes at room temperature. For intracellular staining, surface-stained cells were fixed and permeabilized using a FoxP3 staining buffer kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Multicolor flow cytometry was performed using an LSR II flow cytometer (BD Biosciences) and the data analyzed by FlowJo V10 software (Treestar). The antibodies used for flow cytometry are detailed in Supplementary Table S2, and the gating strategies are summarized in Supplementary Fig. S1.

Drug treatment of patient samples and intracellular cytokine staining

Cryopreserved BMMCs from patients with NDMM were thawed and incubated overnight in RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were resuspended in RPMI1640 medium supplemented with 10% FBS and 2×10^5 cells added to each well of a U-bottom 96-well plate. Plated BMMCs were cultured with bortezomib (Merck), lenalidomide (Sigma-Aldrich), or daratumumab (Creative Biolabs) for 6 hours in the presence of anti-CD107a (BD Biosciences). Brefeldin A and monensin were added 1 hour after the initiation of drug treatment. DMSO or IgG was used as the control for the drugs (lenalidomide and bortezomib) or daratumumab, respectively. Cytokine production was assessed by intracellular staining using antibodies against IFNγ (BD Biosciences) and TNFα (BD Biosciences).

Myeloma cell line and ADCC assay

Cytotoxicity assays were performed using RPMI 8226 myeloma cells (ATCC) cultured in RPMI1640 (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco), which were used at less than five passages. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C and confirmed to be *Mycoplasma* negative (MycoAlert Detection Kit, Lonza). The viability of myeloma cells just before the initiation of the experiment was >95%. Target RPMI 8226 cells were labeled with PKH26 dye (Sigma-Aldrich) according to the manufacturer's protocol, and then cocultured with sorted effector $NKG2C^-$ or $NKG2C^+$ NK cells with various effector to target ratios (0:1, 0.5:1, 1:1, or 2:1) in the presence of IgG (10 μg/mL) or daratumumab (10 μg/mL) for 6 hours. Cells were then harvested and stained with TO-PRO-3 (Thermo Fisher Scientific) to label dead target cells. For intracellular cytokine staining, brefeldin A and monensin were added during 6 hours of incubation.

Statistical analysis

No statistical methods were used to predetermine sample size. The experiments were randomized, and investigators were blinded to allocation during experiments and outcome analyses. All values are presented as mean ± SD. Categorical variables and continuous variables were compared as indicated in the figure legends. Significance was determined by the two-sided Mann–Whitney *U* test to compare two groups or the two-way ANOVA with Holm–Sidak multiple comparisons test for multiple group comparisons. The overall survival (OS) and progression-free survival (PFS) were plotted using the Kaplan–Meier test and compared using the log-rank test. OS was calculated from the date of diagnosis until death from any cause, with surviving patients censored at the last follow-up date. PFS was measured from the date of diagnosis to the date of disease progression, relapse, or death from any cause. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software). Significance was set at $P < 0.05$.

Data and code availability

Source data for all quantifications and R scripts used for analyzing the data and generating figures are available from the corresponding author upon reasonable request. Single-cell RNA sequencing data are available from the National Center for Biotechnology Information's Gene Expression Omnibus under the accession number GSE155795.

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Results

Single-cell RNA sequencing identified a distinct subset of adaptive NK cells

To define the heterogeneity of NK cells and explore their functionality based on the transcriptional profile of CD56$^+$ NK cells in multiple myeloma, we performed droplet-based single-cell mRNA sequencing of CD3$^-$CD14$^-$CD19$^-$CD56$^+$ NK cells isolated from the BMMCs of patients with NDMM (**Fig. 1A**; Supplementary Fig. S1A). After quality control filtering, we had a total of 7,165 cells by pooling the data from 3 patients with NDMM. Initial clustering resulted in seven distinct clusters visualized by a UMAP plot (Supplementary Fig. S2A). Most of the cells expressed high levels of well-defined NK-cell markers (38, 44), such as *CD7, NKG7, NCAM1, KLRD1, KLRF1*, and *GNLY*, and low levels of T cell–specific markers

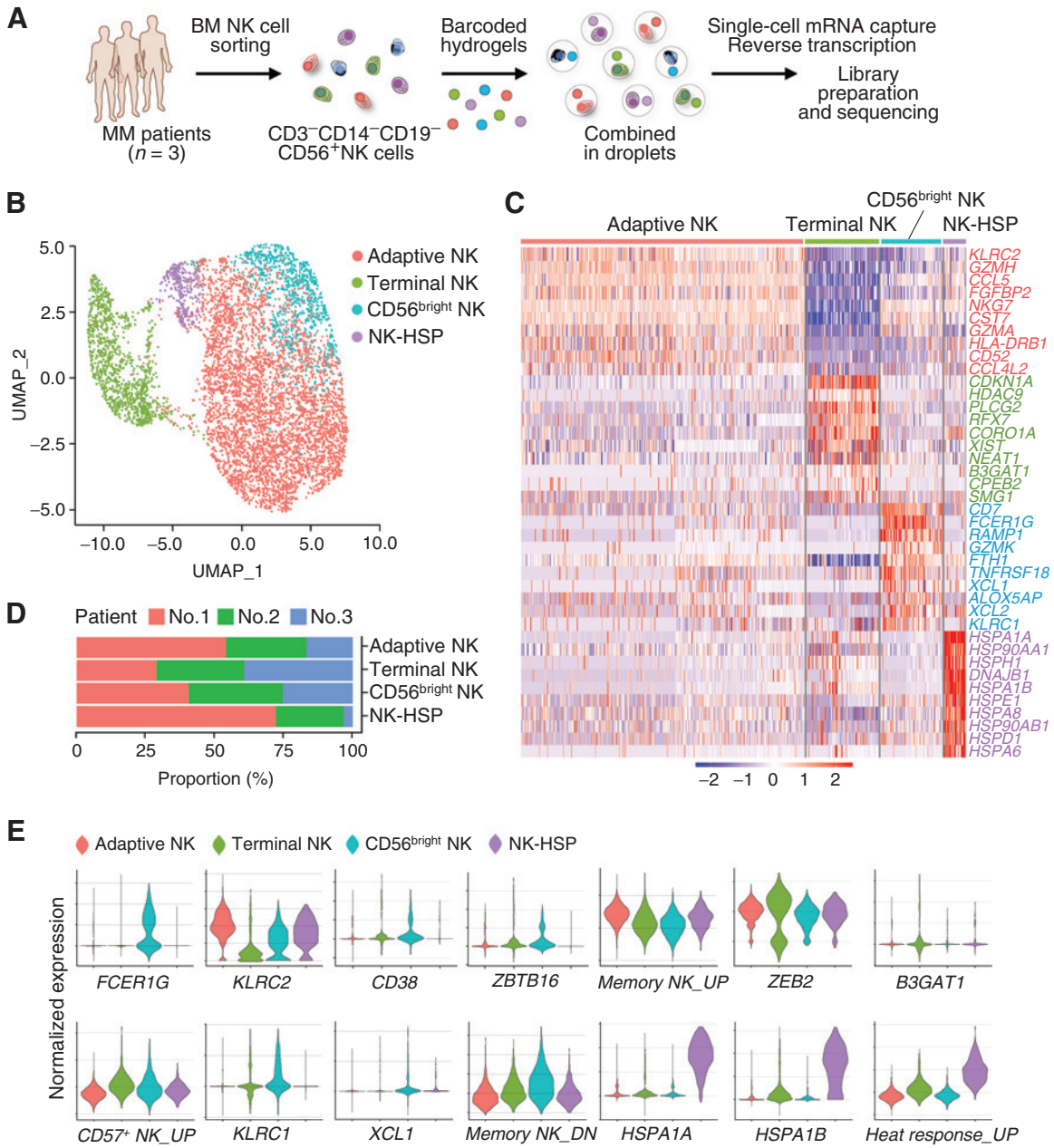

Figure 1.

Single-cell RNA sequencing of BM NK cells from patients with multiple myeloma delineates heterogeneity and identifies adaptive NK cells. **A,** Diagram of droplet-based single-cell RNA sequencing of CD3$^-$CD14$^-$CD19$^-$CD56$^+$ NK cells sorted from the BM of patients with newly diagnosed multiple myeloma. **B,** Visualization of unsupervised clustering of four distinct CD56$^+$ NK-cell clusters by UMAP in the BM of patients with newly diagnosed multiple myeloma. HSP, heatshock proteins. **C,** Heatmap of the scaled expression patterns of the top 10 differentially expressed genes for random sampled cells (maximum thousand cells) for each indicated cluster. **D,** Bar plot showing the composition of the patients within each cluster. **E,** Violin plots showing the expression of top-ranking marker genes and gene sets for each cluster. Log-normalized read counts are given on the *y*-axis (normalized expression).

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(*CD3D/E/G/TRAC*) and myeloid cell–specific markers (*LYZ/MARCO/CD68/FCGR3A*; Supplementary Fig. S2B). However, we noted high expression of B cell–specific markers (*CD79A/IGH/M/G3/A2*) and plasma cell–specific markers (*BACH2/CD38/BCL6/PRDM1/IRF4/XBP1/SDC1/SLAMF7*) in cluster 5 (Supplementary Fig. S2B), which were considered to be non-NK-cell contamination; thus, we excluded these cells in subsequent analyses.

We reanalyzed the 6,819 NK cells, which resulted in four distinct clusters (**Fig. 1B**). When we examined differentially expressed genes (DEG), each NK-cell cluster exhibited substantial cluster-specific gene expression profiles visualized by a heatmap (**Fig. 1C**). On the basis of the cluster-defining DEGs, we termed the four distinct clusters: "adaptive NK," "terminal NK," "$CD56^{bright}$ NK," and "NK-HSP" (**Fig. 1B** and **C**). Notably, we were able to distinguish the adaptive NK-cell cluster based on the high level of *KLRC2* expression and low level of *FCER1G* expression (refs. 33, 36, 39; **Fig. 1C** and **E**). The adaptive NK cluster expressed lower levels of CD38 and PLZF compared with terminal NK or $CD56^{bright}$ NK (**Fig. 1E**). The terminal NK cluster expressed *ZEB2* and *B3GAT1* (39, 45), and the $CD56^{bright}$ NK cluster highly expressed *KLRC1, GZMK, CD7*, and *FCER1G* (**Fig. 1C** and **E**; ref. 39). Finally, a cluster characterized by the high levels of genes for HSP and the gene signature of a cellular response to heat was designated as NK-HSP (**Fig. 1C** and **E**).

The NK-HSP cluster was considered NK cells stressed during freezing and thawing, which was lately identified by single-cell RNA sequencing of fresh and cryopreserved/thawed cells from a healthy identical donor (46). When we examined the relative proportion of each donor within the individual cluster, all of the clusters were found across all donors (**Fig. 1D**). We also validated our clusters with the publicly available sequencing datasets for NK cells derived from healthy donors (34). Gene set enrichment analysis verified upregulation of memory NK genes in the adaptive NK cluster, and upregulation of memory NK-downmodulated genes in the $CD56^{bright}$ NK cluster (**Fig. 1E**). The $CD57^{+}$ NK gene sets from the public data (34) also distinguished the terminal NK cluster (**Fig. 1E**). Moreover, GO suggested possible discrete functions of four distinct BM NK clusters from patients with NDMM (Supplementary Fig. S2C).

Adaptive NK cells have distinct immunophenotypic features

When we analyzed the cellular composition of NK-cell subsets in paired PBMCs and BMMCs from patients with NDMM, we observed an enrichment and a positive correlation of $CD56^{dim}CD16^{+}$ NK cells (Supplementary Fig. S1B; Supplementary Fig. S3A and S3B). As adaptive NK cells can be distinguished by either a lack of *FCER1G* (FcRγ) expression or high expression of *KLRC2* (NKG2C; ref. 36), we defined adaptive NK cells and conventional NK cells as $FcR\gamma^{-}$ and $FcR\gamma^{+}$ NK cells, respectively. We observed a strong correlation in the conventional and adaptive NK-cell composition between paired PBMCs and BMMCs from patients with NDMM (Supplementary Fig. S3C).

Having uncovered adaptive NK cells in the PB and BM of patients with multiple myeloma, we characterized the differential expression pattern of NK inhibitory and activating receptors. First, we examined the expression level of various inhibitory receptors expressed by NK cells, such as killer cell immunoglobulin-like receptor (KIR), NK group 2 member A (NKG2A), T-cell immunoglobulin and ITIM domain (TIGIT), T-cell immunoglobulin mucin receptor 3 (TIM3), lymphocyte activation gene 3 protein (LAG-3), and programmed death 1 (PD-1), which skew NK cells to a hyporesponsive state with decreased functionality (31). Unlike conventional NK cells, adaptive NK cells expressed significantly lower levels of KIR, NKG2A, and TIGIT in both the PB and BM (**Fig. 2A** and **B**). However, LAG-3 and PD-1 were minimally expressed on both conventional and adaptive NK cells from the PB and BM of patients with NDMM (**Fig. 2B**), in line with a recent study (47). There were minor differences in the level of expression of NK-activating receptor NK group 2D (NKG2D) and CD226 (DNAM-1), but natural cytotoxic receptors, NKp46 and NKp30, were decreased on adaptive NK cells compared with conventional NK cells in both the PB and BM of patients with NDMM (**Fig. 2C**), which is consistent with observations in healthy individuals (33). Moreover, in both conventional and adaptive NK cells, a positive correlation was observed between the expression profiles of inhibitory and activating receptors in paired PBMCs and BMMCs from patients with NDMM (Supplementary Fig. S3D), suggesting that adaptive NK cells in PB can mirror those in the BM of patients with NDMM. We further validated the immunophenotypes of conventional and adaptive NK cells in BM samples derived from our large cohort of 157 patients with NDMM with 99.4% human cytomegalovirus (HCMV) seropositivity (Supplementary Table S1), in which we observed consistent results (**Fig. 3A** and **C**).

Daratumumab has been shown to mediate NK fratricide by binding to the heterogeneously expressed CD38 on NK cells (48). We therefore examined the level of CD38 expression on conventional and adaptive NK cells. Of note, adaptive NK cells exhibited a significantly lower level of CD38 expression compared with conventional NK cells in paired PB and BM of patients with multiple myeloma and in BM of healthy donors (Supplementary Fig. S4A and S4B; Supplementary Fig. S5), suggesting that adaptive NK cells may evade daratumumab-induced fratricide. Further analysis revealed that adaptive NK cells express higher levels of Bcl-2 and CD57, but a lower level of PLZF compared with conventional NK cells without significant differences in their expression pattern between patients with multiple myeloma and healthy donors (Supplementary Fig. S4A and S4B; Supplementary Fig. S5), suggesting that adaptive NK cells are indeed differentiated with memory-like properties (36).

Notably, we observed a significantly reduced proportion of adaptive NK cells compared with conventional NK cells in high-risk patients (Supplementary Fig. S6), implying that this is one of the possible explanations for poor responsiveness to daratumumab in this subgroup. However, there were no survival differences according to the level of baseline NK subsets (Supplementary Fig. S7A and S7C), which perhaps owes to the patient and treatment heterogeneity in the absence of daratumumab.

We also detected myeloma cells in the BM of patients with NDMM (Supplementary Fig. S1C). These cells expressed high levels of galectin-9, HLA-ABC, and HLA-E, in contrast to the low expression of poliovirus receptor (Supplementary Fig. S8A and S8C).

Ex vivo treatment of BMMCs with daratumumab discloses superior effector functions of adaptive NK cells

On the basis of the differential expression profile of NK inhibitory and activating receptors between conventional and adaptive NK cells, we sought to understand the functional consequences of these findings related to the current standard treatment approaches for multiple myeloma (1, 2). In this regard, we compared cytokine production (IFNγ and TNFα) and the cytotoxic degranulation activity (CD107a) between conventional and adaptive NK cells in response to the treatment of lenalidomide, bortezomib, or daratumumab for 6 hours to cultured BM cells from patients with NDMM (**Fig. 4A**). Lenalidomide or bortezomib minimally induced cytokine production and degranulation in both conventional and adaptive NK cells from

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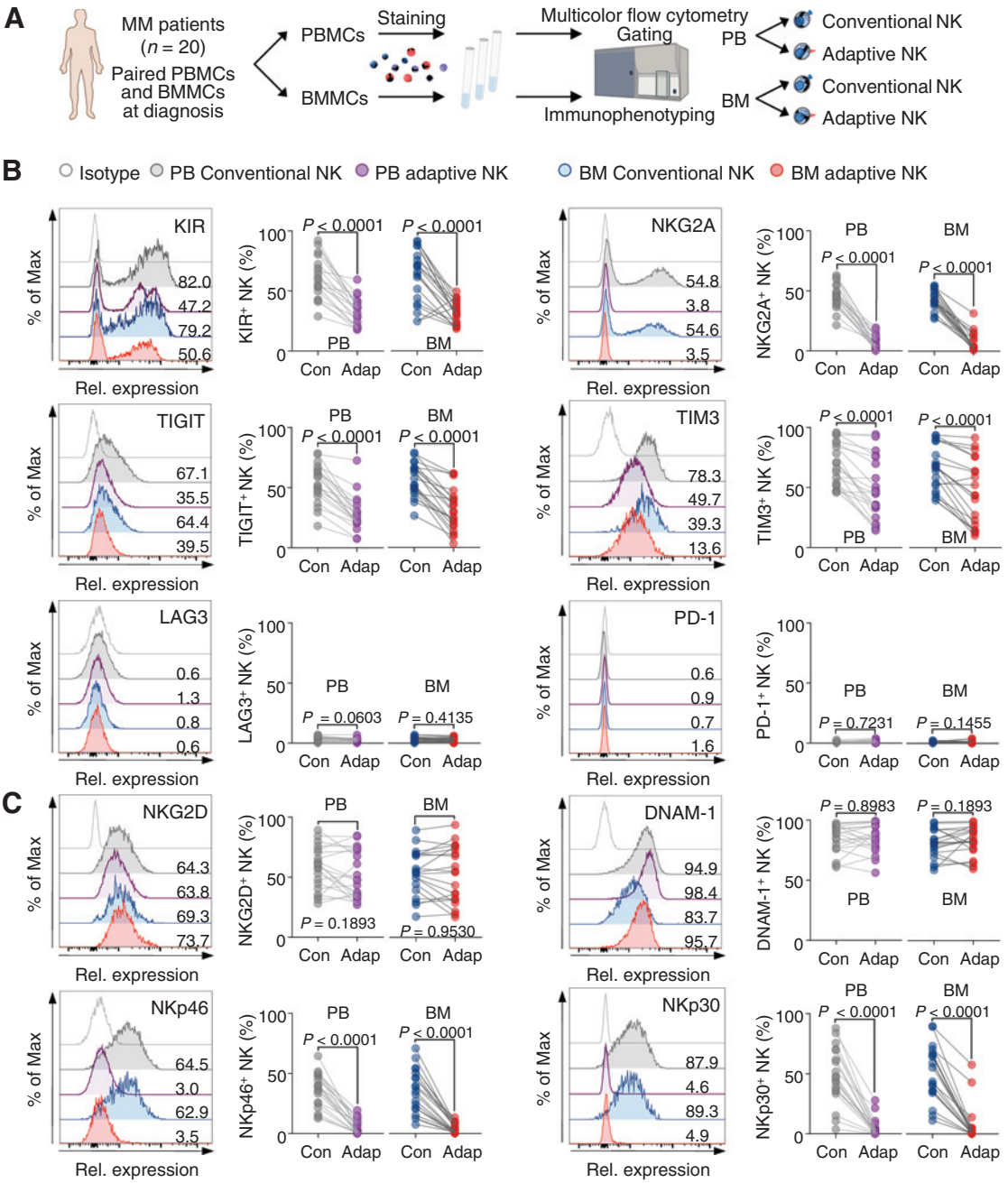

Figure 2.

Distinct immunophenotypic features of adaptive NK cells compared with conventional NK cells in paired PBMCS and BMMCs from patients with newly diagnosed multiple myeloma. **A,** Diagram depicting the immunophenotypic analysis of conventional and adaptive NK cells by flow cytometry in paired PBMCs and BMMCs from patients with newly diagnosed multiple myeloma. **B** and **C,** Representative FACS histograms showing the expression of indicated proteins on gated conventional (Con) or adaptive (Adap) NK cells and percentage of positive cells among the gated cells. Each dot indicates a value obtained from 1 patient and $n = 20$ patients per group from three independent experiments. *P* values were determined versus conventional NK cells using the Mann–Whitney *U* test.

patients with NDMM (**Fig. 4B**). However, when treated with daratumumab, adaptive NK cells from patients with NDMM exhibited strong cytokine production and augmented degranulation with increased polyfunctionality (defined as simultaneous positivity for IFNγ, TNFα, and/or CD107a) in a dose-dependent manner compared with conventional NK cells (**Fig. 4A** and **C**). Higher effector functionality of adaptive NK cells was maintained but not increased when daratumumab was combined with lenalidomide, bortezomib, or lenalidomide plus bortezomib at 6 hours (Supplementary Fig. S9A and S9C), which may require a longer incubation period for their synergism (49).

Next, we analyzed the relative frequencies of NK cells and their subsets in correlation with the functional activity of total BM NK cells from patients with NDMM. The percentage of $CD56^{dim}CD16^{+}$ NK

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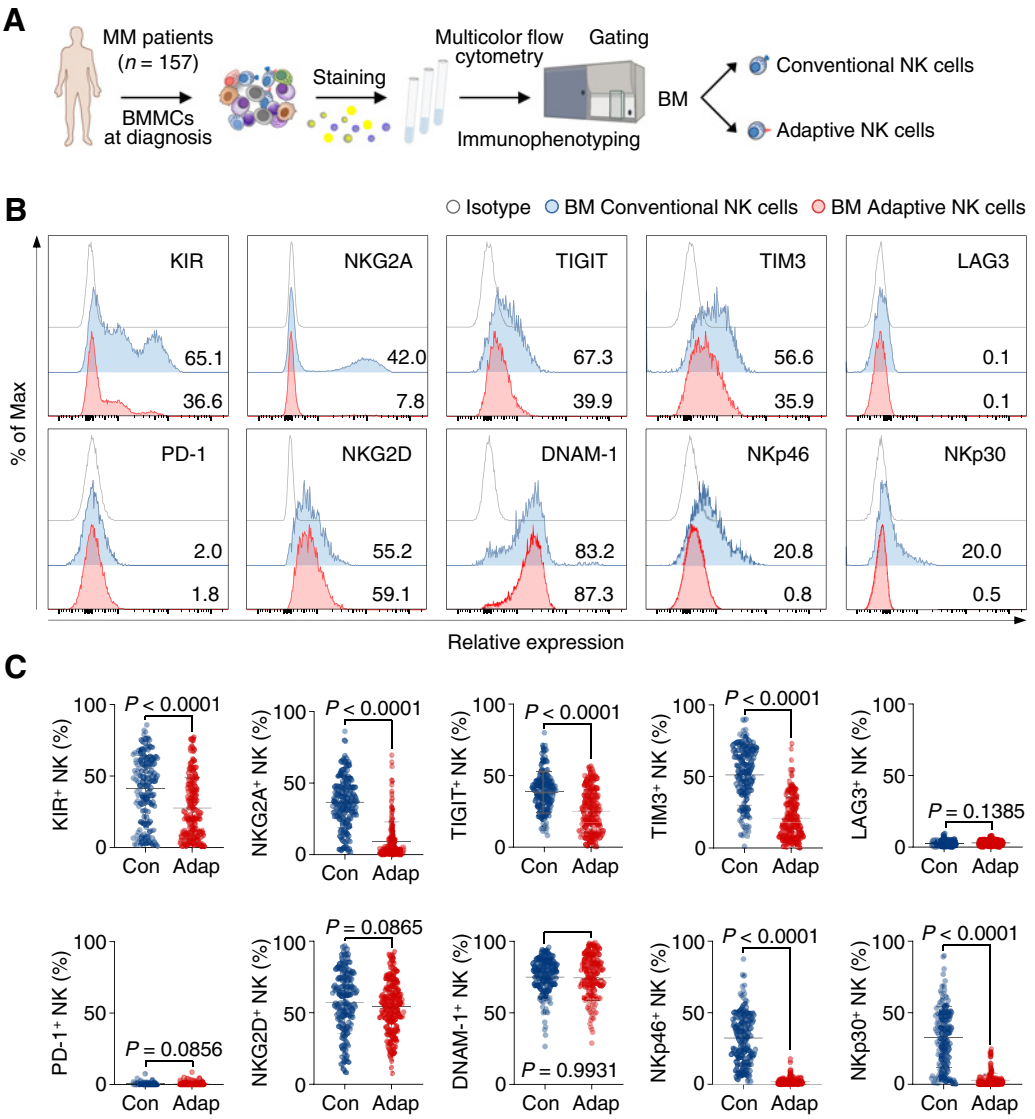

Figure 3.

BM adaptive NK cells have distinct immunophenotypic features compared with conventional NK cells in multiple myeloma (MM). **A,** Diagram depicting the immunophenotypic analysis of conventional and adaptive NK cells by flow cytometry in BMMCs of patients with multiple myeloma at diagnosis. **B,** Representative FACS histograms showing the expression of the indicated proteins on gated conventional (Con) or adaptive NK cells (Adap) among BMMCs from patients with multiple myeloma. **C,** Summary data on the percent of positive cells among the gated cells. Each dot indicates a value obtained from 1 patient and $n = 157$ patients per group from four independent experiments. *P* values were determined versus conventional NK cells using the Mann–Whitney *U* test.

cells among BMMCs showed trend toward correlation with the percentage of cytokine production and degranulation, but did not reach significance (**Fig. 5A**). However, the percentage of adaptive NK cells, but not conventional NK cells, among $CD56^{dim}CD16^{+}$ NK cells weakly but significantly correlated with their effector functions, including cytokine production, degranulation, and polyfunctionality, upon daratumumab treatment to BMMCs from patients with NDMM (**Fig. 5B** and **C**), suggesting the importance of adaptive NK cells in the daratumumab-dependent effector functions of the BM NK-cell population.

To gain further insight into the role of adaptive NK cells in multiple myeloma, we sought to corroborate the correlation between the immunophenotype of adaptive NK cells and their daratumumab-dependent effector functions. Correlation analysis between the percentage of KIR^{+}, $NKG2A^{+}$, $TIGIT^{+}$, $TIM3^{+}$, $NKG2D^{+}$, $DNAM\text{-}1^{+}$, $NKp46^{+}$, or $NKp30^{+}$ cells among adaptive NK cells and their effector functions indicated that the percentage of $TIGIT^{+}$ cells among adaptive NK cells significantly and negatively correlated with their daratumumab-dependent effector functions (Supplementary Fig. S10A and S10B), suggesting a regulatory effect of TIGIT on the antibody-dependent effector functions of adaptive NK cells.

Collectively, our results show that adaptive NK cells mediate superior effector functions upon daratumumab treatment, and that their composition determines the functional activity of total BM NK cells of patients with multiple myeloma.

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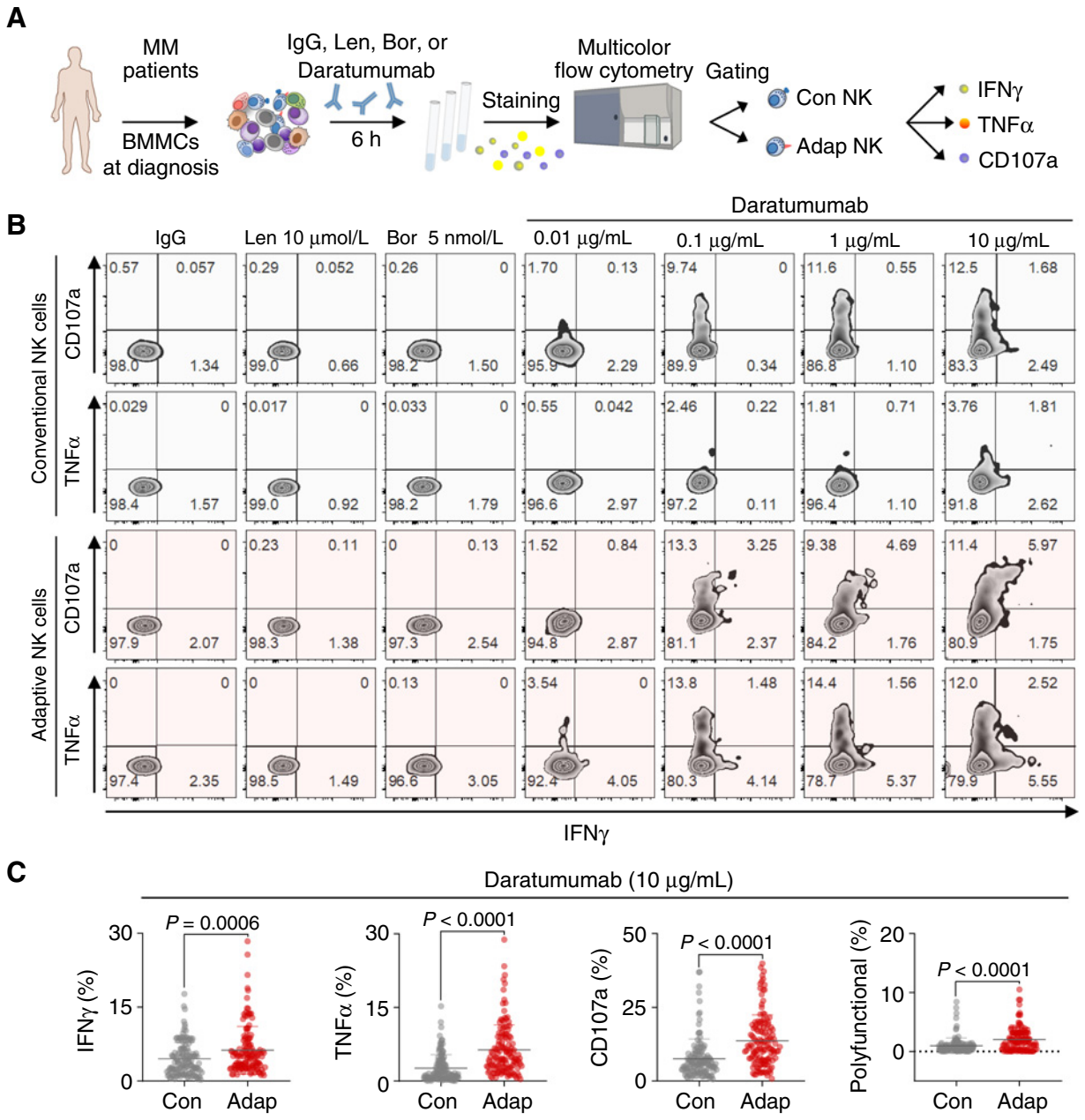

Figure 4.

Adaptive NK cells from patients with multiple myeloma (MM) exhibit robust effector functions upon daratumumab treatment. **A,** Diagram depicting the analysis of effector functions of conventional (Con) and adaptive (Adap) NK cells by flow cytometry after *ex vivo* treatment with IgG (10 μg/mL), lenalidomide (Len, 10 μmol/L), bortezomib (Bor, 5 nmol/L), or daratumumab (0.01–10 μg/mL) for 6 hours using BMMCs from patients with newly diagnosed multiple myeloma. **B,** Representative flow cytometry plots of IFNγ and TNFα production and CD107a expression among the conventional (Con) and adaptive (Adap) NK cells ($n = 3$ patients) post-treatment. **C,** Percentage of IFNγ$^+$, TNFα$^+$, CD107a$^+$, and polyfunctional cells that coexpress IFNγ$^+$, TNFα$^+$, or CD107a$^+$ after daratumumab (10 μg/mL) treatment for 6 hours. Each dot indicates a value obtained from 1 patient and $n = 133$ patients per group from four independent experiments. *P* values were determined versus Con using the Mann–Whitney *U* test.

Adaptive NK cells efficiently kill myeloma cells in the presence of daratumumab

To better delineate the differential functions of NK cells in multiple myeloma, we assessed daratumumab-mediated cytotoxicity against RPMI 8226, a multiple myeloma cell line with high cell surface CD38 expression (50). For this purpose, we sorted conventional or adaptive NK cells from the BMMCs of patients with NDMM according to the cell surface expression of NKG2C (**Fig. 6A**). Sorted conventional or adaptive NK cells were cocultured with multiple myeloma cells at different effector-to-target ratios and treated with either IgG or daratumumab (**Fig. 6A**). The cytotoxic activity against multiple myeloma cells was profound in adaptive NK cells upon daratumumab

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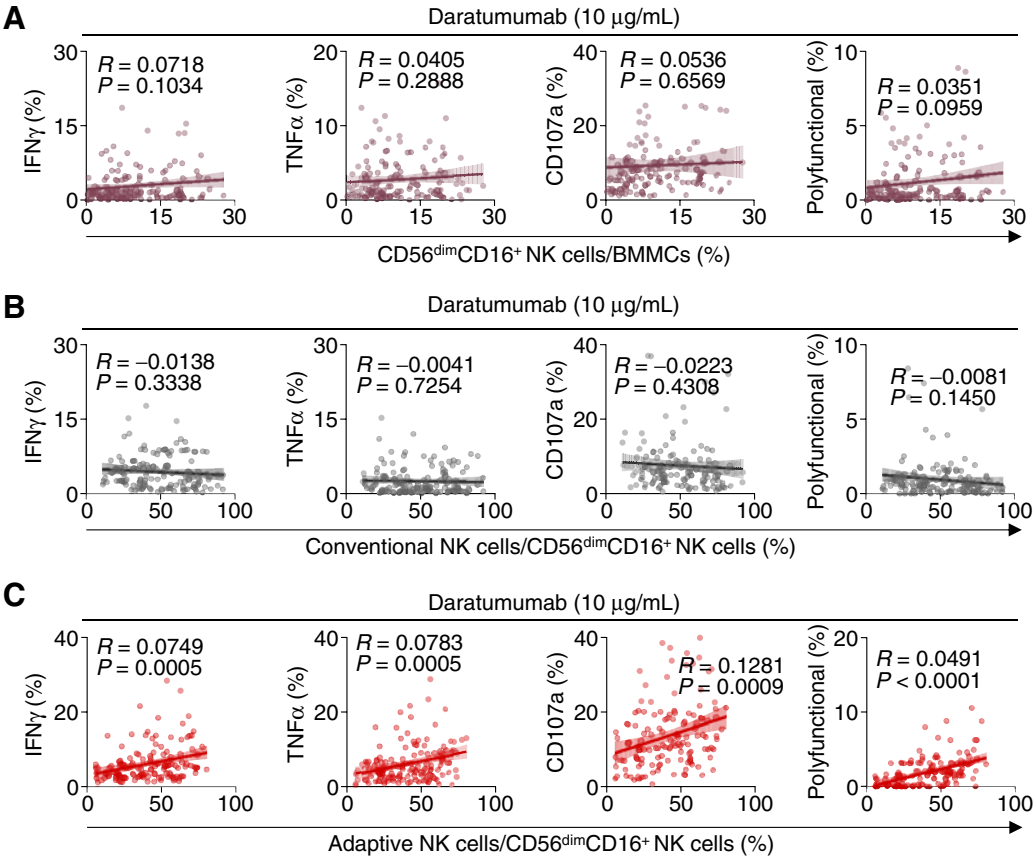

Figure 5.

Frequency of adaptive NK cells, but not conventional NK cells positively correlates with their effector functions upon daratumumab treatment *ex vivo*. Correlation of the frequency of $CD56^{dim}CD16^{+}$ NK among BMMCs (**A**), conventional NK cells among $CD56^{dim}CD16^{+}$ NK cells (**B**), and adaptive NK cells among $CD56^{dim}CD16^{+}$ NK cells and IFN-γ^{+}, TNF-α^{+}, $CD107a^{+}$, and polyfunctional cells (**C**) by flow cytometry after *ex vivo* daratumumab (10 μg/mL) treatment of BMMCs for 6 hours. Each dot indicates a value obtained from 1 patient and $n = 133$ patients per group from four independent experiments. Correlation coefficients (R) and P values were determined by Spearman rank-order correlation test.

treatment, which was further strengthened by increasing the effector-to-target ratio (**Fig. 6A** and **C**). However, daratumumab-dependent cytotoxicity was not pronounced in conventional NK cells cocultured with multiple myeloma cells and unaffected by changes in effector-to-target ratio (**Fig. 6A** and **C**).

Taken together, we propose that adaptive NK cells in patients with multiple myeloma have distinct immunophenotypic features, and that they mediate robust daratumumab-dependent effector functions and direct cytotoxicity against myeloma cells (Supplementary Fig. S11A and S11B).

Discussion

Our results highlight the importance of adaptive NK cells in multiple myeloma by identifying their specialized immunophenotypic features that are distinct from conventional NK cells, including attenuated expression of NK inhibitory receptors, such as KIR, NKG2A, TIGIT, and TIM3, in addition to the diminished expression of NKp46 and NKp30. Functional analysis and cytotoxic assessment using BMMCs from patients with NDMM suggested that adaptive NK cells are an important mediator of daratumumab-dependent ADCC.

We identified adaptive NK cells present in the BM of patients with multiple myeloma through single-cell RNA sequencing and flow cytometry. Although diverse, most patients have a certain portion of adaptive NK cells, which are associated with HCMV infection universally distributed among individuals (39, 40, 51). The characteristics of the adaptive NK cells found in the BM of patients with multiple myeloma in our study were similar to the previously established characteristics of adaptive NK cells in the PB of healthy donors (33, 34). Our data also revealed reduced expression of NK-activating receptors, NKp46 and NPp30, and inhibitory receptors, KIR and NKG2A, on adaptive NK cells compared with conventional NK cells (33, 34). Adaptive NK cells have weaker natural effector functions against tumor cells, while they exert greatly enhanced antibody-dependent effector functions (52). As the therapeutic effect of antibodies that are currently being used to treat a variety of tumors depend on ADCC, adaptive NK cells may be the major NK-cell subpopulation contributing to therapeutic antibody-induced antitumor activity.

Many of the recent studies on improving the outcomes of patients with multiple myeloma have focused on restoration of the exhausted T-cell response with immune checkpoint modulation, such as PD-1, TIGIT, or 4-1BB on $CD8^{+}$ T cells in multiple myeloma (53–57). However, NK cells undergo alterations toward immune dysfunction during cancer progression as well (52, 58–60). In this regard, blockade of NK inhibitory receptors has been shown to have the potential to restore NK-cell dysfunction in various tumors (61–63). In particular,

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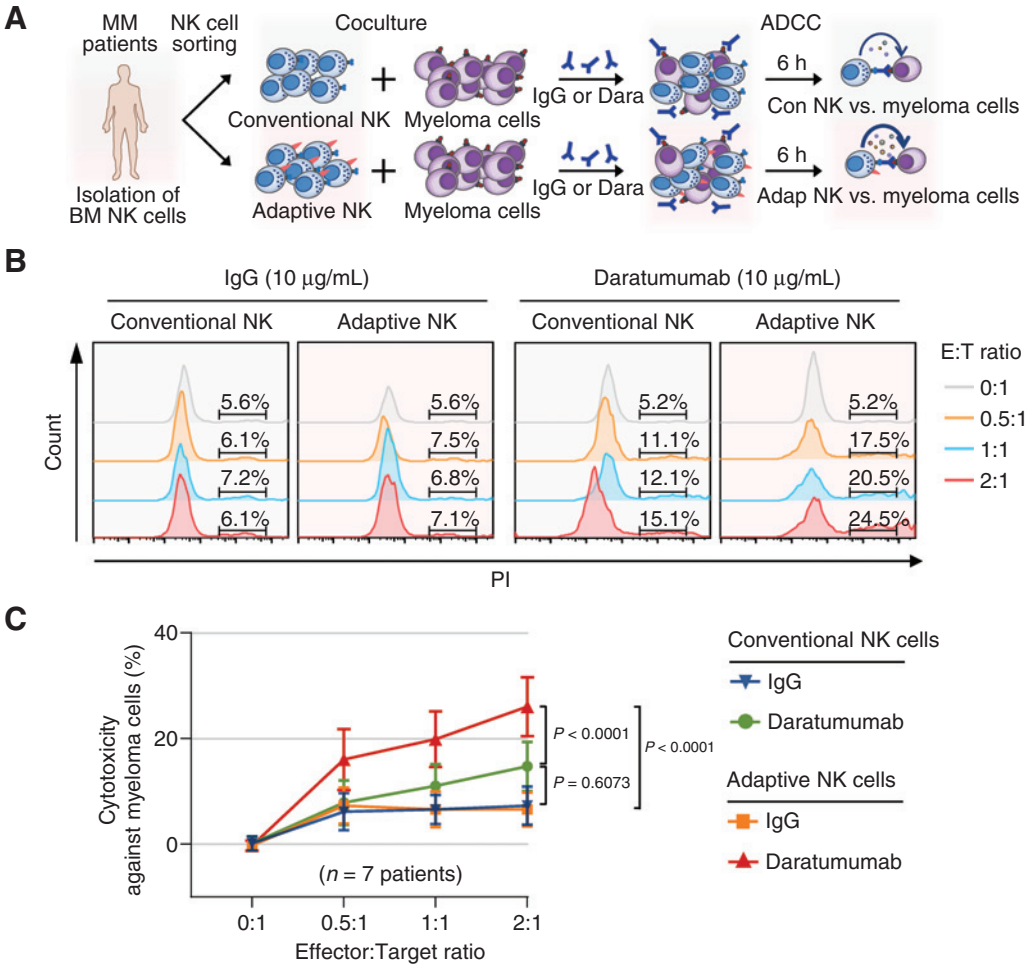

Figure 6.

Adaptive NK cells play a greater role than conventional NK cells in killing myeloma cells upon daratumumab treatment *ex vivo*. **A,** Diagram depicting the analysis of daratumumab-mediated cytotoxicity against PKH26-labeled myeloma cells (RPMI 8226) after coculture and treatment with IgG (10 μg/mL) or daratumumab (10 μg/mL) for 6 hours using FACS-sorted conventional or adaptive NK cells from the BMMCs of patients with multiple myeloma at diagnosis with various effector:target (E:T) ratios. **B,** Cytotoxicity was evaluated by staining with TO-PRO-3-iodide and flow cytometric analysis. Representative histograms are shown. **C,** Summary data showing the cytotoxicity of conventional or adaptive NK cells against myeloma cells in the presence of IgG or daratumumab at the indicated effector:target ratios. Horizontal bars indicate mean ± SD of $n = 7$ patients per group from two independent experiments. *P* values were determined versus IgG-treated conventional NK cells, daratumumab-treated conventional NK cells, or IgG-treated adaptive NK cells using Mann–Whitney *U* test.

tumor-infiltrating NK cells exhibit weak PD-1 expression but increased TIGIT expression (58). In this study, we demonstrate high expression of TIGIT but weak expression of PD-1 on conventional NK cells from patients with NDMM, whereas TIGIT expression is efficiently downregulated on adaptive NK cells. Moreover, TIGIT expression is negatively correlated with the effector functions of adaptive NK cells when treated with daratumumab *ex vivo*. Our data imply that TIGIT downregulation may be involved in the mechanism underlying enhanced functionality of adaptive NK cells during ADCC.

Daratumumab induces multiple myeloma cell death through various mode of action including not only ADCC by NK cells, but also ADCP, CDC, immunomodulatory effects, and direct induction of apoptosis (64). However, elucidation of the mechanism with a dominant influence in multiple myeloma microenvironment needs further investigation. Despite the efficacy and tolerability of daratumumab in the treatment of multiple myeloma, some patients do not respond to daratumumab and have progressive disease for unknown reasons (19–22). The level of CD38 expression on multiple myeloma cells is substantially decreased immediately after daratumumab treatment (6). However, the variable clinical responses after receiving daratumumab cannot be explained solely by differential expression of CD38, as many patients continue to maintain a response to daratumumab despite the low level of CD38 expression on multiple myeloma cells (65). The CD38 expression returns to baseline up to 6 months after the last daratumumab treatment (6). Therefore, some other mechanisms may exist to explain the variability in response to daratumumab. In this study, we showed that the proportion of adaptive NK cells varies among patients with NDMM, and that their frequency significantly correlates with the functional activity of total BM NK cells when treated with daratumumab *ex vivo*. Our data suggest that differences in the composition of the NK-cell subsets, including the proportion of adaptive NK cells, may at least in part contribute to predicting the antibody-dependent response against multiple myeloma.

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Considering the strong ADCC activity of adaptive NK cells, whether the composition of adaptive NK cells can predict the treatment response of daratumumab among patients with multiple myeloma needs to be examined. Importantly, we have shown that the proportion and phenotype of adaptive NK cells significantly correlate between the PB and BM of patients with NDMM, supporting future investigations to assess whether adaptive NK cells in PB may be useful to predict the treatment response to daratumumab in a relatively easier way.

Better understanding of the mechanisms that enable augmented ADCC by adaptive NK cells and how they cooperate with the other immune cells for anti-myeloma activity is still needed. Nevertheless, the identification of adaptive NK cells and demonstration of their important role in ADCC is a crucial step toward predicting and improving the survival of patients with multiple myeloma. Our results highlighting the significance of adaptive NK cell–mediated ADCC may not be limited to multiple myeloma and should support further efforts to predict and promote the efficacy of mAb treatment in cancer.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

H. Cho: Conceptualization, data curation, formal analysis, funding acquisition, investigation, visualization, methodology, writing–original draft. **K.H. Kim:** Conceptualization, data curation, software, formal analysis, writing–original draft. **H. Lee:** Data curation. **C.G. Kim:** Data curation. **H. Chung:** Data curation. **Y.S. Choi:** Data curation. **S.-H. Park:** Data curation. **J.-W. Cheong:** Data curation. **Y.H. Min:** Data curation, supervision. **E.-C. Shin:** Conceptualization, resources, data curation, supervision. **J.S. Kim:** Conceptualization, resources, data curation, supervision.

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Adaptive Natural Killer Cells Facilitate Effector Functions of Daratumumab in Multiple Myeloma

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