Clinical Cancer Research

Fratricide of NK Cells in Daratumumab Therapy for Multiple Myeloma Overcome by *Ex Vivo*–Expanded Autologous NK Cells

Yufeng Wang[1], Yibo Zhang[1], Tiffany Hughes[1], Jianying Zhang[2], Michael A. Caligiuri[1,2,3,4], Don M. Benson[1,2,3,4], and Jianhua Yu[1,2,3,4]

Abstract

Purpose: Daratumumab and its use in combination with other agents is becoming a new standard of care for the treatment of multiple myeloma. We mechanistically studied how daratumumab acts on natural killer (NK) cells.

Experimental Design: Quantities of NK cells in peripheral blood and/or bone marrow of patients with multiple myeloma or healthy donors were examined by flow cytometry. NK-cell apoptosis and the associated mechanism were assessed by flow cytometry and immunoblotting. Patients' NK cells were expanded *in vitro* using feeder cells. Combination treatment of daratumumab and expanded NK cells was performed using an MM.1S xenograft animal model.

Results: $CD38^{-/low}$ NK cells survived, whereas $CD38^{+}$ NK cells were almost completely eliminated, in peripheral blood and bone marrow of daratumumab-treated multiple myeloma patients. NK-cell depletion occurred due to daratumumab-induced NK-cell fratricide via antibody-dependent cellular cytotoxicity. Consequently, $CD38^{-/low}$ NK cells were more effective for eradicating multiple myeloma cells than were $CD38^{+}$ NK cells in the presence of daratumumab. Blockade of CD38 with the $F(ab)_2$ fragments of daratumumab inhibited the antibody-mediated NK-cell fratricide. $CD38^{-/low}$ NK cells displayed a significantly better potential for expansion than $CD38^{+}$ NK cells, and the expanded NK cells derived from the former population were more cytotoxic than those derived from the latter against multiple myeloma cells. Therefore, infusion of *ex vivo*–expanded autologous NK cells from daratumumab-treated patients may improve the antibody therapy.

Conclusions: We unravel a fratricide mechanism for daratumumab-mediated NK-cell depletion and provide a potential therapeutic strategy to overcome this side effect in daratumumab-treated patients with multiple myeloma. *Clin Cancer Res; 24(16); 4006–17. ©2018 AACR.*

Introduction

Multiple myeloma is one of the most frequently diagnosed hematologic cancers occurring in developed countries, accounting for approximately 2% of all cancer-related deaths and 10% to 15% of all hematologic malignancies in the United States (1). The recent development and FDA approval of therapeutic mAbs, including daratumumab (an mAb against CD38) and elotuzumab (an mAb against CS1), is changing the treatment algorithm for multiple myeloma. However, multiple myeloma still relapses and remains incurable, with especially short progression-free survival periods (less than 21 months; refs. 2–5).

As a single agent, elotuzumab is safe but has low efficacy, whereas daratumumab has a response rate of over 30% (6, 7). As with many other cancers, combination therapy has always been more successful compared with single agents in treating multiple myeloma, as each patient has multiple myeloma clones at the time of diagnosis, and more clones develop after relapse; daratumumab combined with lenalidomide and/or dexamethasone is becoming a new standard-of-care treatment for multiple myeloma (2). Recent clinical trials of bortezomib and dexamethasone with daratumumab showed a significant improvement in the rate of progression-free survival (2, 8). It was also shown that this combination treatment lacked dose-limiting toxic effects and had a greater than 80% overall rate of response (6, 7).

Daratumumab binds the CD38 molecule and mediates tumor cell killing via various mechanisms of action, including complement-dependent cytotoxicity, antibody-dependent cellular phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and direct induction of tumor cell apoptosis (9). ADCC, including that induced by daratumumab, is mediated by natural killer (NK) cells. Moreover, accumulating evidence implicates NK cells as an indispensable component of immunosurveillance preventing tumor occurrence as well as relapse (10, 11).

In the current study, we demonstrate that peripheral blood as well as bone marrow NK cells were depleted in multiple myeloma patients who have undergone daratumumab therapy. This NK-cell depletion occurs as a result of daratumumab-induced fratricide among NK cells, due to high levels of CD38 surface

[1]The Ohio State University Comprehensive Cancer Center, Columbus, Ohio. [2]Center for Biostatistics, Department of Bioinformatics, Columbus, Ohio. [3]The James Cancer Hospital, Columbus, Ohio. [4]Division of Hematology, Department of Medicine, College of Medicine, The Ohio State University, Columbus, Ohio.

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Y. Wang and Y. Zhang contributed equally to this article.

Corresponding Author: Jianhua Yu, Division of Hematology, Department of Internal Medicine, The Ohio State University, 460 West 12th Ave, BRT 816, Columbus, OH 43210. Phone: 614-293-1471; Fax: 614-688-4028; E-mail: jianhua.yu@osumc.edu

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Translational Relevance

We highlight that daratumumab-mediated natural killer (NK) cell depletion in patients with multiple myeloma occurs via a mechanism of NK-cell fratricide. This side effect of daratumumab for patients with multiple myeloma may disrupt NK-mediated antibody-dependent cellular cytotoxicity against multiple myeloma cells and subsequently influences the efficacy of daratumumab therapy and also increases the risk of multiple myeloma relapse. We further demonstrate that *ex vivo*–expanded autologous NK cells have the potential to overcome daratumumab-induced NK-cell depletion to improve daratumumab therapy for multiple myeloma.

expression on these cells. We observed that the remaining NK cells in these patients with multiple myeloma are $CD38^{-/low}$ and *ex vivo*–expanded NK (eNK) cells from daratumumab-treated patients with multiple myeloma are highly proliferative and have the potential to rescue this daratumumab-induced NK-cell depletion in multiple myeloma patents. We believe that our study is clinically significant as daratumumab-treated patients with multiple myeloma frequently relapse, an observation that may be at least partially explained by daratumumab-induced NK-cell depletion.

Materials and Methods

Mice

NOD.Cg-prkdcscid IL2rgtm1Wjl/szJ (NSG) mice (6–8 weeks old) were purchased from The Jackson Laboratory. All experiments were approved by The Ohio State University Animal Care and Use Committee. Mice were checked once a day for signs of discomfort, weight loss, ataxia, and paralysis to measure multiple myeloma progression.

Cell lines

Multiple myeloma cell lines (MM.1S) were obtained from the ATCC and were maintained in RPMI1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Sigma). The NK-92 cell line and K562 feeder cells were received from Dr. Michael A. Caligiuri's laboratory and cultured in RPMI1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Sigma). IL2 (100 U/mL) was also included in the culture of the NK-92 cell line. These cell lines have not been authenticated since receipt but were routinely tested to ensure they are negative for mycoplasma using MycoAlert PLUS Mycoplasma Detection Kit from Lonza.

Patient and healthy donor samples

Peripheral blood and bone marrow samples were collected from patients with multiple myeloma who had undergone treatment with or without daratumumab at the James Cancer Hospital (Columbus, OH). All patient samples were obtained with an IRB approval. Peripheral blood mononuclear cells (PBMCs) of healthy donors were derived from leukopaks obtained from the American Red Cross.

NK-cell expansion

Primary NK cells were purified using a negative selection method, as detailed in Supplementary Information. NK cells were expanded using PBMCs or purified NK cells cultured in the presence of IL2 (100 U/mL) and K562 feeder cells expressing membrane-bound IL21, as described previously (12). In brief, NK cells or PBMCs and irradiated feeder cells (1:1 ratio for NK: feeder cells and 1:2 for PBMCs:feeder cells) were cocultured in RPMI1640 supplemented with 20% heat-inactivated FBS, L-glutamine, and IL2 (100 U/mL) at 37°C in a 5% CO_2 incubator. Media were changed on the basis of cell density, and an equal number of irradiated feeder cells were added every 7 days. Cells preserved for future use were stored at −80°C in a solution of FBS containing 10% DMSO at a maximum density of 2.5×10^7 cells per vial.

Multiple myeloma mouse model and bioluminescence imaging

Firefly luciferase–expressing MM.1S cells were established using a Pinco-pGL3-luc/GFP virus as described previously (13), and GFP-positive cells were purified by FACS. An orthotopic xenograft multiple myeloma model was then established using NSG mice injected intravenously with 8×10^6 cells in 200 μL of saline on day 0 (13). A pilot study was then performed to measure the anti–multiple myeloma activity of *ex vivo*–expanded NK cells. In particular, on days 7 and 14 after tumor inoculation, mice were injected with 5×10^6 NK cells expanded from the PBMCs of daratumumab-treated patients with multiple myeloma. To determine whether NK cells expanded *ex vivo* from the PBMCs of daratumumab-treated patients with multiple myeloma were able to improve the outcome of daratumumab therapy, on days 14, 21, and 28 after tumor inoculation, mice were also injected intravenously with daratumumab at a dose of 8 mg/kg, as described previously (14), followed by intravenous injection with 5×10^6 eNK cells on the following days (i.e., on days 15, 22, and 29). To monitor tumor growth, mice were infused intraperitoneally with D-luciferin (150 mg/kg; Gold Biotechnology; ref. 13) for *in vivo* bioluminescence imaging by In Vivo Imaging System (IVIS-100) with Living Image software (PerkinElmer; ref. 13).

Statistical analysis

Student *t* test or paired *t* test was used to compare two independent or paired groups. Linear or linear mixed models were used to compare multiple groups and account for the covariance structure due to repeated measures. Kaplan–Meier method was used to estimate survival functions, and log-rank test was used to compare any two survival curves. *P* values were corrected for multiple comparisons. A *P* value less than 0.05 was considered statistically significant.

See Supplementary Materials and Methods for additional details.

Results

Daratumumab-induced NK-cell activation

Both daratumumab and NK cells have been shown to play roles in eradicating multiple myeloma cells. For this reason, we set out to determine whether daratumumab activates NK cells and to characterize potential mechanisms by which these effects may occur. We found that daratumumab indeed stimulates NK cells, as evidenced by an increase in expression of *IFNG* mRNA and protein (Supplementary Fig. S1A and S1B). To assess whether daratumumab can also promote NK-mediated ADCC against MM.1S target cells, which robustly express CD38 (Supplementary Fig. S2), we performed standard ^{51}Cr release assays using primary

NK cells from healthy donors as effectors and the MM.1S multiple myeloma tumor cell line as targets. Results suggested that daratumumab can indeed significantly enhance NK cell–mediated cytotoxicity against MM.1S targets (Supplementary Fig. S3A; ref. 9). In particular, this enhanced cytotoxicity seemed to be occurring via ADCC, as the addition of an anti-CD16 blocking Ab greatly diminished the effects of daratumumab (Supplementary Fig. S3A). These daratumumab-mediated effects on NK-cell activation occurred concomitantly with induction of STAT1 phosphorylation and activation of NF-κB p65 (Supplementary Fig. S3B). Notably, even a low dose of daratumumab (1 μg/mL) was sufficient to trigger phosphorylation of STAT1 and activation of NF-κB (Supplementary Fig. S3B). Thus, the aforementioned finding lends further support to the data depicted in Supplementary Fig. S1, which shows that an increase in NK-cell *IFNG* mRNA expression occurs in response to treatment with the same doses of daratumumab. NF-κB and STAT1 activation occurs downstream of factors containing immunoreceptor tyrosine-based activation motifs (15, 16), which are recruited by CD16 in NK cells (17). Accordingly, we found that daratumumab was able to induce *IFNG* expression in NK-92 cells that were CD16 (158V/F) positive, but not in those that were CD16 negative. Because both of the aforementioned populations expressed similar levels of CD38 (Supplementary Fig. S4A and S4B), our findings together implicate CD16 as a factor necessary for daratumumab-triggered activation of NK cells.

$CD38^{+}$ but not $CD38^{-/low}$ NK cells are depleted in daratumumab-treated patients with multiple myeloma

The above data together demonstrate that daratumumab is indeed capable of activating NK cells *in vitro*. Therefore, we set out to next determine how daratumumab affects NK cells in daratumumab-treated patients with multiple myeloma. To this end, we assessed serial samples taken from patients with multiple myeloma undergoing treatment at the James Cancer Hospital. Daratumumab treatment was administered once per week (16 mg/kg) for 8 weeks, then once every other week (8 mg/kg) for 16 weeks, and then once per month (16 mg/kg). Of note, it was reported that after the first 16 mg/kg dose was administered, the mean serum concentration of daratumumab was >250 μg/mL (8). We found that daratumumab treatment significantly reduced the relative abundance of NK cells in the peripheral blood of patients with multiple myeloma, whose NK cells comprised approximately 2% of peripheral blood lymphocytes; in contrast, NK cells represented approximately 10% of peripheral blood lymphocytes in healthy donors or patients with multiple myeloma that had not undergone daratumumab treatment (Fig. 1A and B). NK-cell presence was also diminished within the bone marrow of daratumumab-treated compared with non–daratumumab-treated patients with multiple myeloma (Supplementary Fig. S5A and S5B). In contrast, we did not observe any significant differences in the relative abundance of peripheral blood T or B lymphocytes among healthy donors, non–daratumumab-treated patients with multiple myeloma, and daratumumab-treated patients with multiple myeloma (Fig. 1C and D). NK cells typically express high levels of CD38 in healthy donors (18) and in non–daratumumab-treated patients with multiple myeloma (Fig. 1E); however, the peripheral blood NK-cell population in daratumumab-treated patients with multiple myeloma was composed almost entirely of $CD38^{-/low}$ NK cells (Fig. 1E and F; Supplementary Fig. S5). We found that daratumumab binds to CD38 in a manner that prevents detection with many of the individual anti-CD38 antibodies that are commercially available, while a multi-epitope polyclonal anti-CD38 antibody can be used to detect CD38 expression in daratumumab-treated patients. Accordingly, we made use of this multi-epitope polyclonal anti-CD38 antibody to stain peripheral blood and bone marrow NK cells from daratumumab-treated patients with multiple myeloma as described in Fig. 1E and F; Supplementary Fig. S5C. The above data suggest that $CD38^{+}$ NK cells were susceptible to daratumumab-mediated depletion, whereas $CD38^{-/low}$ NK cells were resistant.

Daratumumab induces NK-cell fratricide via NK-to-NK ADCC

To explore the mechanism by which daratumumab induces NK-cell depletion as shown in Fig. 1, we first tested the effects of daratumumab on NK-cell death. We showed that daratumumab triggers NK-cell apoptotic activity in a dose-dependent manner, which results in a significant reduction in the absolute quantity of primary NK cells (Fig. 2A and B; Supplementary Fig. S6). In addition, we observed that treatment with daratumumab also significantly increased the rate of NK-cell degranulation (Fig. 2C). Interestingly, this daratumumab-induced degranulation did not require the presence of target cells (i.e., tumor cells), as it could be observed when NK cells were treated in the absence of target cells. On the basis of this observation, we predicted that daratumumab may be inducing NK-cell apoptosis through a mechanism involving NK-cell fratricide (NK-mediated cytotoxicity against neighboring NK cells) occurring via ADCC, as a majority of NK cells highly express CD38 (18, 19). To this end, we performed a ^{51}Cr release assay and a flow cytometry–based cytotoxicity assay, both of which indicated that NK cells were indeed able to lyse one another in the presence of daratumumab (Fig. 2D and E). The low percentages of killing in both the daratumumab-treated and untreated groups in the ^{51}Cr release assay could be due to the fact that the ^{51}Cr uptake by primary NK cells tends to be very low [e.g., 1,054 counts per minutes (cpm) for primary NK vs. 10,998 cpm for MM.1S tumor cells in our experiment]. In contrast, we did not observe NK-cell fratricide by instead trying to target CS1 on NK cells (20) with elotuzumab (an anti-CS1 mAb; Fig. 2E). Moreover, we found that when daratumumab was digested into Fc and $F(ab)_2$ fragments using an IgG-specific protease (IdeZ; Supplementary Fig. S7), the fragments failed to increase NK-cell apoptosis (Fig. 2F and G), indicating that daratumumab-augmented NK-cell apoptosis requires the integrity of daratumumab, as depicted in Fig. 2H. NK-92 cells, an NK-cell line that is naturally CD16 deficient but robustly expresses CD38 (Supplementary Fig. S4A), were resistant to daratumumab-induced apoptosis; however, this effect was reversed in NK-92 cells engineered to overexpress native CD16 or a high-affinity version of CD16 (158V/F; Supplementary Fig. S4C). These results indicate that in addition to being required for NK-cell activation (Supplementary Fig. S4B) and being essential for NK-mediated ADCC (21), the presence of functional CD16, also called FcγRIIIA, may also be required for daratumumab-induced NK-cell apoptosis. Furthermore, although the digested $F(ab)_2$ and Fc fractions of daratumumab were still individually able to recognize CD38 and CD16 molecules, respectively, the enzyme-digested version of daratumumab was unable to form a molecular bridge between the CD38 and CD16 receptors on neighboring NK cells, thus lacking a necessary condition for ADCC, and then were incapable of inducing apoptosis of primary NK cells (Fig. 2F and G). These results demonstrate that

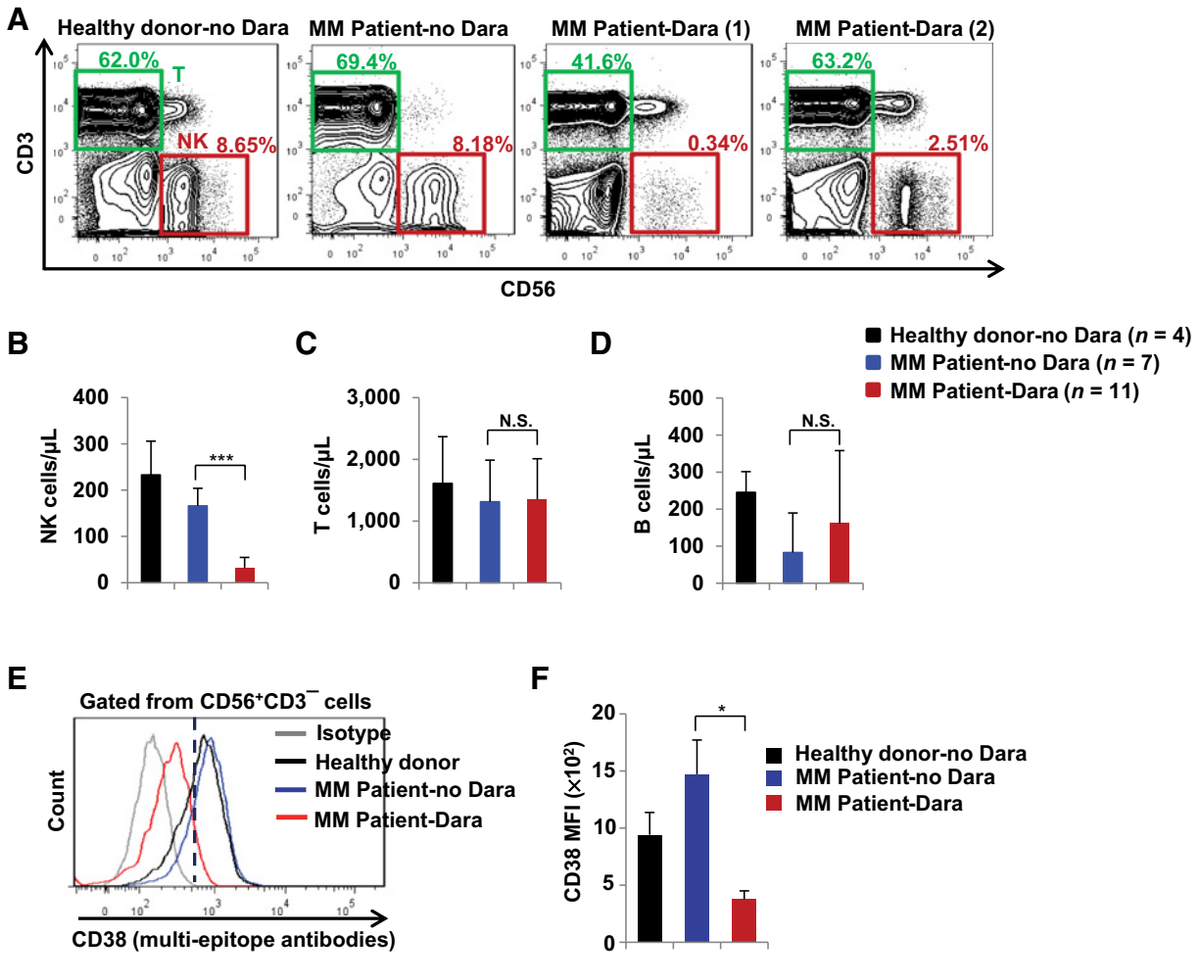

Figure 1.

Daratumumab (Dara) depletes CD38$^+$ NK cells in the peripheral blood of patients with multiple myeloma (MM). **A,** Flow cytometric analysis of NK cells (CD56$^+$CD3$^-$) and T cells (CD56$^-$CD3$^+$) in PBMCs from healthy donors ($n = 4$), patients with multiple myeloma (MM) without daratumumab treatment (MM patient-no Dara, $n = 7$), and patients with multiple myeloma treated with daratumumab (MM patient-Dara, $n = 11$). The representative MM patient-Dara (1) sample was collected after the patient was treated with daratumumab once per week for 3 weeks. The representative MM patient-Dara (2) sample was collected after the patient was treated with daratumumab once per week for 8 weeks followed by every other week for 3 weeks. **B–D,** Quantitative assessments of NK cells (CD56$^+$CD3$^-$), T cells (CD56$^-$CD3$^+$), and B cells (CD3$^-$CD19$^+$) in the peripheral blood of healthy donors-no Dara, MM patients-no Dara, and MM patients-Dara were analyzed by flow cytometry. **E** and **F,** Flow cytometric analysis of CD38 surface expression, as determined by flow cytometric analysis in samples stained with a multi-epitope anti-CD38 antibody, in NK cells from healthy donors-no Dara, MM patients-no Dara, and MM patients-Dara ($n = 3$ for each group). MFI, mean fluorescence intensity. Error bars, SD; N.S., not significant. *, $P < 0.05$; ***, $P < 0.001$.

daratumumab-mediated NK-cell apoptosis occurs through a mechanism of NK-cell fratricide.

CD38$^{-/low}$ NK cells are resistant to daratumumab-induced NK-cell fratricide and thus are better at killing multiple myeloma cells via daratumumab-mediated ADCC

Because daratumumab-induced NK-cell fratricide (Fig. 2) depletes only the CD38$^+$ subset of NK cells in multiple myeloma patients (Fig. 1), we next tested whether CD38$^{-/low}$ NK cells are resistant to daratumumab-induced apoptosis. Approximately 15% of NK cells in human peripheral blood are CD38$^{-/low}$ (Fig. 3A) and, remarkably, daratumumab-induced apoptosis was frequently observed in CD38$^+$, but rarely detected in CD38$^{-/low}$ NK cells (Fig. 3B and C). We also observed that in the absence of daratumumab, the survival rate of CD38$^{-/low}$ NK cells was significantly higher than seen in CD38$^+$ NK cells (Supplementary Fig. S8). Interestingly, although eradication of MM.1S targets was enhanced when CD38$^{-/low}$ and CD38$^+$ NK cells were combined with daratumumab, this effect was stronger within the CD38$^{-/low}$ NK subset (Fig. 3D and E). Presumably, this difference may be attributable, at least in part, to the daratumumab-mediated induction of NK-cell apoptosis as we reported above, which would be happening concurrently in the CD38$^+$ population but not in the CD38$^{-/low}$ population. In accordance with this presumption, the rate of apoptosis was indeed higher within the CD38$^+$ NK-cell subset than in the CD38$^{-/low}$ NK-cell subset during daratumumab-mediated ADCC against MM.1S targets (Fig. 3F). Together, these results indicate that compared with

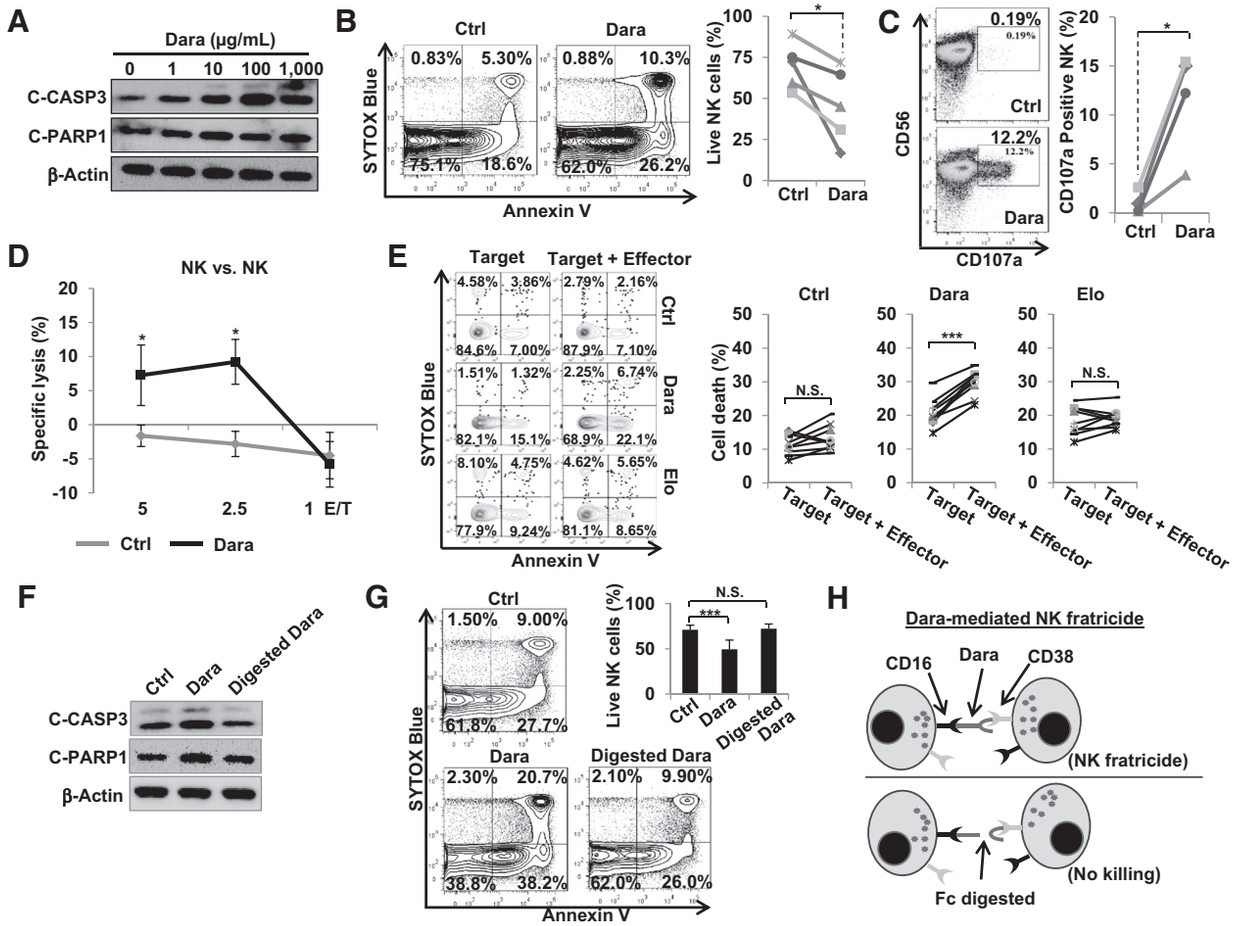

Figure 2.

Daratumumab (Dara) induces primary NK-cell fratricide, which occurs via nontraditional ADCC between NK cells in the absence of tumor targets. **A,** The expression of cleaved(C)-CASP3 and C-PARP1 was detected by immunoblotting after NK cells were treated for 16 hours with various doses of daratumumab as indicated. **B,** NK cells were treated for 16 hours with 10 μg/mL of daratumumab, then stained with an anti-Annexin V antibody and SYTOX Blue viability dye. NK-cell apoptosis was analyzed by flow cytometry ($n = 5$). Each line in the panel on the right indicates survival of daratumumab-treated versus untreated NK cells from the same individual donors. **C,** NK cells were treated for 4 hours with 10 μg/mL of daratumumab; then, expression of CD107a, an NK degranulation marker, was analyzed by flow cytometry ($n = 4$). Each line in the panel on the right indicates daratumumab-treated versus untreated NK cells from the same individual donors. **D,** Daratumumab-mediated NK-cell fratricide (both effector cells and target cells are NK cells) was performed using a standard 4-hour ^{51}Cr-release assay ($n = 3$). **E,** A 4-hour flow cytometry–based cytotoxicity assay was performed (effector/target is 1:1; $n = 9$). NK cells serving as target cells were labeled with CFSE and pretreated with or without daratumumab or elotuzumab (Elo), an anti-CS1 mAb, for 30 minutes. Effector NK cells (effector) were pretreated with F(ab)$_2$ fragments of daratumumab or elotuzumab for 30 minutes to block binding of intact daratumumab or elotuzumab to CD38 or CS1, respectively, thus preventing fratricide among the effector cells. Target cells were gated from CFSE$^+$ events, and cell death was detected by flow cytometric analysis via staining with an anti-Annexin V antibody and SYTOX Blue viability dye. Each line in the panel on the right compares target alone versus target + effector group from the same individual donors. **F,** Expression of cleaved(C)-CASP3 and C-PARP-1 was detected by immunoblotting after 16-hour treatment with 10 μg/mL of intact daratumumab or daratumumab enzyme digested by an IgG-specific protease (IdeZ). **G,** NK cells were treated for 16 hours with 10 μg/mL of daratumumab or enzyme-digested daratumumab; then, apoptosis was analyzed ($n = 9$) as described in **B**. **H,** Schematic detailing mechanism for daratumumab-induced NK-cell fratricide. CASP3, caspase-3. Error bars, SD; N.S., not significant; *, $P < 0.05$; ***, $P < 0.001$.

CD38$^+$ NK cells, CD38$^{-/low}$ NK cells are superior at acting cooperatively with daratumumab to eradicate multiple myeloma cells via ADCC.

CD38$^{-/low}$ NK cells from healthy donors are more proliferative than their CD38$^+$ counterparts, and expanded cells from the former population are more cytotoxic than those from the latter

The NK cells that remain in patients with multiple myeloma following daratumumab treatment are primarily CD38$^{-/low}$ and resistant to daratumumab-induced fratricide, and we show above that compared with CD38$^+$ NK cells, CD38$^{-/low}$ NK cells have the better potential to cooperate with daratumumab to eradicate multiple myeloma tumor cells. However, CD38$^{-/low}$ NK cells represent a relatively minor population in healthy donors, accounting for approximately 15% of their peripheral blood NK cells (Fig. 3A). We therefore investigated the potential to expand these cells *in vitro*, and then, we characterized the expanded cells. For this purpose, peripheral blood NK cells from healthy donors were FACS-purified into two separate populations: CD38$^{-/low}$ or CD38$^+$. Both of these NK-cell subsets were then expanded on a

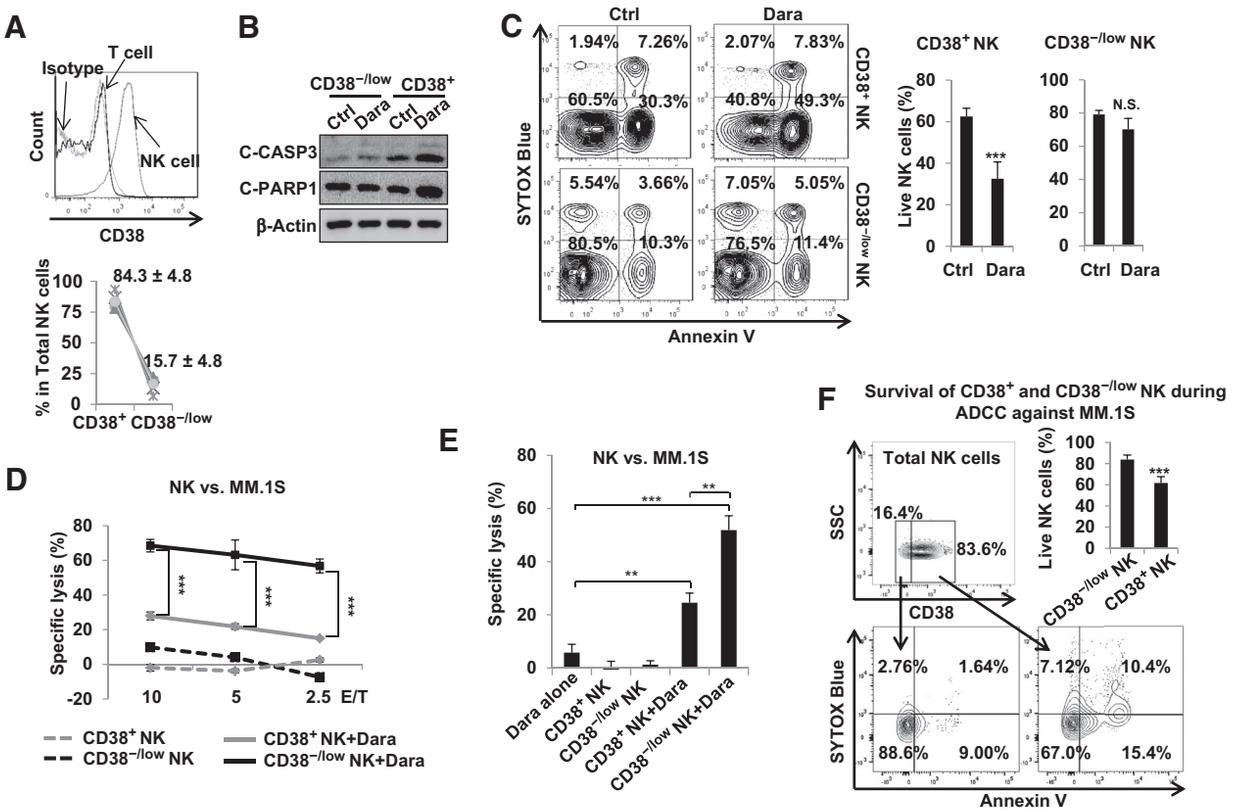

Figure 3.

$CD38^{-/low}$ primary NK cells are resistant to daratumumab-induced apoptosis and, compared with $CD38^{+}$ NK cells, show better efficacy at daratumumab-mediated ADCC against multiple myeloma (MM) cells. **A,** Expression of CD38 was detected by flow cytometry using healthy donor PBMCs. Percentages of $CD38^{-/low}$ and $CD38^{+}$ populations among total NK cells were analyzed ($n = 6$). Each line in the bottom indicates proportion of $CD38^{+}$ and $CD38^{-/low}$ NK cells within the same individual donors. **B,** Immunoblotting analysis was performed after $CD38^{-/low}$ or $CD38^{+}$ NK cells were cultured for 16 hours in the presence or absence of 10 μg/mL of daratumumab. **C,** $CD38^{-/low}$ or $CD38^{+}$ NK cells were treated for 16 hours with 10 μg/mL of daratumumab, then stained with an anti-Annexin V and SYTOX Blue viability dye ($n = 9$). **D,** Daratumumab-mediated ADCC against MM.1S target cells, assessed by a 4-hour standard ^{51}Cr-release assay. Effectors were $CD38^{-/low}$ or $CD38^{+}$ NK cells FACS-sorted from healthy donors ($n = 3$). **E,** Comparison of synergistic effect on tumor eradication, as assessed by a 4-hour standard ^{51}Cr-release assay, between daratumumab and $CD38^{-/low}$ or $CD38^{+}$ NK cells FACS-sorted from healthy donors (effector/target ratio is 5:1, $n = 3$). **F,** Purified NK cells were cocultured with MM.1S cells in the presence of daratumumab for 16 hours. Cells were stained with anti-Annexin V and SYTOX Blue dye ($n = 9$). Error bars, SD; N.S., not significant; **, $P < 0.01$; ***, $P < 0.001$.

K562 feeder cell line modified to express membrane-bound IL21, as reported previously (22). We observed a significant increase in proliferation of NK cells that were derived from $CD38^{-/low}$ progenitors ($CD38^{-/low}$ exp. cells) compared with those derived from $CD38^{+}$ NK progenitors ($CD38^{+}$ exp. cells; Fig. 4A; Supplementary Fig. S9A). Both $CD38^{-/low}$ exp. and $CD38^{+}$ exp. cells had some capacity to kill multiple myeloma target cells; however, when compared with $CD38^{+}$ exp. cells, $CD38^{-/low}$ exp. cells were more cytotoxic against the MM.1S cell line (Supplementary Fig. S9B, left) in the absence of daratumumab. Interestingly, the previous observation occurred despite the fact that each of the two expanded subsets expressed similar levels of GZMB protein (Supplementary Fig. S9C). Moreover, although treatment with daratumumab was able to further increase cytotoxicity against the MM.1S cell line in both $CD38^{-/low}$ exp. and $CD38^{+}$ exp. NK-cell subsets, daratumumab-mediated killing of the cell line remained higher in the $CD38^{-/low}$ exp. cells relative to that seen in $CD38^{+}$ exp. cells (Supplementary Fig. S9B, right). Likewise, when challenged with primary multiple myeloma cells as targets, in the presence of daratumumab, $CD38^{-/low}$ exp. cells displayed higher levels of cytotoxicity than that of $CD38^{+}$ exp. cells (Fig. 4B). We speculated that this may be occurring due to differential expression of CD38 on the progeny of these two eNK cell subsets, as we found that CD38 expression could lead to NK-cell fratricide in the presence of daratumumab. Indeed, we observed that the eNK cells derived from $CD38^{-/low}$ NK cells expressed lower levels of CD38 than those derived from $CD38^{+}$ progenitors at all tested time points (Fig. 4C). Although $CD38^{-/low}$ exp. cells have higher levels of daratumumab-mediated ADCC against multiple myeloma cells than $CD38^{+}$ exp. cells (Fig. 4B), the former subset also acquired lower levels of CD38 expression on the cell surface than the latter subset. We then tested whether daratumumab $F(ab)_2$ fragments can block surface CD38, and this blockade may prevent fratricide and improve daratumumab-mediated ADCC in eNK cells. For this purpose, we first tested the blockade effect using $CD38^{+}$ exp. NK cells through preincubation with varying concentrations of daratumumab $F(ab)_2$ fragments. Preincubation with $F(ab)_2$ daratumumab fragments was indeed sufficient for

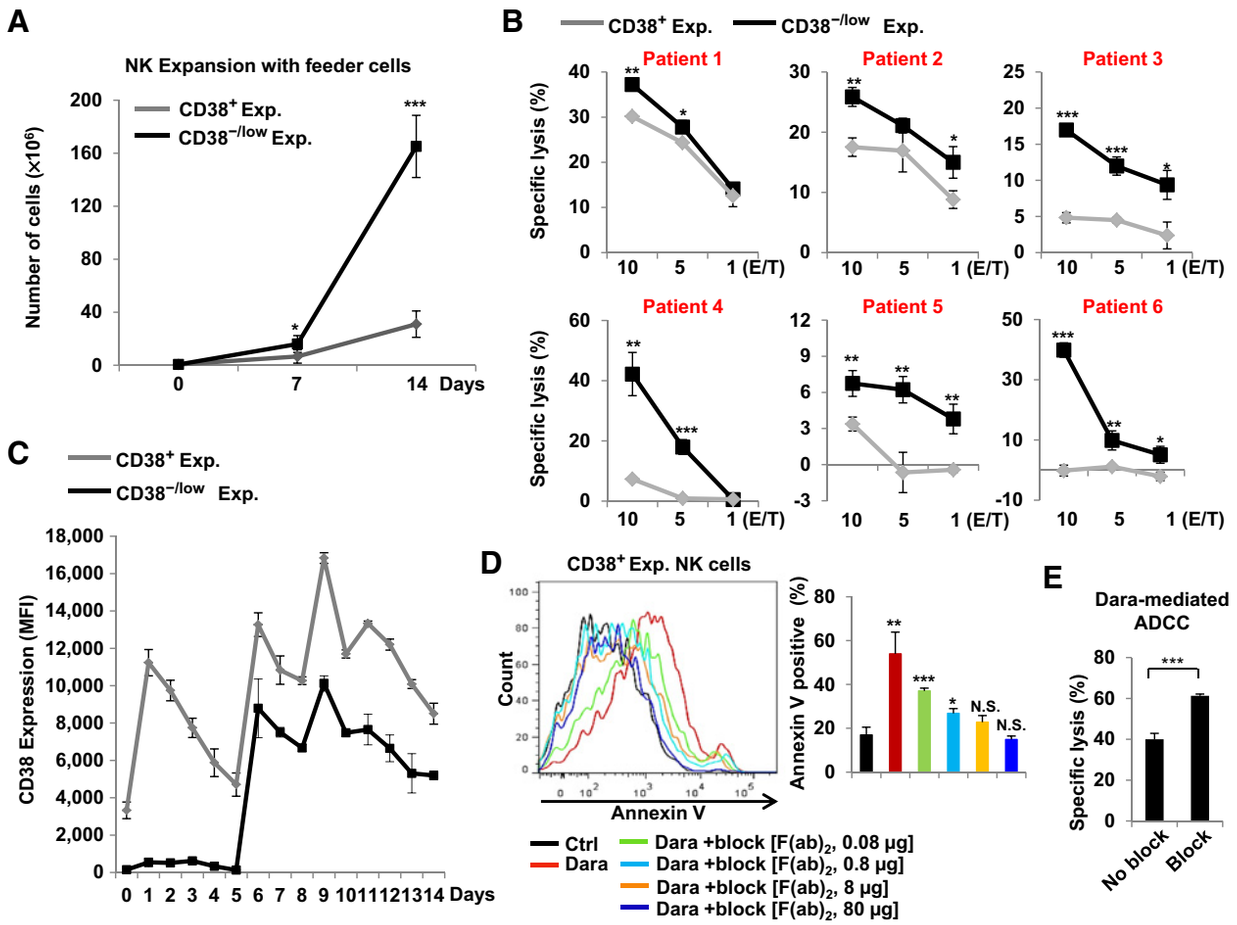

Figure 4.

Compared with their CD38$^+$ counterparts, CD38$^{-/low}$ primary NK cells are more proliferative and more cytotoxic against multiple myeloma (MM) target cells. **A,** A total of 0.5 × 10^6 NK cells were expanded on K562 feeder cells. At days 7 and 14, cells were counted and analyzed to compare CD38$^{-/low}$ expression and CD38$^+$ exp. cells ($n = 5$). **B,** A standard 4-hour ^{51}Cr-release assay was used to measure daratumumab (Dara)-mediated ADCC capacity of CD38$^{-/low}$ exp. and CD38$^+$ exp. NK cells against primary multiple myeloma cells, isolated from bone marrow of patients with multiple myeloma ($n = 6$). **C,** Expression of CD38 on CD38$^{-/low}$ exp. and CD38$^+$ exp. NK cells was detected by flow cytometry ($n = 3$). **D,** A total of 0.5 × 10^6 CD38$^+$ expanded NK cells were pretreated for 1 hour with the indicated doses of F(ab)$_2$ daratumumab fragments, followed by 4-hour treatment with 10 μg/mL of intact daratumumab. An anti-Annexin V antibody was then used to analyze apoptosis by flow cytometry ($n = 3$). **E,** CD38$^+$-expanded NK cells were pretreated for 1 hour with F(ab)$_2$ fragments of daratumumab. A standard 4-hour ^{51}Cr-release assay was then used to assess daratumumab-mediated ADCC against MM.1S target cells (effector/target ratio is 5:1, $n = 3$). MFI, median fluorescence intensity. Error bars, SD; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

preventing the increased apoptosis mediated by daratumumab in CD38$^+$ exp. NK cells, which occurred in a dose-dependent manner (Fig. 4D). Furthermore, this blockade of CD38 indeed significantly enhanced the capacity of CD38$^+$ exp. NK cells for daratumumab-mediated ADCC against MM.1S targets (Fig. 4E).

Alternatively, it is possible that, in the absence of CD38 blockade, CD38$^+$ exp. NK cells compete with multiple myeloma cells for the pool of available daratumumab, and coculture with CD38$^+$ exp. NK cells would lead to lower levels of daratumumab bound to CD38 on the surface of multiple myeloma cells; whereas the higher levels of daratumumab available to multiple myeloma cells could help explain the increase in ADCC observed when multiple myeloma cells were instead cocultured with CD38$^{-/low}$ exp. NK cells. However, we found that this problem of NK-cell competition with multiple myeloma cells for daratumumab binding could be eliminated through administering daratumumab at 10 and 100 μg/mL doses in our cytotoxicity experiments because all tumor cells were bound with daratumumab at these concentrations in the presence of the potential competitor, CD38$^+$ exp. NK cells (Supplementary Fig. S10). This also strengthens our hypothesis that NK-cell fratricide occurs *in vitro* and in patients because the 10 μg/mL concentration used in our culture system and the >250 μg/mL serum concentration achieved in patients treated with daratumumab at a dose 16 mg/kg (8) are either within or above the range of daratumumab concentrations (i.e., 10–100 μg/mL) where there is no antibody binding competition between NK cells and multiple myeloma cells.

Because the data above suggest that CD38$^{-/low}$ NK cells and CD38$^+$ NK cells appear to be two functionally different subsets, we used freshly isolated bulk NK cells to further characterize each

of these subsets. We showed that levels of CD16 and NKp46 expression were lower, whereas CXCR4, KLRG1, CD69, and CD96 expression were higher in $CD38^{-/low}$ NK cells than in $CD38^{+}$ NK cells (Supplementary Fig. S11). The expression of NKG2D, TIGIT, CD94, and CD226 was not significantly different between the two subsets of NK cells (Supplementary Fig. S11). Furthermore, we found that freshly isolated $CD56^{dim}$ and $CD56^{bright}$ NK cells both contained $CD38^{+}$ and $CD38^{-/low}$ subsets (Supplementary Fig. S12A). Consistent with previous studies (23, 24), although GZMB expression was not detected in $CD56^{bright}$ NK cells, $CD56^{dim}$ NK cells expressed GZMB in abundance (Supplementary Fig. S12C). Among $CD56^{dim}$ NK cells, the $CD56^{dim}CD38^{-/low}$ NK-cell subset expressed GZMB at levels significantly lower than those found in the $CD56^{dim}CD38^{+}$ NK subset (Supplementary Fig. S12B and S12C). Previous reports have indicated that high levels of GZMB expression occur following NK-cell terminal maturation (25), which would suggest that $CD38^{+}$ NK cells may be more mature than $CD38^{-/low}$ NK cells; however, it has also been reported that $CD38^{-/low}$ and $CD38^{+}$ NK-cell subsets may be generated from distinct populations of hematopoietic stem cells that are $CD38^{-}$and $CD38^{+}$, respectively (26). Thus, further in-depth studies characterizing the nature of the developmental relationship between $CD38^{-/low}$ and $CD38^{+}$ NK cell subsets are warranted.

NK cells from daratumumab-treated patients with multiple myeloma are more proliferative than those from non–daratumumab-treated patients with multiple myeloma or healthy donors

Because only a small number of $CD38^{-/low}$ NK cells can be found in the peripheral blood of daratumumab-treated patients with multiple myeloma, we set out to determine whether NK cells, when freshly isolated or from samples that had been previously frozen, can be expanded from multiple myeloma patient PBMCs *ex vivo* on K562 feeder cells. For this reason, multiple myeloma patient PBMCs (stored at −80°C) were thawed and expanded with the aforementioned feeder cells plus IL2. We observed that both non–daratumumab-treated and daratumumab-treated multiple myeloma patients' NK cells had rapidly expanded by day 7 (Supplementary Fig. S13). The purity of these eNK cells reached approximately 90% at day 7 and further increased to more than 95% by day 19 (Supplementary Fig. S13; Fig. 5A). Moreover, we observed that NK cells from the PBMCs of daratumumab-treated patients with multiple myeloma expanded at a significantly higher rate than NK cells from the PBMC of non–daratumumab-treated patients with multiple myeloma ($P < 0.05$) or healthy donors ($P < 0.01$; an average of 60,000-fold increase vs. 10,000-fold and 3,100-fold at day 19, respectively; Fig. 5B). This finding was consistent with experiments performed using NK cells from healthy donors, in which we compared expansion capacity of $CD38^{-/low}$ with $CD38^{+}$ and total NK cells (Supplementary Fig. S9A), because the majority of NK cells from daratumumab-treated patients with multiple myeloma are $CD38^{-/low}$, whereas NK cells from non–daratumumab-treated mimic total NK cells of healthy donors in terms of NK-cell subsets defined by CD38 surface expression (Fig. 1E). Daratumumab-treated multiple myeloma patients' eNK cells had remarkable cytotoxic activity against multiple myeloma target cells, killing at much higher rates than eNK cells from patients with multiple myeloma without daratumumab treatment or from healthy donors (Fig. 5C). Together, these data demonstrate that daratumumab-treated multiple myeloma patients' NK cells, which are largely $CD38^{-/low}$, have an outstanding capacity for proliferation, and the eNK cells derived from PBMCs of these patients are potent killers of multiple myeloma cells.

NK cells expanded from daratumumab-treated multiple myeloma patient PBMCs display better efficacy following combination treatment with daratumumab than with single agents *in vivo*

We first tested whether eNK cells from multiple myeloma patients possess the capacity to kill multiple myeloma. For this and the remaining *in vivo* experiments, we used the MM.1S xenograft model that we described previously (27). Indeed, we observed that eNK cells from daratumumab-treated multiple myeloma patients' cells have significant antitumor activity in multiple myeloma tumor-bearing mice (Supplementary Fig. S14). We applied a combination treatment consisting of daratumumab with eNK derived from daratumumab-treated multiple myeloma patients' PBMCs to test whether eNK cells from peripheral blood of daratumumab-treated multiple myeloma patients synergize with daratumumab to kill multiple myeloma and provide a survival advantage. Tumor growth was monitored by bioluminescence imaging twice a week, starting at day 14 after tumor inoculation. A diagram illustrating the scheme for treatment is shown in Fig. 6A. We found that, compared with the control group and groups treated with only a single agent, combination treatment significantly improved tumor growth suppression (Fig. 6B and C; Supplementary Fig. S15A and S15B). Likewise, survival of multiple myeloma tumor-bearing mice was improved to a greater degree in the group receiving combined treatment than in any other treatment groups (Fig. 6D; Supplementary Fig. S15C). Thus, combination treatment with daratumumab and eNK cells from daratumumab-treated patients with multiple myeloma displayed more effective and potent antitumor activity compared with treatment with daratumumab alone. However, as the aforementioned effects of combination treatment on prolonging survival may not be durable, subsequent injections with eNK cells should be performed frequently following daratumumab treatment.

Discussion

Daratumumab's efficacy has been proven in a series of clinical trials, both as a single agent to target the CD38 molecule, and as part of combination treatments for multiple myeloma (2, 6, 8, 28). Daratumumab works to eliminate tumor cells via several mechanisms, including ADCC and direct induction of tumor cell apoptosis (9, 29). ADCC is mediated by NK cells (30, 31); however, as we demonstrate in this study, NK-cell depletion in peripheral blood and bone marrow can simultaneously occur in patients with multiple myeloma who are undergoing daratumumab therapy, consistent with previous studies (32, 33). Although the mechanism by which daratumumab depletes NK cells was previously unclear, in this study, we revealed that daratumumab can enhance NK-cell apoptosis through NK-to-NK ADCC, wherein fratricide occurs among NK cells without the involvement of tumor cells. Furthermore, the $CD38^{-/low}$ NK cells remaining in daratumumab-treated patients with multiple myeloma are highly proliferative *ex vivo*, and following expansion, these NK cells can acquire potent *in vivo* anti–multiple myeloma activity. Therefore, we propose combined treatment with daratumumab and eNK

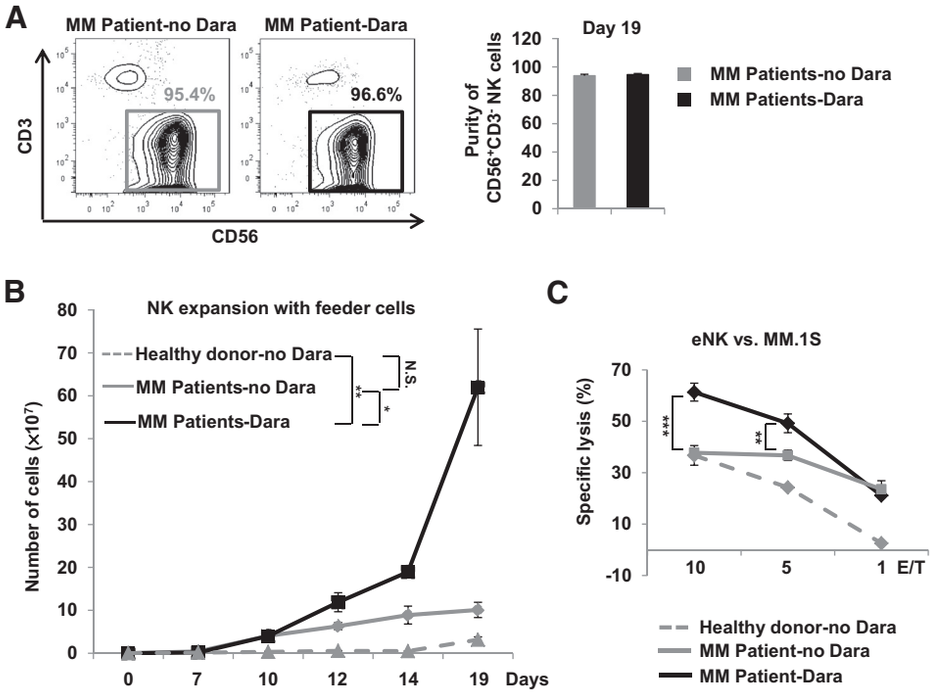

Figure 5.

Expansion of primary NK cells from multiple myeloma (MM) patients' PBMCs. **A,** Purity of NK cells expanded (eNK) from non–daratumumab (Dara)-treated or daratumumab-treated patients with multiple myeloma assessed by flow cytometry at day 19 and presented as the percentage of $CD56^+CD3^-$ lymphocytes among total lymphocytes ($n = 3$). **B,** Expansion of NK cells derived *in vitro* from the PBMCs of healthy donors and patients with multiple myeloma treated with or without daratumumab (Dara and no Dara, respectively), as assessed on days 7, 10, 12, 14, and 19 ($n = 3$). **C,** A 4-hour standard ^{51}Cr-release assay was performed using NK effector cells expanded from the PBMCs of healthy donors or patients with multiple myeloma treated with or without daratumumab ($n = 3$). MM.1S served as targets. Error bars, SD; N.S., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cells as a potential therapeutic strategy for treatment of patients with multiple myeloma. To the best of our knowledge, this combination renders our study novel compared with previous studies (32, 33).

NK-cell activation can either be induced by receptor recognition between effector cells and target cells (34), or directly stimulated through treatment with antibodies directed against markers, such as CD16 and/or NKG2D (35), which represent examples of cytotoxicity-related receptors on NK cells (36). Although CD38 is also expressed on the surface of NK cells, anti-CD38 $F(ab)_2$ fails to trigger NK-cell activation in freshly isolated human NK cells (19), consistent with our findings that the $F(ab)_2$ fragment of daratumumab does not trigger NK activation or cell death. Thus, CD38 may not be a direct transducer of cell signaling that controls NK-cell activation. Cytokine-mediated activation and antibody-mediated ADCC enhance NK-cell activation, which can promote killing of tumor target cells or virally infected cells (37, 38); however, both of the aforementioned processes rely on the presence of target cells (37, 38). Interestingly, our observations indicate that daratumumab [but not the $F(ab)_2$ part] is able to directly trigger NK-cell activation even in the absence of target cells. During this process, NK cells not only became activated, but also killed each other. Therefore, we believe the daratumumab-induced NK-cell death we described occurs at least partially through NK-mediated nonclassical ADCC directed against neighboring NK cells, a process termed fratricide. During this process, $CD38^+$ NK cells serve as both "target cells" and effector cells. Interestedly, another FDA-approved antibody, elotuzumab directed against CS1, does not induce NK-cell fratricide, although CS1 is expressed on NK cells (39, 40). In addition, we concede that we cannot formally exclude the possibility that this phenomenon may also be attributed to activation-induced cell death (AICD), particularly because during the process of daratumumab-mediated NK-cell fratricide, we indeed observed NK cells acquiring an activated phenotype. However, to our knowledge, whether ADCC in NK cells also induces AICD, a process that is more typically induced by cytokines, has not yet been explored. Thus, it would be difficult to formally test whether the daratumumab-induced NK-cell death occurring through ADCC-mediated NK-cell fratricide might also be accompanied by AICD-mediated NK-cell suicide.

The effect of daratumumab on NK-cell death requires the coexpression of CD38 and the Fc receptor, CD16, on the surface of NK cells. Findings in previous studies are consistent with our observations reported here, indicating that NK cells highly express CD38, while T cells are nearly CD38 negative (41). This detail can help explain why daratumumab treatment results in depletion of NK cells but not T cells in patients with multiple myeloma. Lack of daratumumab-mediated NK cell to NK-cell engagement through the use of an IgG-specific protease IdeZ, which digests daratumumab into $F(ab)_2$ and Fc fragments, completely diminishes daratumumab-induced NK-cell death. On the basis of this finding, the $F(ab)_2$ fragments of daratumumab may be useful for blocking the CD38 receptor on NK cells, which will prevent NK cells from succumbing to daratumumab-induced apoptosis. Indeed, blockade of CD38 with daratumumab $F(ab)_2$ is sufficient to prevent daratumumab-induced NK-cell death. Importantly, we found that this method simultaneously works to enhance daratumumab-mediated cytotoxicity of NK cells against multiple myeloma cells. Thus, blocking CD38 with daratumumab $F(ab)_2$ prevents daratumumab-induced apoptosis in $CD38^+$ NK cells, suggesting that CD38 blockade with daratumumab $F(ab)_2$ in NK cells isolated or expanded from peripheral blood of multiple myeloma patients or allogeneic donors may represent a useful strategy for improving the outcome of daratumumab therapy in multiple myeloma.

CD38 is a glycoprotein and a multifunctional enzyme that catalyzes the synthesis and hydrolysis of the reaction from NAD^+

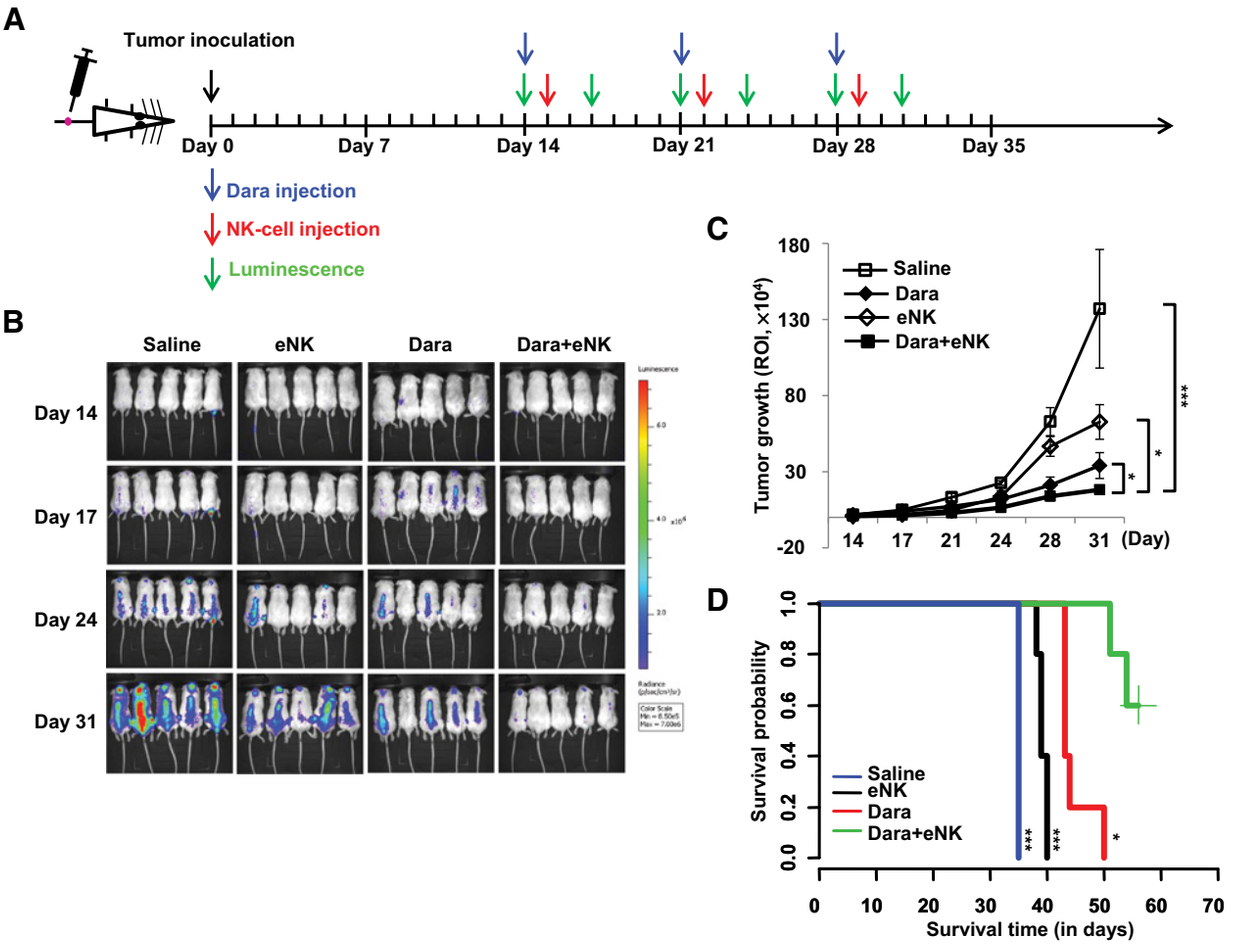

Figure 6.

Treatment of multiple myeloma–bearing mice with or without daratumumab, expanded NK cells (eNK), or their combination. **A,** Treatment and imaging schedules. **B,** Images of mice in each treatment group at 14, 17, 24, and 31 days after MM.1S cell inoculation. **C,** Bioluminescent quantification of tumor growth ($n = 5$; **, $P < 0.01$; ***, $P < 0.001$, combination vs. saline, daratumumab, or eNK), indexed by region of interest (ROI). **D,** Survival analysis ($n = 5$ for each group). *, $P < 0.05$; ***, $P < 0.001$, combination versus saline, daratumumab, or eNK.

to ADP-ribose (41), a process with important functions in cell adhesion and the calcium signaling pathway (42, 43). Loss of CD38 is associated with impaired immune responses (41). CD38, a receptor that lacks an intracellular domain (41), also positively regulates cytokine release and cytotoxicity in activated NK cells, likely through interacting with CD16, an event that may trigger activation of the calcium signaling pathway (44). As mentioned above, the $F(ab)_2$ fragment of daratumumab, without an Fc proportion, failed to stimulate NK-cell activation, indicating that the binding of the daratumumab-recognized epitope of CD38 did not activate downstream signaling pathways. Our data are in keeping with this concept, as we show that daratumumab fails to activate NK-92 cells that are $CD16^-$, whereas the antibody is able to activate a variant of the NK-92 cell line that has been engineered to ectopically express CD16, an Fc receptor. Consistently, when the $F(ab)_2$ fragment of daratumumab was used on $CD16^+$ NK cells, activation was not observed. These data suggest that activation of CD38 signaling in NK cells may require an anti-CD38 antibody that is capable of bridging CD38 and CD16 on the same NK cell via the $F(ab)_2$ and the Fc portion of the antibody, respectively. Indeed, this is an attractive and logical hypothesis; however, whether daratumumab bridges CD38 and CD16 on the same cell to induce NK-cell suicide remains to be proven.

$CD38^{-/low}$ NK cells were more proliferative than $CD38^+$ NK cells. NK cells remaining in daratumumab-treated multiple myeloma patients are mainly $CD38^{-/low}$ and have much higher capacity for expansion than NK cells isolated from healthy donors or from patients with multiple myeloma who have not undergone daratumumab treatment. We also observed that freshly isolated $CD38^{-/low}$ NK cells expressed much lower levels of granzyme B than did $CD38^+$ NK subsets. Alternatively, the $CD38^+$ subset may represent NK cells that are at a more senescent stage than the $CD38^{-/low}$ subset. In support of this, expanded cells from $CD38^+$ NK cells are less proliferative and less cytotoxic than those from $CD38^{-/low}$ NK cells. However, the relationship between these two subsets of NK cells as it pertains to differentiation and/or maturation status has not yet been characterized in the literature. It will be of great interest to determine whether peripheral blood

$CD38^{-/low}$ NK subset represents a more immature developmental stage, or whether the development of the $CD38^{-/low}$ and $CD38^{+}$ NK-cell subsets occurs entirely independently from distinct subsets of hematopoietic progenitors. Indeed, if proven to be true, either of the aforementioned hypotheses might lead to the observed functional differences between the two NK cell subsets.

Interestingly, our data also showed that $CD38^{-/low}$ NK cells acquire a certain level of increased CD38 expression during *in vitro* expansion. If this occurs *in vivo*, it may explain why $CD38^{-/low}$ NK cells, with greater proliferative ability, do not expand *in vivo* in daratumumab-treated patients with multiple myeloma to reconstitute the NK-cell compartment after depletion of $CD38^{+}$ NK cells by daratumumab, namely because the acquisition of CD38 expression might result in daratumumab-induced apoptosis of these cells. For the same reason, in addition to the sequential treatment with daratumumab and $CD38^{-/low}$ exp. NK cells, a pretreatment of $CD38^{-/low}$ exp. NK cells with $F(ab)_2$ fragments of daratumumab to prevent fratricide of these NK cells may represent a promising alternative approach for the use of combinational therapy with expanded NK cells and daratumumab. Although the latter approach allows for administration of daratumumab and expanded NK cells simultaneously, the potential rebinding of $F(ab)_2$ fragments shed from NK cells onto multiple myeloma cells may also result in multiple myeloma cells that are resistant to daratumumab.

In conclusion, we highlight that daratumumab depletes NK cells in patients with multiple myeloma through a mechanism involving NK-cell fratricide. This side effect of daratumumab for multiple myeloma patients may influence the efficacy of daratumumab therapy, particularly because NK-mediated ADCC against multiple myeloma cells could become diminished and may subsequently increase the risk of multiple myeloma relapse. To address these issues, we propose a novel therapeutic strategy for the treatment of multiple myeloma, which combines daratumumab treatment with eNK cells expanded from daratumumab-treated patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Wang, M.A. Caligiuri, J. Yu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zhang, J. Yu
Writing, review, and/or revision of the manuscript: Y. Wang, T. Hughes, M.A. Caligiuri, J. Yu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.M. Benson, J. Yu
Study supervision: J. Yu
Other (performed experiments): Y. Wang, Y. Zhang

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