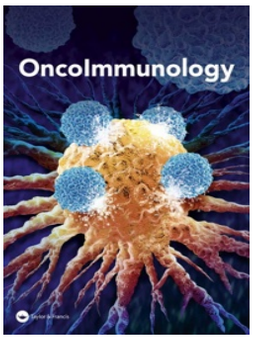

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NK92 cells stably transfected with CD16 are efficient against multiple myeloma cells *ex vivo* and *in vivo*, especially if combined with daratumumab

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ABSTRACT

Adoptive cell therapy and the use of monoclonal antibodies are two therapeutic modalities implemented in the treatment of multiple myeloma (MM). In this study, we combined the anti-CD38 therapeutic mAb daratumumab with different types of NK cells, leveraging the antibody-dependent cell-mediated cytotoxicity (ADCC) performed by these immune cells. Daratumumab was initially combined with activated and expanded NK cells (eNK), resulting in significant cytotoxic activity against human MM cell lines. As an alternative model to minimize the variability among donors of NK cells, the NK92 cell line was used, which showed greater cytotoxic activity than eNK cells against MM cell lines. However, since NK92 cells lacked CD16 receptor expression, they could not be used in combination with mAbs. To circumvent this, we performed a CD16 transfection on NK92 cells, generating the stable NK92-CD16 cell line. These cells were tested in combination with daratumumab against human MM cell lines with excellent results under various conditions, such as 2D and 3D cultures, even at very low effector-to-target ratios. NK92-CD16 cells were then tested in the presence of daratumumab against plasma cells from MM patients, with anti-myeloma activity even against cells from relapsed patients. *In vivo* experiments using MM xenografts or intravenous injection of MM cells in NGS mice, followed by treatment with NK92-CD16 cells in the presence or absence of daratumumab showed tumor regressions, especially in the second model, with nearly complete elimination of the MM cells when NK92-CD16 cells were combined with daratumumab.

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Introduction

Although the primary function of NK cells is the control viral infections, particularly those that down-regulate MHC-I expression such as members of the *Herpesviridae* family, they have also been recognized for their role in antitumor immunity.[1–4] Initially, NK cells were considered specialized in eliminating tumor cells, as they demonstrated their ability to kill them *in vitro*,[5,6] and their contribution to cancer immune surveillance has been clearly demonstrated afterward.[7–9] As part of the innate immune system, NK cells lack of specific antigen receptors and do not require prior exposure to a target to become activated.[9–11] They are also capable of executing rapid immune responses, and, under some circumstances, can acquire functional features resembling immune-like memory.[12,13] The fact that NK cells elicit potent antitumor responses selectively respecting healthy cells makes NK cell-based immunotherapy a powerful and promising

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approach.[10,14,15] However, significant challenges remain. NK cells constitute a minor cell population in the bloodstream, and their expansion and activation require complex conditions.

In order to obtain large quantities of effective NK cells, various protocols have been developed to activate and expand them *in vitro*, and then use *in vivo*.[16–20] The expansion protocol developed by our research group, based on culturing peripheral blood mononuclear cells or umbilical cord blood cells in the presence of IL15 plus IL2 and inactivated feeder cells, results in a more than 50-fold expansion rate and a significant increase in cytotoxicity against cancer cells.[19,21–23] Moreover, in most cases, the expanded cells do not express CD279 (PD-1), which prevents them from receiving inhibitory signals if the target cells express the co-inhibitory ligand CD274 (PD-L1).[19,21–23] Another approach is to use immortalized NK cell lines, which offer a more stable source of cells that are independent of the donor and that can be efficiently transfected and expanded.[24,25] For instance, the NK92 cell line has an unlimited proliferation capacity as long as it is supplemented with IL2.[26–28]

Multiple myeloma (MM) is a malignancy characterized by the uncontrolled proliferation of abnormal plasma cells.[29,30] It is the second most common hematological cancer in developed countries and globally it accounts for around 1% of all malignancies.[31] Although clinical outcomes and survival of MM patients have improved considerably thanks to new therapeutic agents such as proteasome inhibitors and immune modulatory drugs,[29,30] no treatment for MM is truly curative yet. Recently, two CD269 (also known as BCMA)-targeting CAR-T cell therapies have been approved for the treatment of relapsed MM.[32] These CAR-T cells have proved very efficient in the treatment of the disease, but relapses have been also frequently detected. It highlights the need to explore alternative cellular therapies, such as NK cell-based immunotherapies to this pathology. Several clinical trials using different sources of NK cells, often in combination with other treatments, are currently underway.[10,33] In the present study, we compared the antitumor activity of expanded NK (eNK) cells generated with our expansion protocol and NK92 cells. To enhance their action on tumor cells, NK cells were combined with the approved therapeutic anti-CD38 mAb daratumumab to induce antibody-dependent cell-mediated cytotoxicity (ADCC). Since NK92 cells do not express the CD16 receptor and are unable to perform ADCC,[13,24,26,27] we first generated NK92 cells stably transfected with CD16. We show that these cells are extremely efficient against MM cell lines and also against plasma cells from bone marrow aspirates of MM patients in short-term assays, especially when combined with daratumumab. The great efficiency of NK92-CD16 cells in combination with daratumumab was also demonstrated *in vivo*, in NSG mice xenotransplanted with human MM cells.

Materials and methods

Cells and cell lines

The NK92 cell line (DMSZ, Germany) is a highly cytotoxic immortalized cell model of human NK cells established from a rapidly progressive non-Hodgkin's lymphoma. NK92 cells were cultured in Minimal Essential Medium Alpha (Gibco, Invitrogen) containing 12.5% FBS (Sigma), 12.5% Horse serum (Gibco, Invitrogen), 1% GlutaMAX^{TM} (Gibco, Invitrogen), and 1% antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml, PAN Biotech) in the presence of 25 IU/ml of IL2 (Peprotech). Medium and IL2 were refreshed each 48–72 h, maintaining cell density between $2x10^5$ – $6x10^5$ cells/ml.

MM.1S, NCI-H929 and RPMI 8226 cells were used as models of multiple myeloma. RPMI-1640 medium (Gibco, Invitrogen) supplemented with 1% GlutaMAX^{TM} (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) and 1% antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml, PAN Biotech), termed as complete RPMI-1640 medium from now on, was used for cell culture. Subcultures were performed every 48–72 hours at a density of 2×10^5 cells/ml.

HEK-293T cells were normally cultured in DMEM medium (Gibco, Invitrogen) supplemented with 1% GlutaMAX^{TM} (Gibco, Invitrogen) 10% FBS (Gibco, Invitrogen) and 1% antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml, PAN Biotech). Opti-MEM^{TM} (Gibco, Invitrogen) medium with 10% FBS (Gibco, Invitrogen) was used for transfection.

PMBC isolation and lymphocyte activation

PMBCs were isolated from leukopacks diluted at 1:1 with PBS and obtained from Banco de Sangre y Tejidos de Aragón by Ficoll®-Paque (Sigma) density centrifugation, essentially as described in.[34] Isolated PBMC cells were cultured at 10^6 cells/ml in complete RPMI-1640 medium. After overnight incubation with 10 µg/ml of phytohemagglutinin (Ibian, Medicago), cells were washed and seeded again in fresh complete RPMI-1640 medium supplemented with 100 IU/ml of IL2. Culture medium and IL2 were renewed each 48 h and cells were used on the 5th day

eNK generation and expansion

eNK cells were generated as described previously in.[21] Using the EasySep Human CD3 Positive Selection Kit II from StemCell Technologies we performed initially a partial T cell depletion. Then, cells were cultured at 2×10^6 cells/ml in complete RPMI-1640 medium in the presence of 25 IU/ml of IL15 (Miltenyi Biotec) and 100 IU/ml of IL2. Cells were cultured in the presence of the HLA-I negative, EBV-transformed, human B-cell lymphoblastoid cell line 721.221 at a primary cell:feeder ratio of 5:1. These feeder cells were previously treated with 0,5 mg/ml mitomycin C (Biorbyt) for 3 hours at 37°C to prevent their proliferation. Co-cultures were maintained between 20 and 25 days, with changes of medium to add fresh cytokines and feeder cells every 5 days. Culture viability and eNK cell expansion, defined as $CD3^-CD56^+$ cells by flow cytometry, were also determined. At the end of the expansion period (from the 18th day), eNK cells were isolated by positive selection using anti-CD56 magnetic beads (Miltenyi Biotec).

Lentiviral vector production

cDNA of the human high-affinity CD16 polymorphism (V at position 158; Genbank Accession No BC036723.1) was cloned into EcoRI-XbaI sites within the pHIV-Luc-ZsGreen vector replacing cDNA of Luciferase, under the transcriptional control of the EF-1α promoter. This new vector was renamed as pHIV-CD16-ZsGreen. $3{,}8 \times 10^6$ HEK-293T cells were seeded in 10 ml DMEM medium 24 h prior to transfection. At the moment of transfection, medium was replaced with OptiMEM and chloroquine 25 µM was added and incubated for 10 minutes. After that, cells were transfected by adding 1 ml of Opti-MEM with a mixture of 92 µg polyethylenimine (PEI, Sigma-Aldrich), 10 µg of pHIV-CD16-ZsGreen vector, 3 µg MDG2 and 10 µg PAX2 plasmid DNA. Medium was replaced 24 h after transfection, and the conditioned medium with lentiviral supernatants was collected 72 h post-transfection and filtered through 0,45 µm pore-size filters (Acrodisc®, Pall Corporation).

NK92 cell line transduction using lentiviral supernatant

NK92 cell line was resuspended in αMEM culture medium and seeded at 10^6 cells/ml and 2 ml of lentiviral supernatant were added. Incubation was done firstly by spinoculation (2400xg, 90 minutes, 32°C) in the presence of 100 UI/ml of IL2 and 8 µg/ml dextran (Sigma-Aldrich) and finally at 37°C during 24 h. The process was repeated twice. Once the infection was completed, cells were washed and seeded at $4x10^5$ cells/ml in their culture medium supplemented with 100 UI/ml of IL2. Transduction efficiency was assessed using the fluorescence of ZsGreen protein, and positive cells were enriched by several rounds of fluorescence-activated cell sorting (SH800S, Sony Biotechnology). CD16 surface expression was also determined by flow cytometry using an anti-CD16 mAb labeled with Alexa Fluor® 647 (BD Biosciences). A table with the antibodies used in this study is available as Suppl. Table I.

Cytotoxic assays of eNK and NK92-CD16 cells on MM cell lines, PBMCs and activated lymphocytes

These assays were done essentially as described in.[21] eNK cells were previously labeled with 1 µM Cell Tracker Green (CTG, Invitrogen). CTG-labeled eNK cells or NK92-CD16 cells were mixed with target cells reaching a final volume of 200 µl per well. Cells were co-cultured at different

effector:target ratios (from 1:10 to 5:1) in the presence or absence of daratumumab at 5 μg/ml. After incubating for 4 h at 37°C, cells were labeled with annexin V-DY634 (obtained following the protocol described in[35] to determine cell death in the target population. NK cells were excluded from the gating by their green fluorescence (CTG or ZsGreen; see an example of the gating strategy in Suppl. Figure S1). Flow cytometry analysis was performed with a FACScalibur cytometer. Cytometry data and results were collected via CellQuestPro software, and analyzed using FlowJo vX.0.7 software.

Cytotoxic assays of NK92-CD16 cells in 3D cell culture conditions

GrowDex® 1,5% hydrogel (UPM, Biomedicals) was used to imitate 3D conditions. MM cells were resuspended at 10^6 cells/ml in 0.25% GrowDex® in RPMI-1640 medium and seeded into 96-well plates (50 μl/well). In some cases cells were pre-incubated with 5 μg/ml Daratumumab for 15 minutes. NK92-CD16 cells were added over the hydrogel reaching a final volume of 200 μl per well and co-cultured at 1:1 and 3:1 effector target ratios during 24 h at 37°C. Finally, the three-dimensional matrix was degraded using GrowDaseTM (UPM Biomedicals) as indicated by manufacturer instructions and cell death analysis was performed as previously described.

NK92-CD16 cells irradiation

8×10^6 NK92-CD16 cells were placed inside a T25 culture flask completely filled with αMEM medium. Cells were resuspended and the flask set inside a water phantom to be exposed to different radiation doses. Simulation was performed on a GE High Speed CT, and dose plan (2 opposed 10FFF beams from a Varian TrueBeam HD120) was calculated with AccurosXB in Eclipse.

After exposure, excess of medium was removed and cells were cultured again as usually. In parallel, a non-exposed control sample was also cultured at the same initial cell density. Cell density and viability were monitored during the following days. Irradiated NK92-CD16 cells were also incubated with MM cells to assess their cytotoxic capability after irradiation.

Bone marrow aspirates from multiple myeloma patients and cytotoxicity assays with NK92-CD16 cells and Daratumumab

Bone marrow samples from MM patients were collected at the Hospital Clínico Lozano Blesa and Hospital Miguel Servet, Zaragoza, Spain. Most samples were collected at diagnosis, although a few were collected at the time of relapse. Fresh MM samples were diluted 1:1 with complete RPMI-1640 medium and processed to isolate the mononuclear cell fraction from the bone marrow aspirate by density gradient centrifugation as previously described for blood samples. Cells were then washed and the expression of CD38 and CD138 were analyzed using specific antibodies (BD Biosciences) and flow cytometry, to calculate the percentage of MM cells present in each medullar aspirate.

5×10^4 bone marrow mononuclear cells (BMMC) from the medullar aspirates were seeded in 50 μl per well into 96-well plates and incubated at 37°C for 15 minutes with daratumumab at 5 μg/ml prior to use in the experiments. NK92-CD16 cells were added reaching a final volume of 200 μl per well and co-cultured at 1:1 and 3:1 effector:target ratios. After 4 h of incubation at 37°C, cells were marked with mouse anti-human CD138 mAb labeled with PE (BD Biosciences) and annexin V-DY634 to determine cell death only in the MM population (see an example of the gating strategy in Suppl. Figure S2). This gating in fact underestimates cell death, since a fraction of dead cells turns to cell debris and are not analyzed. An alternative quantification of target cell death, which we have used for this type of samples, can be achieved by, using the same gating, estimating the absolute numbers of $CD138^+$ cells in the control (9954, a 24.9% of the total in the example shown) and those remaining after the 4 h incubation with the effector cells (1628, a 3.48% of the total in the example).

Solid subcutaneous in vivo *tumor model*

Immunosuppressed 6-week male NSG (NOD Scid gamma) mice, obtained from Charles River, were inoculated with 5×10^5 MM.1S-GFP-Luc cells subcutaneously in the dorsal region and mice randomly subdivided in three experimental groups of 5 mice each. MM.1S-GFP-Luc cells were a kind gift from Dr. Ignacio Melero and Dr. M. Carmen Ochoa from CIMA, Pamplona, Spain. On days 16 and 30 post-injection, 5×10^6 NK92-CD16 cells were administered in 200 µl of PBS intravenously into the tail vein, alone or in combination with daratumumab (50 µg/mouse). IL2 calculated at a final blood concentration of 100 IU/ml was included in this volume to allow survival of the therapeutic cells. On days 0 (5 hours after inoculation), 6, 13, 16, 20, 27 and 36, 200 µl luciferin at 1 mg/ml was administered intraperitoneally before sedation with isofluorane, to monitor the progress of tumor development through the bioluminescence of MM.1S-GFP-Luc cells captured on an IVIS® Lumina III. Mice that remained alive on day 36 post-inoculation were sacrificed. After sacrifice, solid tumors were removed, sieved with a sterile syringe and passed through a 70 µm cell strainer (Corning, FisherScientific). Cells collected were finally resuspended for 10 min in erythrocyte lysis buffer (NH_4Cl 154 mM, $KHCO_3$ 10 mM, and EDTA 0.1 mM, pH 7.4) and centrifuged for 5 minutes at 300xg. Cells were then resuspended in RPMI-1640 medium and analyzed by flow cytometry to determine the amount of tumor and therapeutic cells present in the excised tumors. The distinction between the two cell types was made through the difference in fluorescence intensity of the GFP or ZsGreen signal, respectively, as described in the gating strategy shown in Suppl. Figure S3.

Intravenous in vivo *tumor model*

Immunosuppressed 6-week male NSG mice were inoculated intravenously into the tail vein with 3×10^5 MM.1S-GFP-Luc cells in 200 µl of PBS and mice subdivided in three experimental groups of 5 mice each. On day 7, 14 and 21 post-injection, 5×10^6 NK92-CD16 cells were administered in 200 µl of PBS intravenously into the tail vein, alone or in combination with daratumumab (50 µg/mouse). IL2 calculated at a final blood concentration of 100 IU/ml was included in this volume and was additionally administered intraperitoneally on days 10, 17 and 24. On days 0 (5 hours after inoculation), 7, 14, 21 and 28, 200 ul luciferin at 1 mg/ml was administered intraperitoneally before sedation with isofluorane, to monitor the progress of tumor development as indicated above. Mice were sacrificed on day 29 post-inoculation. After sacrifice, spleens and bone marrow were removed, cells collected and tumor and therapeutic cells infiltrated analyzed as described above for the solid tumor model.

Statistical analysis

Graphical representation, interpretation and statistical analysis of the results were performed using GraphPad Prism v9.0.2 (GraphPad Software, Inc., San Diego, CA, USA). Depending on the type of data and the experiment, one of the following statistical tests was performed: t-test, multiple t-test, Mann-Whitney test, two-way ANOVA or Nested t-test. Results were expressed as mean ± standard deviation (SD).

Results

Generation of NK92-CD16 cells

Lentiviral particles produced by transfection of HEK-293T packaging cells were used to transduce the CD16 gene into the human NK92 cell line, which does not express this receptor. The efficiency was determined by flow cytometry according to the signal of the ZsGreen reporter protein included in the construction. The efficiency of the initial transfection was low, approximately of 0.07% (Suppl. Figure S4A). However, after several cycles of immune selection using a flow cytometry-based sorter, the percentage of transduced cells was increased, achieving an enrichment of over 95%, as shown in Suppl, Figures S4(B). The expression of both CD16 and ZsGreen proteins remained stable for nearly two years (Suppl. Figure S4C), with minor fluctuations.

Comparative cytotoxicity of eNK and NK92-CD16 cells on MM cell lines

Once the NK92-CD16 cell line was established, we sought to compare its cytotoxicity to that exerted by expanded NK cells (eNK) derived from healthy donors following an activation protocol previously described by our group.[19,21,22] The level of CD16 expression in a representative eNK preparation is compared with the level of expression achieved in NK92-CD16 cells in Suppl Figure 5, showing a compact pattern of expression in close to 100% cells, and being higher in eNK than in NK92-CD16 cells. In our previous works, we demonstrated that PBMC NK cells maintained CD16 expression in a high percentage of cells during the eNK cell generation process, with the observed level of expression slightly higher in day 0 NK cells.[22]

eNK cells and NK92-CD16 cells can exert cytotoxicity against MM cells both independently and in combination with therapeutic antibodies, such as the anti-CD38 mAb daratumumab. Hence, we determined initially the membrane expression of CD38 in different MM cell lines (Figure 1(A)). The MM.1S and NCI-H929 cell lines showed a high expression level, being higher and more homogeneous in NCI-H929 cells. In contrast, the RPMI 8226 cell line had a lower and heterogeneous expression, with around a third of cells being CD38 negative.

To analyze the cytotoxic potential of both eNK cells and NK92-CD16 cells, we comparatively tested them against the same MM targets in the presence or absence of daratumumab. Previously, we demonstrated that daratumumab at the concentration used in our study did not induce any direct cytotoxic effect on a panel of MM cell lines (Suppl. Figure 6).

As shown in Figure 1(B), both types of effector cells had a similar effect on RPMI 8226, but the cytotoxic capacity of NK92-CD16 cells was significantly higher than that of eNK cells on the other two cell lines. In fact, at an effector:target ratio of 3:1, NK92-CD16 cells were able to kill all MM1.S or NCI-H929 MM cells when combined with daratumumab in 4 h assays. The increase in cytotoxicity due to the combination with daratumumab correlated with the level of CD38 expression determined for each target cell and shown in Figure 1(A). The higher increase in ADCC observed in NK92-CD16 cells could be related with the presence of the high-affinity CD16 polymorphism in our transfected cells (V at position 158).

We also determined CD38 expression on the surface of NK92-CD16 or eNK cells. While CD38 expression was consistently high in NK92-CD16 cells, it was variable in eNK cells derived from different donors (Suppl Figure 7). This could mean that eNK and especially NK92-CD16 cells would commit fratricide through ADCC when combined with daratumumab. To test this hypothesis, we measured fratricide levels and found that NK92-CD16 cells exhibited a lower rate of fratricide compared to the average rate observed in the different eNK expansions (Suppl. Figure 8). Nevertheless, fratricide did not prevent these cells to induce cytotoxicity against MM cell lines, as demonstrated in Figure 1(B).

To confirm that NK92-CD16 cells maintain their efficient activity against MM cells under more conditions better mimicking an *in vivo* situation, MM cells were embedded in a three-dimensional matrix using GrowDex® hydrogel and NK92-CD16 cells were externally added to this 3D culture. These experiments were prolonged for 24 h to allow NK cells to infiltrate in the 3D gel, and were analyzed in parallel with conventional two-dimensional (2D) cultures. No significant differences were observed in the extent of MM cell death induced in 2D or 3D cultures (Figure 1(C). Therefore, NK92-CD16 cells also show the ability to infiltrate tumors and kill MM cells.

Cytotoxicity assays of NK92-CD16 cells on plasma cells from MM patients

The next step was to evaluate the cytotoxic potential of NK92-CD16 against bone marrow mononuclear cells (BMMC) from MM patients, and we performed these assays on a total of 20 patients in duplicate. Either NK92-CD16 cells or daratumumab separately induced to some extent specific MM cell death (around 20% as a mean value; Figure 2(A)). The cytotoxic effect of daratumumab alone suggests the presence of active NK cells and/or macrophages among the purified BMMCs from the patients. However, when NK92-CD16 cells were combined with daratumumab, cytotoxicity increased significantly at all effector:target ratios tested. A 3:1 effector:target ratio in the presence of daratumumab eliminated approximately 80% of MM cells in 4 h, with higher cytotoxicity observed in some patients. It is interesting to note that several of the samples analyzed corresponded to patients who had suffered a relapse after previous treatments

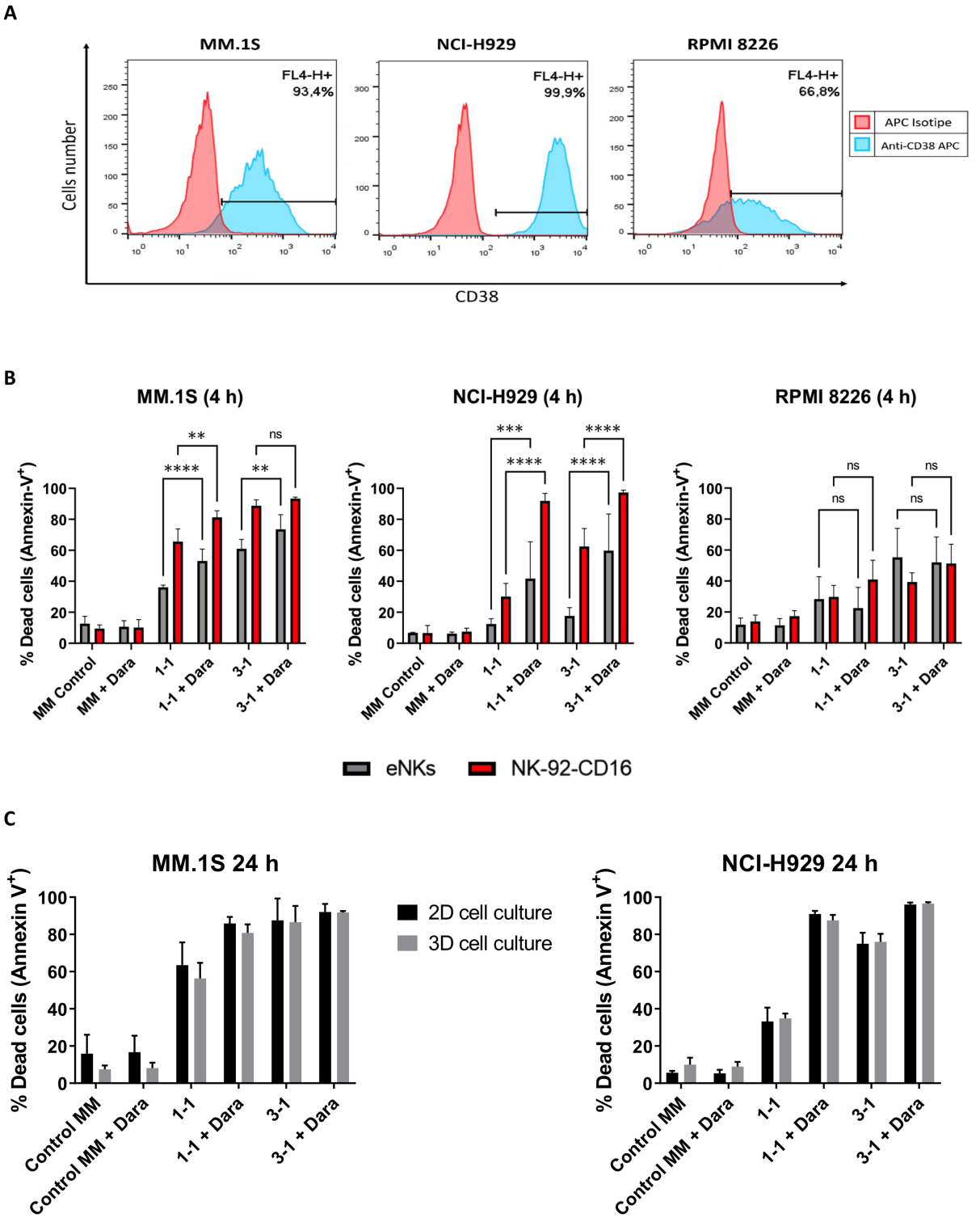

Figure 1. (A) The expression of CD38 on the plasma membrane of MM cell lines was determined by flow cytometry using a specific anti-CD38 mAb labeled with APC. Red histograms show the background fluorescence signal analyzed using an isotype control. Blue histograms represent the CD38 labeling for each cell line. The percentages indicate the fraction of the population of each cell line that is considered positive. Histograms are representative of at least 4 different determinations. (B) Cytotoxicity of NK92-CD16 (red bars) or expanded NK (eNK) cells (gray bars) on MM cell lines. MM cells were pre-incubated for 15 minutes with daratumumab (Dara, 5 µg/ml) where indicated. Effector and target cells were incubated for 4 h at different ratios, as indicated, and cell death on gated target cells, following the gating strategy indicated in Suppl. Fig 1, was determined by analyzing PS translocation using annexin-V labeled with DY634. Results are the mean ± SD of 3 different experiments. *$p < 0.05$; ****$p < 0.0001$, analyzed by 2-way ANOVA. (C) Cytotoxicity of NK92-CD16 cells in 2D and 3D cultures. The cytotoxicity tests in the 2D cultures were performed as indicated in B, increasing the incubation time to

(indicated in red). In some of these patients, the initial sensitivity of MM cells to NK92-CD16 was low, but it was greatly increased by the addition of daratumumab. The relapsed patients had not been treated before with daratumumab, but two of them were treated with daratumumab-dependent regimes afterward, experienced partial remissions, and are still alive.

We also determined the expression of CD38 on the surface of the plasma cells present in the patients' samples, which was variable (see Suppl. Figure S9). It is interesting to note that the sample with the higher level of expression (patient MM-18–22) corresponded to one of the relapsed patients mentioned above, and was very sensitive to the cytotoxicity of NK92-CD16 cells combined to daratumumab (90% of specific cell death both at the 1:1 and 3:1 effector to target ratios). However, other samples with low levels of CD38 expression were also sensitive to ADCC, with intermediate values of cell death, indicating that the level of CD38 alone is not predictive of the response to the treatment.

To ascertain their selectivity against tumor cells, we tested the cytotoxicity of NK92-CD16 cells on quiescent PBMCs and activated T cell blasts obtained from healthy donors (Figure 2(B)). Despite their intrinsic cytotoxic activity, the average cell death induced by NK92-CD16 cells on healthy cells was lower than 20%. In fact, the percentage of normal cells killed at the highest effector-target ratio tested (18.2% on average) was lower than the cell death induced at a 1:1 ratio on the MM cell lines or on plasma cells from MM patients.

Cytotoxicity assays using irradiated NK92-CD16 cells

Since NK92-CD16 cells are tumor cells, irradiation before their use in patients would prevent their proliferation.[36] Hence, we analyzed how irradiation would affect their viability and cytotoxic potential. NK92-CD16 cells were subjected to different doses of radiation in a linear electron accelerator. Specifically, a 10 Gy was applied, a radiation dose that had been used in clinical trials with cell therapies based on NK92 cells,[36,37] and also a higher dose, 15 Gy. NK92-CD16 cells were cultured immediately after irradiation and monitored. As shown in Figure 3(A), during the first 24 h post-irradiation, viability remained high. However, over time, the irradiated cells began to die, reaching approximately a 40% cell death after about 48 h. Later, the amount of dead cells rapidly increased, reaching 100% of cell death at day 6. No differences were observed between the irradiation doses. Therefore, 10 Gy was chosen as radiation dose to inhibit the growth of NK92-CD16 cells.

Next, we compared the cytotoxicity exerted by NK92-CD16 cells, either irradiated or not, against the MM.1S and NCI-H929 cell lines. Cell death induced by non-irradiated effector cells was considered as 100% cytotoxicity for each condition tested. In the case of irradiated NK92-CD16 cells, they were collected during the first 48 h post-irradiation. As shown in Figure 3(B), NK92-CD16 cells irradiated at 10 Gy maintained around an 80% of their cytotoxic potential. In contrast, cells irradiated at 15 Gy, except at the 1:1 ratio with NCI-H929 cells, lost more than 50% of their cytotoxic potential. In summary, irradiation of NK92-CD16 cells at 10 Gy would be sufficient to ensure safe and effective application in clinic, since in spite of the loss of their replicative potential, they retain considerable anti-tumor activity during the first 48–72 hours post-irradiation.

Adoptive transfer in vivo *assays*

In order to determine the *in vivo* anti-tumor activity of NK92-CD16 cells, MM.1S-GFP-Luc cells were inoculated subcutaneously into NSG immune-deficient mice. Once the presence of solid tumors was evident, NK92-CD16 cells were intravenously administered, either alone or in combination with daratumumab. Tumor development was monitored by luciferase bioluminescence produced by MM.1S-GFP-Luc

24 h. In the 3D determinations, cells from the indicated MM cell lines were resuspended in GrowDex® hydrogel and seeded embedded in it. Where indicated, MM cells were pre-incubated with daratumumab (Dara, 5 µg/ml). NK92-CD16 cells were added in suspension on the corresponding hydrogel at the indicated effector to target ratio. After 24 h incubation, the 3D matrix was degraded using GrowDase and cell death determined by flow cytometry as indicated in B. Results are the mean ±SD of three different experiments.

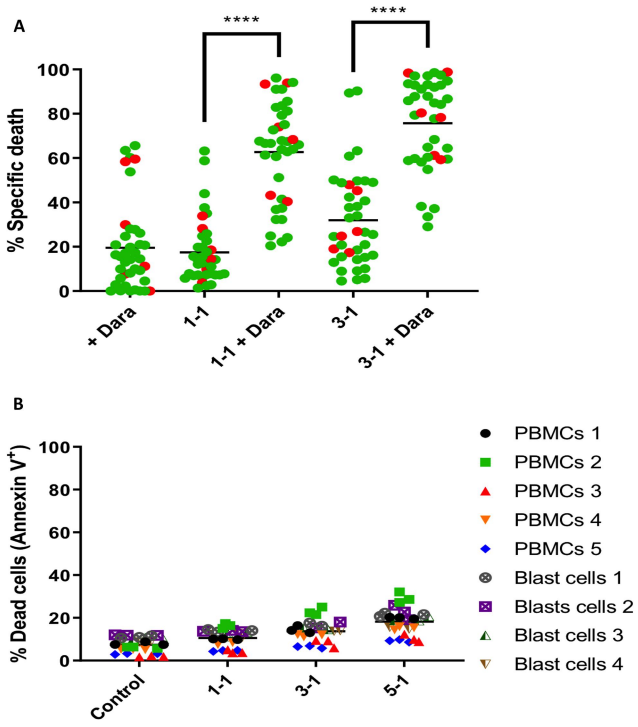

Figure 2. Cytotoxicity of NK92-CD16 cells on MM cells from patients and on PBMC from healthy donors. (A) Bone marrow mononuclear cells obtained from MM patients' medullar aspirates were co-incubated with NK92-CD16 cells at the indicated effector-target ratios for 4 h, and apoptosis was determined by flow cytometry assessing PS translocation using annexin-V DY634^{+} labeling in myeloma cells following the gating strategy shown in Suppl Fig 2 and the alternative method explained in materials and methods. Samples from patients who had suffered a relapse are shown in red. Where indicated, MM cells where pre-incubated for 15 min with 5 µg/ml daratumumab. The graphs represent specific cell death, calculated for each sample by subtracting the percentage of annexin-V DY634^{+} MM cells from the control from the percentage of total annexin-V DY634^{+} MM cells in each condition. (B) NK92-CD16 cells were incubated during 4 h with PBMC from 5 healthy donors or with T cell blasts obtained from them at the indicated effector:target ratios. After the incubation, cell death in the gated primary cells was tested by Annexin-V^{+} labeling and flow cytometry. Cell death observed in PBMC or T cell blasts cultured in the absence of effector cells is shown as "control." The results in A and B are shown as the individual values for each experiment and the mean of each group of values. ****$p < 0.0001$, analyzed by 2-way ANOVA.

cells in the presence of luciferin, until mice were sacrificed. The ratio of the luminescent signal at a given time point and for each experimental group related to the initial signal was calculated and results are shown in Figure 4(A). The two groups that received the treatment had lower mean tumor growth. However, the high variability within the control group prevented these results from being statistically significant. Additionally, solid tumors from the sacrificed mice were extracted and analyzed by flow cytometry, determining the relative percentage of true tumor cells (positive for GFP fluorescence) present in them. As shown in Figure 4(B), the control group was mostly homogeneous, with an average of 42.4% ± 3% tumor cells. In the treated groups, the amount of tumor cells present in the excised tissue tended to decrease, in agreement with the bioluminescence data. In the group treated with NK92-CD16 cells in combination with daratumumab, the reduction was fairly consistent (mean 33.1% ± 0.3%) and the differences were statistically significant with respect to the control. Given the difference in fluorescence emission intensity, we were also able to distinguish between tumor GFP^{+} cells from therapeutic Zs-Green^{+} cells in the tumor explants. Although the amount of NK cells inside tumors was low (around 1%; Suppl. Figure 10), their detection evidenced that NK92-CD16 cells were able to infiltrate into the solid tumors.

To generate a condition more closely resembling the real pathology of multiple myeloma, we conducted a second *in vivo* experiment by injecting MM1.S-GFP-Luc cells intravenously and making slight adjustments to the treatment protocol schedule. Using this approach, MM cells migrate to their more usual localizations, bone marrow and spleen, and they can be tracked using the luminescence signal in the IVIS instrument. Therapeutic NK92-CD16 cells, alone or in the presence of daratumumab,

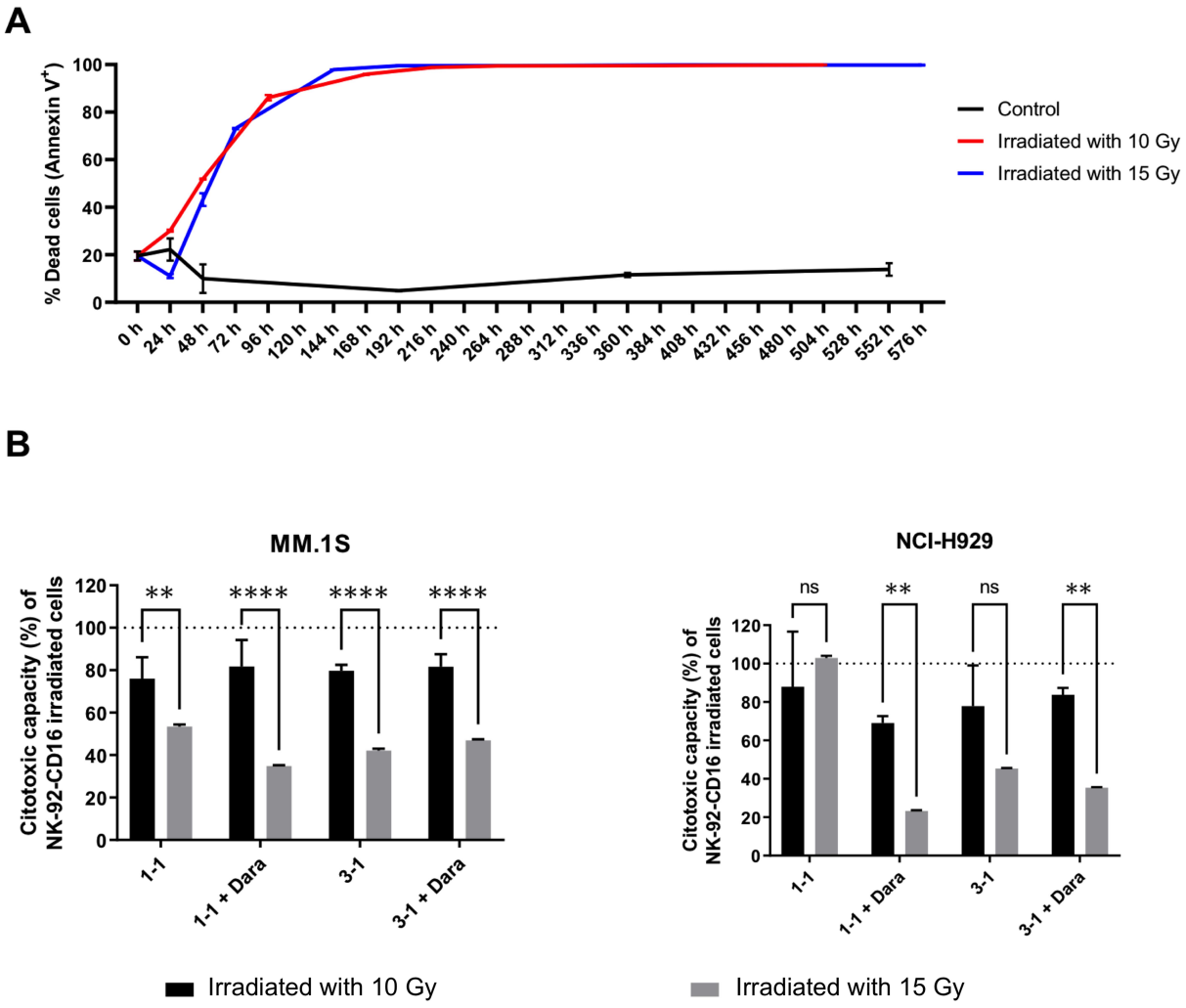

Figure 3. Effect of irradiation on NK92-CD16 cell viability and functionality. (A). Cells were irradiated with 10 or 15 Gy, as indicated, and put again in culture. The x-axis represents the time elapsed since the moment of cell irradiation, while the y-axis indicates the percentage of dead cells/annexin-V DY634^{+}. Results are represented as the mean ± SD of the corresponding values. (B). Cellular cytotoxicity experiments were performed on MM cell lines for 4 h, as indicated in the Figure legend of Figure 1(B) using NK92-CD16 irradiated with 10 or 15 Gy, as indicated. Data are shown as percentage of apoptosis induced by non-irradiated NK92-CD16 cells. The results are shown as the mean ± SD of 3 different experiments in the case of cells irradiated at 10 Gy, and the mean ± SD of 2 individual determinations of 1 experiment in the case of cells irradiated at 15 Gy. $^{**}p < 0.001$; $^{****}p < 0.0001$, analyzed by 2-way ANOVA.

were injected at day 7, 14 and 21, and IL2 was additionally administered on days 10, 17 and 24. As shown in Figure 4(C), the treatment with NK92-CD16 cells alone reduced MM development by 60% at day 28, but this reduction was not statistically significant. The combination of NK92-CD16 cells with daratumumab reduced MM development by 60% already at day 21, and at day 28 the effect was more pronounced, significantly reducing the BLI signal by 97% with respect to that obtained in the control group, almost completely eliminating MM cells. Spleens or bone marrows from the sacrificed mice were extracted and tumor cells detected by flow cytometry using their GFP fluorescence, while NK92 cells infiltrated were detected by their Zs-Green fluorescence. As shown in Figure 4(D), MM cells constituted an 80% of the total bone marrow cells in the control group, while being a 20% of the cells in the spleen. In the spleens of mice treated with NK92-CD16 cells alone this percentage was partially reduced and it was completely abrogated when using NK92-CD16 cells in combination with daratumumab, in agreement with results shown in Figure 4(C) for whole body luminescence. Regarding bone marrow, the therapeutic cells did apparently not affect the high percentage of MM cells. Taking into account the result for the same mice in Figure 4(C), this could mean that MM cells are still in the bone marrow at

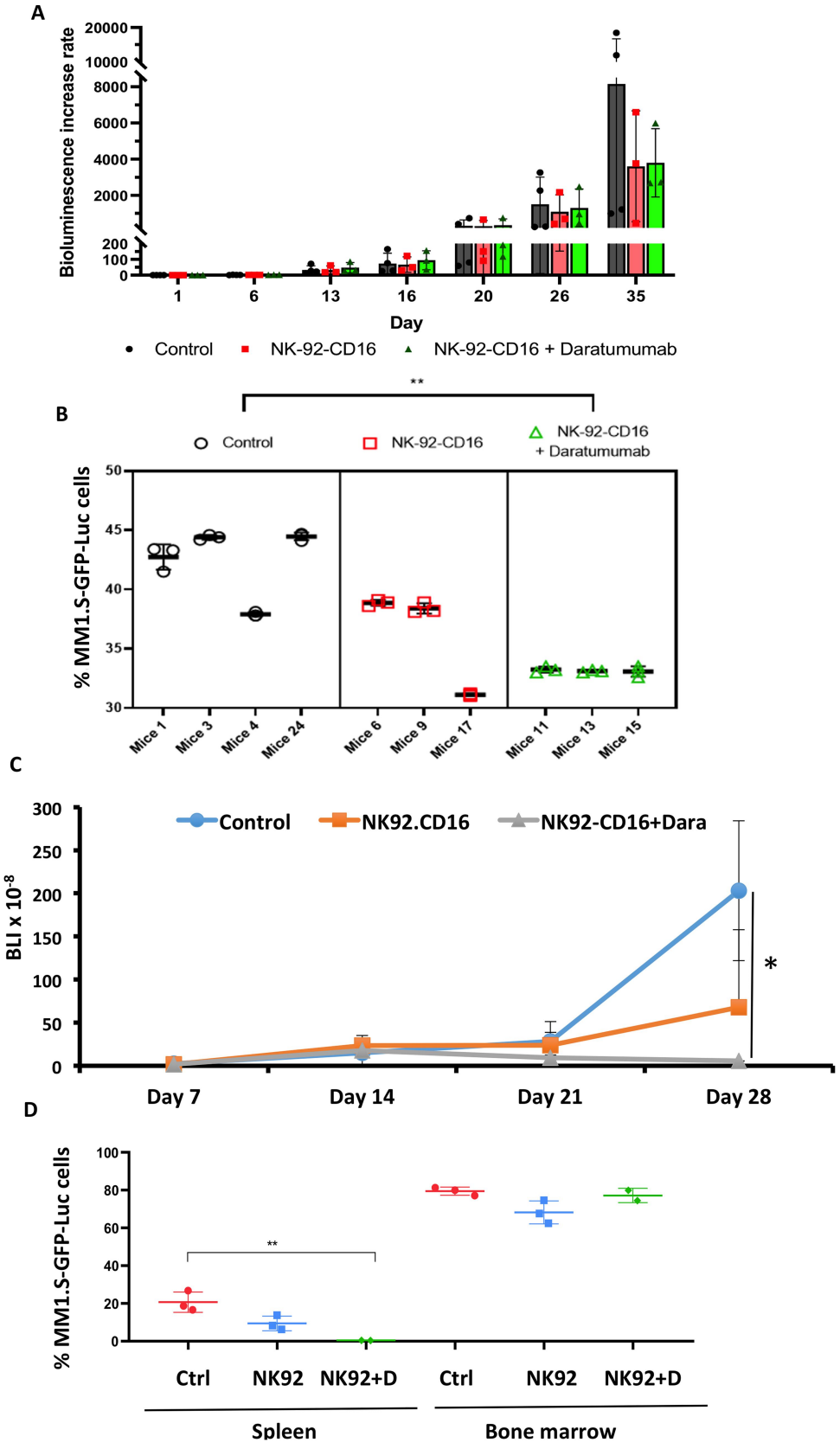

Figure 4. *In vivo* experiments. (A). MM.1.S-GFP-Luc cells were injected subcutaneously and tumor growth followed by luminescence emission. In the treated groups, NK92-CD16 cells were injected intravenously at day 16 and day 30, in the presence or absence of 50 µg daratumumab per mice, as indicated. Data show the increase in bioluminescence in each experimental group, calculated as the ratio of the BLI values at each time and those at day 0. Results are the mean ± SD of

the 29-day time point, with passive fluorescence emission from the GFP protein, but that they are no longer metabolically active, since they do not give luminescence signal mediated by the enzymatic action of luciferase. Regarding NK92-CD16 infiltration in the bone marrow and spleen, we could readily detect their infiltration and persistence in mice treated with NK92-CD16 cells alone (15% of the total cells in the spleen, and 6% in the bone marrow; Suppl. Figure 11). Curiously, this infiltration was much lower in the case of mice treated with the combination of NK92-CD16 cells with daratumumab, being undetectable in the spleen, and only 1% in the bone marrow. This could indicate that, although this combination is of great therapeutic value, as demonstrated in the MM eradication shown in Figure 4(C), it also results in NK cell fratricide, especially at long times.

Discussion

Although treatments for multiple myeloma have made great progress in recent decades, leading to an increase in the life expectancy of patients, MM is still ultimately an incurable pathology.[30] An emerging therapy that has proven useful in hematological malignancies is immunotherapy. In particular, adoptive cell therapy based on CAR-T cells is the latest treatment approved by regulatory agencies for this disease, specifically CAR-T cells targeting the BCMA antigen.[38] However, MM patients with negative or low BCMA expression relapse after receiving BCMA-targeted CAR T-cell therapy and the treatment can also result in BCMA antigen loss.[39] Therapy based on syngeneic or allogeneic NK cells, activated or genetically modified to express a CAR, could be an alternative in cases of relapse.[40] The choice of NK cells instead of T lymphocytes for this type of therapy has advantages, such as lower costs and fewer adverse effects,[24,25,40] and opens the possibility of combining them with another therapeutic modality such as monoclonal antibodies already approved for the treatment of MM.

In this regard, the anti-CD38 mAb daratumumab has been approved for the treatment of MM and exhibits beneficial effects in a high percentage of cases.[30,41] This antibody enhances NK cell cytotoxicity through ADCC, in addition to having additional effects.[30,41] Despite the existence of studies and clinical trials on this subject, the availability of NK cells in sufficiently large quantities for therapeutic use remains a major problem.[25,28] Initially, the possibility of using activated and expanded NK cells (eNK), produced from PBMC from healthy donors or UCB, was analyzed using a protocol previously developed in our laboratory.[19,21,22] The results obtained demonstrated the ability to produce large quantities of activated cells in each expansion, with ratios of expansion between 50 and 200-fold.[21,22] These eNK cells do not express PD-1 in most of the expansions performed,[21,22] and their cytotoxic action against MM is increased when combined with daratumumab, being this enhancement greater the more CD38 the target cells express.

Among the negative aspects found are the limited life span of eNK cells and the fact that expansions must be carried out continuously, as well as the costs that this entails. In addition, the great variability of the different expansions is evident, due to the intrinsic genetics of each donor. This is why, as an alternative, we also used NK92 cells. This cell line, being of tumor origin, has a practically unlimited expansion capacity and, since they are

the values for the mice in each experimental group, with the individual values for each mouse indicated as a dot. (B) Quantification of the percentage of MM cells present in the excised tumors in the experiment described in A. Cell suspensions obtained from the excised tumors were analyzed by flow cytometry, and MM.1S-GFP-Luc cells were detected and quantified by fluorescence of the GFP protein. The x-axis shows the identification of the mice belonging to each experimental group. The y-axis represents the percentage of MM.1S-GFP-Luc cells detected in each case. Each sample was analyzed in triplicate and the results are shown as the individual values for each mouse, plus their mean ± SD. $^{**}p < 0.01$, analyzed by nested t-test. C. MM.1.S-GFP-Luc cells were injected intravenously and tumor growth followed by luminescence emission. In the treated groups, NK92-CD16 cells were injected intravenously at day 7, 14 and 21, in the presence or absence of 50 µg daratumumab per mouse, as indicated. Data show the net increase in bioluminescence as a function of time and are the mean ± SD of the values for the mice in each experimental group. *, $p < 0.05$, analyzed by the Student's t test D. Quantification of the percentage of MM cells present in spleen or bone marrows of sacrificed mice at day 29 in the experiment described in (C) Cell suspensions obtained from spleens or bone marrows were analyzed by flow cytometry and MM.1S-GFP-Luc cells were detected and quantified by fluorescence of the GFP protein. The x-axis shows the identification of the experimental groups and the spleen of bone marrow localization. The y-axis represents the percentage of MM.1S-GFP-Luc cells detected in each case, and data are expressed as mean ± SD**. $p < 0.01$, analyzed by 2-way ANOVA.

constitutively activated, they exhibit high cytotoxicity against a broad spectrum of tumor targets. [13,24,26–28, 37] However, although NK92 cells showed a greater cytotoxicity than eNK cells, their lack of CD16 expression prevented them from being combined with therapeutic mAbs, such as daratumumab. [13,24,26–28, 37] To correct this situation, we generated a line derived from NK92 cells that overexpressed this receptor, NK92-CD16 cells. These cells have maintained CD16 expression in a functional and stable manner for more than two years.

Once the NK92-CD16 cells were characterized, the work focused on evaluating their potential use for cell therapy, showing excellent results against MM cell lines, even at low effector:target ratios and in 2D or 3D conditions, especially if combined with daratumumab. When challenged alone and in combination with daratumumab against plasma cells from bone marrow aspirates of MM patients, the results obtained were very promising. In many samples, tumor death was even higher than 80% in the 4 h assays, especially if NK92-CD16 cells were combined with daratumumab. This combination was also effective against cells from patients who had shown several lines of resistance or relapses. These results suggest that combining NK92-CD16 cells with the corresponding mAbs could improve their therapeutic potential against other types of tumors, including the elusive solid tumors.

In order to use NK92-CD16 cells as a therapy, it was necessary to guarantee strict safety requirements due to its tumor origin.[24,26,28] For this reason, we irradiated them and subsequently tested their viability and cytotoxic potential. We found that an irradiation dose of 10 Gy, used in previous clinical studies,[36,37] was effective in ensuring safe administration. Irradiation creates a framework for action during the first 48–72 hours after the process, but, at longer times, the irradiated NK92-CD16 cells lose their viability, assuring patient safety.

In the solid tumor *in vivo* model we observed indications of the antitumor effect of NK92-CD16 cells, with a lower rate of tumor growth in the treated groups compared to the control group, as well as a significant reduction in the number of malignant cells found in the tumor, the best treatment group being the one that combined NK92-CD16 cells with daratumumab, In the *in vivo* model more similar to MM, the combination of NK92-CD16 cells with daratumumab resulted in an almost complete tumor eradication, as judged by the tumor bioluminescence data.

In the clinical trial described in Williams et al.,[37] NK92 cells were used as a safe therapy in different types of cancer patients, including 5 MM patients. In these last patients, 1 experimented a complete remission, while the other 4 patients relapsed and disease progressed. NK92 cells were also used to generate NK-CAR against MM, including a CAR directed against the SLAMF7/CS1 antigen, which demonstrated an efficient anti-tumor effect in a preclinical model.[42] More recently, anti-BCMA NK92-based CARs[43,44] and also a dual anti-CD19 and anti-BCMA NK92-based CAR[45] have been generated and tested in *in vivo* assays.

NK92 cells transfected with CD16 to allow ADCC were first generated using a murine CD16,[46] but afterward the same group also transfected these cells with human CD16, showing their efficacy in an *in vivo* human mammary tumor model when combined with trastuzumab.[47] A similar result was obtained by another group combining commercial NK92-CD16 cells with an anti-CD123 mAb in an *in vivo* acute myeloid leukemia model.[48] No clinical trials with NK92-CD16 cells have been performed to date, but the data presented in our study regarding MM development, together with the reported previous studies in other tumor models, predict that their action would be greatly improved if combined with therapeutic mAbs already approved for cancer treatment.

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Author contributions

CRediT: **David Giraldos:** Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft; **Evelyn Galano-Frutos:** Data curation, Formal analysis, Investigation; **Laura Cambronero-Arregui:** Investigation, Writing – review & editing; **Manuel Beltrán Visiedo:** Investigation, Writing – review & editing; **Eduardo Romanos:** Methodology; **Chantal Reina-Ortiz:** Investigation, Methodology, Resources; **Gemma Azaceta:**

Investigation, Resources; **Beatriz Martínez-Lázaro:** Investigation, Resources; **Bárbara Menéndez-Jándula:** Investigation, Resources; **Alejandro García-Romero:** Investigation, Methodology; **Francisco Javier Jiménez-Albericio:** Methodology; **Isabel Marzo:** Conceptualization, Formal analysis, Project administration, Supervision, Writing – review & editing; **Javier Naval:** Conceptualization, Formal analysis, Supervision, Writing – review & editing; **Alberto Anel:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data available Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

ARRIVE guidelines

The authors have adhered to ARRIVE guidelines and uploaded a completed checklist

Ethics of experimentation

Animal experiments were performed according to the European recommendations on animal ethics and the University of Zaragoza Animal Experimentation Ethical Commission (CEAEA) previously approved the housing and experimental protocols, with permission numbers PI39_19 and PI10_23.

Informed consent

The study adheres to the Declaration of Helsinki regarding the use of human samples. Patient samples were obtained after written consent by them, and the approval of the protocol by the Committee on Ethics in Research from the Government of Aragón (CEICA) with permission numbers PI19/452 and PI23/031.

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