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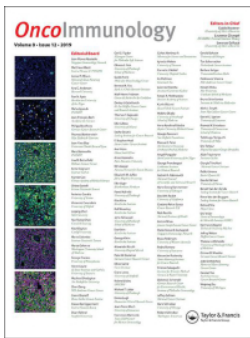
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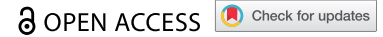


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ORIGINAL RESEARCH



Expanded NK cells from umbilical cord blood and adult peripheral blood combined with daratumumab are effective against tumor cells from multiple myeloma patients

Chantal Reina-Ortiz^a, Michael Constantinides^b, Alexis Fayd-Herbe-de-Maudave^b, Jessy Présume^b, Javier Hernandez^b, Guillaume Cartron^c, David Giraldo^a, Rosana Díez^d, Isabel Izquierdo^d, Gemma Azaceta^e, Luis Palomera^e, Isabel Marzo^a, Javier Naval^a, Alberto Anel^{a*}, and Martín Villalba^{b,f*}

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ABSTRACT

In this study we evaluated the potential of expanded NK cells (eNKs) from two sources combined with the mAbs daratumumab and pembrolizumab to target primary multiple myeloma (MM) cells *ex vivo*. In order to ascertain the best source of NK cells, we expanded and activated NK cells from peripheral blood (PB) of healthy adult donors and from umbilical cord blood (UCB). The resulting expanded NK (eNK) cells express CD16, necessary for carrying out antibody-dependent cellular cytotoxicity (ADCC). Cytotoxicity assays were performed on bone marrow aspirates of 18 MM patients and 4 patients with monoclonal gammopathy of undetermined significance (MGUS). Expression levels of PD-1 on eNKs and PD-L1 on MM and MGUS cells were also quantified. Results indicate that most eNKs obtained using our expansion protocol express a low percentage of PD-1⁺ cells. UCB eNKs were highly cytotoxic against MM cells and addition of daratumumab or pembrolizumab did not further increase their cytotoxicity. PB eNKs, while effective against MM cells, were significantly more cytotoxic when combined with daratumumab. In a minority of cases, eNK cells showed a detectable population of PD1⁺ cells. This correlated with low cytotoxic activity, particularly in UCB eNKs. Addition of pembrolizumab did not restore their activity. Results indicate that UCB eNKs are to be preferentially used against MM in the absence of daratumumab while PB eNKs have significant cytotoxic advantage when combined with this mAb.

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1. Introduction

The immune system largely prevents the development of tumors but clinical cancers evade this immune surveillance. Multiple pathways help create an immunosuppressive tumor environment, interfering with tumor antigen presentation to cytotoxic T cells.¹ Tumor cells hide the expression of tumor-specific antigens, limiting the specific T cell anti-tumor response.² NK cells, which are not antigen specific, provide a potent antitumor response and are a source of powerful immunotherapies.³ NK cells are used in the clinic, especially in hematological cancers with poor prognosis, and can safely be used in allogeneic settings.^{4–6} Their combination with anti-tumor antibodies, through antibody-dependent cellular cytotoxicity (ADCC), offers interesting therapeutic opportunities.^{7,8}

The immune checkpoints refer to molecules that inhibit or modulate immune responses, being CTLA-4 and PD-1 the two most studied, especially in the context of T cell activation.^{9–11} The use of anti-PD1 blocking antibodies such as pembrolizumab and nivolumab has become a first-line treatment in tumors with poor prognosis.^{12,13} Some reports indicate that

PD-L1 expression in tumor cells results in functional impairment of PD-1⁺ NK cells.^{14–16} While certain reports show that NK cell function can be partially restored using blocking mAbs,^{15,16} others show a more acute impairment that requires cytokines such as IL-2 and IL-15 for functional restoration.¹⁴ Regarding NK cells, additional molecules act as checkpoint inhibitors, such as the inhibitory NK cell receptor NKG2A, which inhibits NK cell activity when ligated by HLA-E, expressed on the surface of tumor cells. The use of a NKG2A blocking mAb, monalizumab, offers promise as a new tumor immunotherapy.¹⁷ Other NK cell checkpoint mechanisms include blocking the activating receptor NKG2D by tumor shedding of their MIC ligands,¹⁸ or the negative action of LAG-3 and TIM-3.¹⁹

Multiple myeloma (MM) arises from uncontrolled proliferation of abnormal plasma cells and accounts for 10–20% of all hematological neoplasms and 0.9% of all newly diagnosed cancer cases worldwide.²⁰ MM is normally preceded by a premalignant phase, termed monoclonal gammopathy of undetermined significance (MGUS). MGUS is found in 3% of

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the population above the age of 50, but the rate of progression from MGUS to MM is very low.²¹ Over the past two decades, treatment regimens and survival rates of MM patients have witnessed a radical improvement, with proteasome inhibitors and monoclonal antibodies (mAbs) as the main contributors.²² However, although overall survival and patient outcomes have considerably improved, drug resistance is still a major concern,²³ mainly in high-risk patients, such as patients with t(4;14) or del17p. For this reason, novel and more efficient therapeutic approaches are needed.

NK cell-based immunotherapy could have a positive impact in MM treatment. One obstacle is the immunosuppressive tumor microenvironment present in MM patients, in whom almost all arms of the immune system are subverted.²⁴ For example, NK cells from MM patients are ineffective against disease progression.²⁵ Hence, allogeneic activated and expanded NK cells could be an interesting approach. In fact, NK-based treatments have been recently optimized for its use in MM,^{26–30} including a combination of a tumor NK cell line with the anti-CD38 mAb daratumumab.³¹

CD38 is highly expressed in aberrant MM cells while its expression on normal lymphoid cells, including plasma cells, is relatively low. Daratumumab shows efficacy in relapsed and refractory MM patients treated with at least two prior lines of therapy.^{32–34} NK cell-mediated cytotoxicity seems to be one of the main mechanisms for its anti-MM activity. Since patient NK cell status is variable, this could explain difference in responses among patients.³⁵

Certain reports indicate PD-L1 expression in MM cells which correlates with disease progression from the MGUS stage.^{36,37} However, clinical trials using PD-1 or PD-L1 blockers, alone or in combination with other treatments have failed in MM.³⁸

The potent cytotoxicity of activated NK cells from PB^{39–41} or UCB cells^{7,42} against a variety of malignancies has previously been published by our laboratories. In one study, the expansion of these UCB NK cells was also reported.⁷ In the present work, we compare the cytotoxic capacity of PB and UCB eNKs against MM patient samples.

2. Results

2.1. Peripheral and umbilical cord blood NK expansion

NK cells were expanded using two different protocols. UCB NK cells, due to their need for both KIR and KAR signals to reach a mature phenotype, were cultured with PLH, an EBV-transformed HLA-I⁺ B lymphoblastoid cell line which works as an accessory cell with both required signals.⁷ Conversely, PB NKs are fully mature and need only activating signals thus the EBV+ HLA-I negative cell line 721.221 was employed.⁴³ As a first step, T cells and NKT cells were depleted from the cultures using anti-CD3 mAb, to favor NK cell expansion (see Figure 1(a), d 0). Each protocol required different ratios of accessory cells, while IL-2 and IL-15 were added at the same concentrations. UCB NKs were treated with accessory cells and cytokines every 3 d and benefited from little manipulation. PB

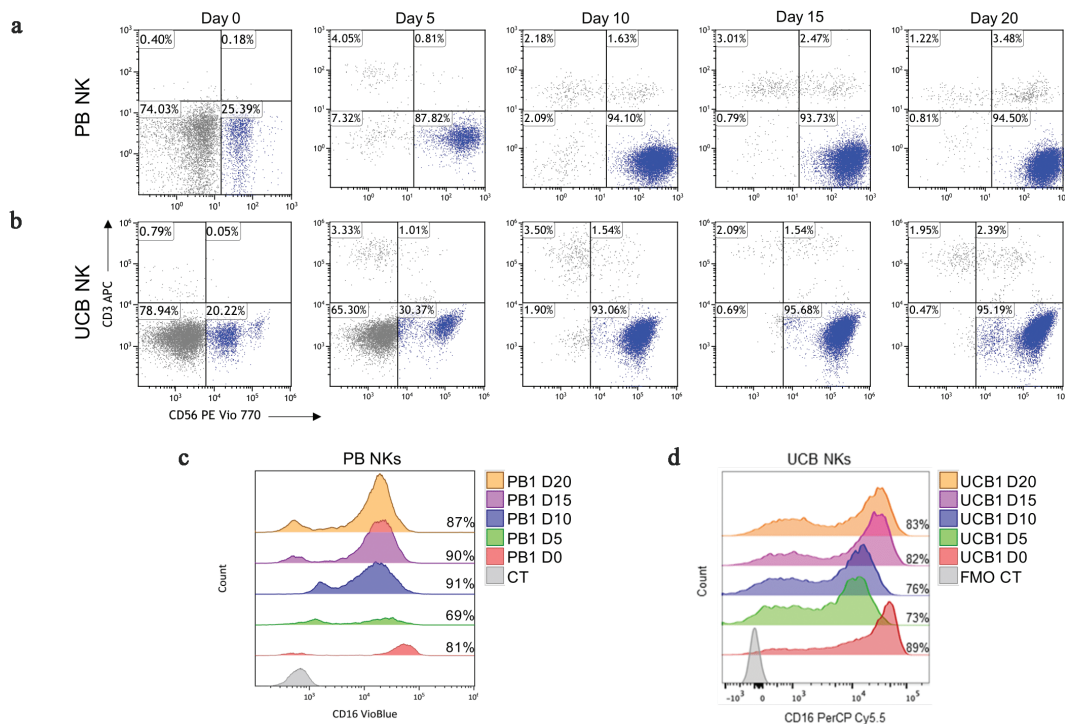


Figure 1. Progression of NK phenotypes through the expansion protocol. Flow cytometry dot plots showing CD3 APC (y-axis) and CD56 PE Vio 770 (x-axis) staining of PB (a) and UCB (b) NKs from d 0 through d 20 of expansion protocol indicated in materials and methods. Histograms depict the progression of PB (c) and UCB (d) NK cells expressing CD16 throughout the 20-d expansion. Numbers indicate the percentage of CD16⁺ NK cells. Cells were stained with CD16 VioBlue or PerCP, as indicated. Lines correlate to testing every 5 d.

NKs were sustained for 5–6 d before culture renewal and withstood daily manipulation seen in [Figure 1](#). By d 20, almost all cells from both sources present in the culture were CD56⁺CD3⁻ NK cells (see [Figure 1\(a,b\)](#)). The purity of NK cells was $94.28 \pm 2.08\%$ for UCB and $95.8 \pm 1.46\%$ as an average (see Suppl. [Figure 1](#) and Suppl. Table I). The CD3⁺ fraction, which was successfully depleted at d 0, did not overtake the CD56⁺ population, allowing for successful expansion of NK cells ([Figure 1\(a,b\)](#)). Beginning with 1E6 NKs in each expansion, PB NKs reached an average of 240E6 cells by d 20 and UCB NKs averaged a 700-fold expansion (Suppl. Fig 2).

The initial characterization of the phenotype of NK cells expanded using EBV-transformed LCL as feeders has been performed in previous studies of our laboratories. A detailed description of all genes and miRNA expressed upon a 5-d activation is found in.⁴⁴ The expression of specific activating and inhibitory NK cell receptors was studied following this 5-d activation protocol, showing increases in the percentage of NKP30⁺ and especially NKP44⁺ populations.⁴⁰ The phenotype of eNK cells following these expansion and activation protocols as compared to freshly isolated NK cells has also been studied in previous work from our groups. eNK cells from UCB show increased expression of activation markers such as CD69 and reduced that of CD45RA, becoming CD45RA^{dim} cells, keeping high CD16 expression.⁷ Regarding eNK cells obtained from PB, the significant increase in the NKP44⁺ population previously described in Sanchez-Martinez et al.⁴⁰ was clearly confirmed in our latest study.⁴⁵ Consequently, we have determined the expression of NKP44 ligands on the surface of patient MM cells and found faint levels of expression (Suppl. Fig 3). Their scant expression level does not justify the increase in cytotoxicity of eNKs. Rather, this increase in cyto-

toxicity could more closely be related to the previously demonstrated increase in granzyme B expression observed in activated NK cells.⁴⁰

Regarding CD16 expression, PB NKs presented high levels at d 0, which was maintained throughout the expansion protocol (see a representative expansion in [Figure 1\(c\)](#) and a summary of data on all donors in Suppl. Table II). In general, fresh UCB NKs also showed CD16 expression, and this level of expression was also maintained during the expansions (see a representative expansion in [Figure 1\(d\)](#) and a summary of data on all donors in Suppl. Table II). The maintenance of CD16 expression in the expanded NK cells is important in order to combine them with therapeutic antibodies.

As the average purity of NK cells at d 20 averaged 95%, we did not proceed with further NK cell isolation before the cytotoxicity experiments. We estimated that both types of eNK cells could be used in cytotoxicity experiments from d 10 on, though expansion continued until d 20 to obtain a maximum number of eNK cells.

2.2. Limited fratricide of eNKs in the presence of daratumumab

As NK cells can express CD38, we have analyzed this point in our expanded NK cells. We show in [Figure 2\(a\)](#) the CD38 expression in the 10 expansions performed. All expansions expressed CD38 to varying degrees. While most expansions had CD38 expression in the majority of their NK cell population, a few expansions from both PB and UCB expressed CD38 in only a minority of the eNK cell population. As daratumumab binds to CD38, when CD38⁺ NKs are in the presence of daratumumab, cross-linking can occur between NK cells

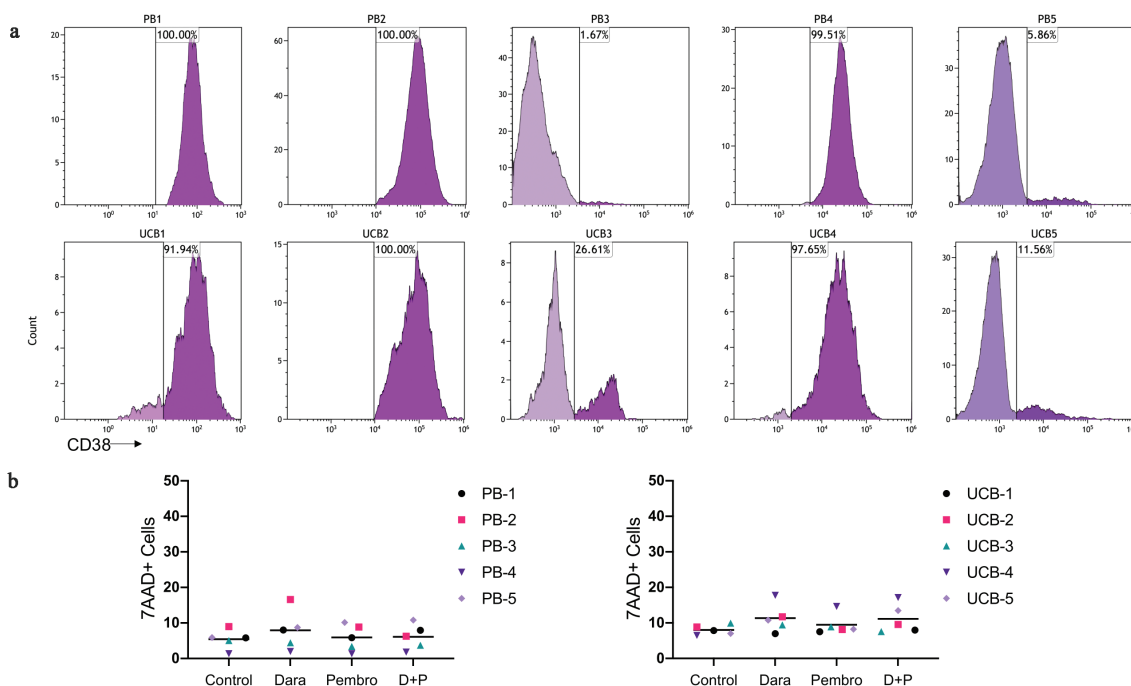


Figure 2. A, CD38 expression determined by flow cytometry on d-20 expanded NK cells (eNK), obtained from PB or from UCB; B, D-20 expanded NK cells (eNK) obtained from PB (left panel) or from UCB (right panel) were left untreated (Control), or they were incubated for 4 h with 5 μ g/ml of daratumumab and/or 10 μ g/ml of pembrolizumab, as indicated. After the incubations, eNK cell death was estimated by 7-ADD labeling. Horizontal lines indicate the mean cell death in each experimental condition.

leading to cell killing via ADCC.⁴⁶ The daratumumab-induced fratricide among NK cells could deplete the number of functional eNK cells available to act against MM cells. To examine the extent of fratricide in our eNK cells, we cultured the eNKs from PB or UCB with daratumumab and/or pembrolizumab, as a secondary control. Daratumumab exhibited a limited cytotoxic effect, averaging less than 5% of specific cell death (Figure 2(b)). Higher levels of CD38 expression on NK cells did not correlate with increased fratricide. Pembrolizumab did not have a cytotoxic effect alone or when combined with daratumumab. We conclude that daratumumab-induced fratricide would not significantly impair eNK cytotoxic potential, although this should be tested in each eNK preparation before use.

2.3. PD-1 expression on eNK cells

Next, we tested PD-1 expression in the 5 PB and 5 UCB eNK used in our study. As shown in Figure 3, most eNK cells obtained (4 out of 5 both in the case of PB and UCB), showed a very limited population positive for PD-1 expression: between 3.9% and 8.3% of the total eNK population in the case of eNK derived from PB and between a 6.5% and a 12.7% in eNK cells derived from UCB. However, eNK cells from PB2 and UCB1 showed the presence of a defined and significant population positive for PD-1 expression, which accounted for 30.9% in PB2 and 23.13% in UCB1. The MFI of the whole eNK population in UCB1 was 4146 while the average MFI in the other 4 UCBs was 1615. In the PB expansions, the MFI value for PB2 was 1481, while the average of the other 4 donors was 766. The population of eNK cells positive for PD-1 expression was never higher than 31%, remaining a minor subset of the whole eNK

population. This result is in agreement with the reported low PD-1 expression in most eNK cells obtained from PB in our previous study.⁴⁵

2.4. eNK cytotoxicity assays against MM and MGUS patient samples

We first obtained bone marrow aspirates of MM and MGUS from 22 patients at different stages of disease. Half of the MM patients had undergone at least one previous treatment and had experienced recurrence. Patient 1 (MM1) underwent six previous treatments and had again relapsed. MGUS patients were monitored but had received no treatment at the time of the bone marrow biopsies. Their clinical data are presented in Table 1. All samples were frozen in liquid nitrogen at the moment of extraction and thawed before being used in the cytotoxicity assays.

To analyze the therapeutic potential of both PB and UCB eNKs, we tested them against the same patient samples in the presence or in the absence of the mAbs. In order to better understand the effect of PD-1 expression on the cytotoxic ability of eNKs, results were stratified attending to PD-1 expression in eNKs.

We first focused on results obtained using PD-1 negative eNK cells on MM patient samples (Figure 4). Neither daratumumab nor pembrolizumab, alone or in combination, had a significant cytotoxic effect on MM samples (Figure 4(a)).

On average, UCB eNKs were more cytotoxic on MM cells than PB eNKs when used as lone treatment (Figure 4(a,b)). UCB eNKs cytotoxicity was not significantly increased with the addition of daratumumab. This was not due to low CD16 expression, which was observed in more than 80% of the different UCB eNKs population (see Figure 1(d) and Suppl.

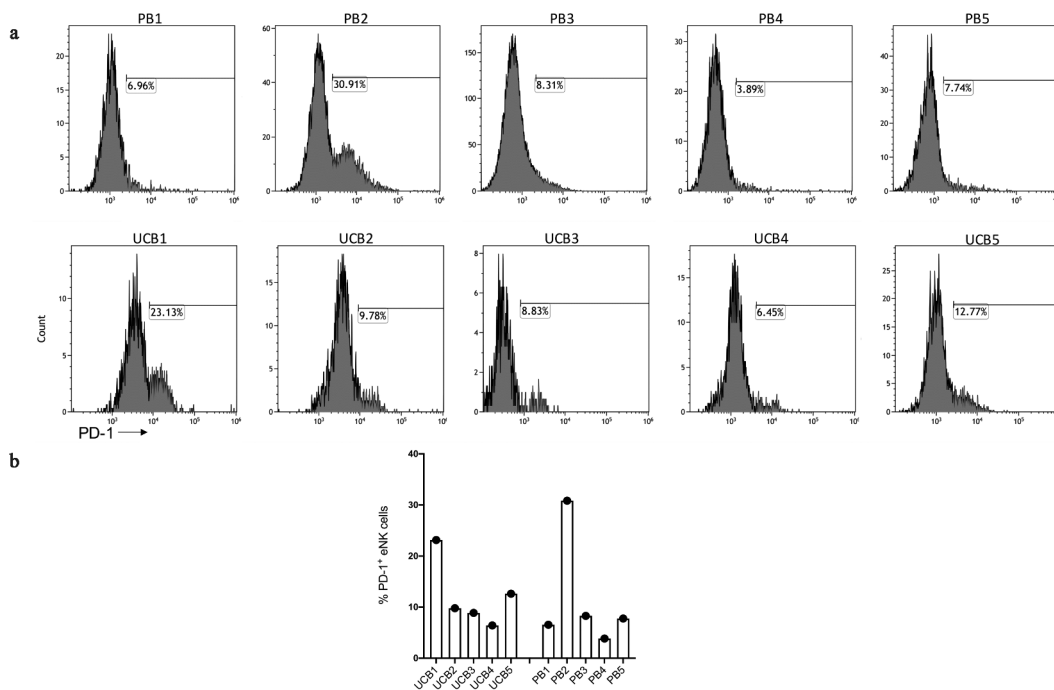


Figure 3. A, Pattern of PD-1 expression on all d-20 expanded NK cells (eNK) used in the study, obtained from PB (upper panels) or from UCB (lower panels). B, Summary of the percentages of PD-1⁺ eNK cells obtained from UCB or from PB, as indicated.

Table 1. MM and MGUS patient clinical data. (A) Age and previous treatment data were calculated based on the date of patient bone marrow biopsy. For MGUS patients, no treatment was given. The Durie-Salmon score is not applicable (NA) to MGUS patients. PD-L1 expression is based on our data. Patients with 0 previous treatments indicate newly diagnosed MM. (B) Numbers of MM patients treated with each of the UCB or PB NK cell expansions. (C) Numbers of MGUS patients treated with each of the UCB or PB NK cell expansions.

A	MM 18 (82%)	MGUS 4 (18%)
Age (at time of sample)		
Mean, years (range)	68 (45–83)	59 (49–77)
Gender		
Male	12 (67%)	3 (75%)
Female	6 (33%)	1 (25%)
Durie Salmon score		
I	5 (28%)	NA
II	4 (22%)	NA
III	9 (50%)	NA
Previous treatments (at time of sample)		
0	9 (50%)	NA
1	4 (22%)	NA
2	2 (11%)	NA
3+	3 (17%)	NA
PD-L1 Expression		
+ (>50% cells+)	9 (50%)	0 (0%)
- (<50% cells+)	9 (50%)	4 (100%)
B		
MM PTS	UCB eNK	PB eNK
1,2,3,4	1	1
8,9,10	2	2
11,13,14,15	3	3
16,17,18,19	4	4
20,21,22	5	5
C		
MGUS PTS	UCB eNK	PB eNK
5	–	1
6,7	2	–
12	3	3

Table II). In contrast, PB eNKs increased their specific cytotoxicity when combined with daratumumab from 25% to 38% on average (Figure 4(b)), close to the level obtained by using UCB eNKs alone. Finally, as expected, pembrolizumab had no significant effect on these PD-1–negative eNKs (Figure 4(a,b)).

Neither daratumumab nor pembrolizumab exhibited appreciable cytotoxicity when used alone or in combination on MGUS samples (Figure 5(a)). When eNKs were tested on MGUS cells, we observed again a significantly higher cytotoxicity of UCB eNKs than PB eNKs on the same samples (Figure 5(a,b)). Cytotoxicity of UCB eNKs was slightly higher on MGUS cells than on MM cells. Again, the combination with daratumumab did not further increase the high level of UCB eNK cytotoxicity. Although we did not find any effect of pembrolizumab on MM cells, we observed a small average increase of cytotoxicity of PB eNKs on MGUS samples, but it was not statistically significant. The increase in cytotoxicity when combining PB eNKs with daratumumab, although observed, was also not statistically significant. Pembrolizumab had no further effect. Cytotoxicity of PB eNKs was also higher on MGUS than on MM cells. These data, obtained with eNKs from both PB and UCB, could indicate that MGUS patients may benefit from eNK treatment prior to disease progression.

2.5. Cytotoxicity of eNK cells with a significant PD-1⁺ population

Figure 6(a,c) show results obtained with samples from the three patients treated with the only eNK obtained from PB that contained a significant PD-1⁺ population (PB2). Curiously, the eNK from PB2 showed a higher overall average cytotoxicity than that observed using PD-1–negative PB eNKs, around 40% of specific cell death (Figure 6(c)). However, the increase in cytotoxicity when PB eNKs were combined with daratumumab, and previously observed in Figure 4, was lost.

For the four patient samples treated with the only eNK obtained from UCB that contained a significant PD-1⁺ population (UCB1), we observed a dramatic decrease in cytotoxicity when compared to results obtained from PD-1–negative UCB eNKs (Figure 6(b,d)). The specific cell death average dropped from 42% (see Figure 4(b)) to 20%. This low cytotoxicity level was not due to resistance of the patient samples themselves, as cells from these same patients were sensitive to PD-1–negative PB1 eNKs, as shown in Figure 4. Addition of daratumumab did not increase the cytotoxicity of these eNKs, even though CD16

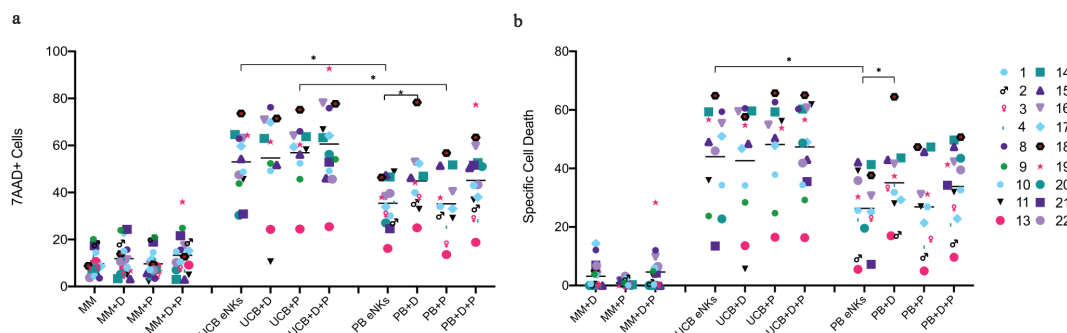


Figure 4. eNK cytotoxicity assays on samples from MM patients. Cells from MM patients were left untreated (MM), or they treated with daratumumab at 5 μ g/ml (d), with pembrolizumab at 10 μ g/ml (p), or with their combination (D + P), or incubated with PD-1–negative UCB or PB eNKs for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. After the incubations, cell death was estimated by 7-AAD labeling in gated target cells (a), as indicated in Material and Methods. In (b) results are shown as the percentage of specific cell death induced, after subtracting basal cell death in each sample. Horizontal lines indicate the mean cell death in each experimental condition. One way ANOVA, ANOVA post hoc Tukey's analysis were applied for multiple comparisons among various groups. * indicates p value less than 0.05 and considered to be significant.

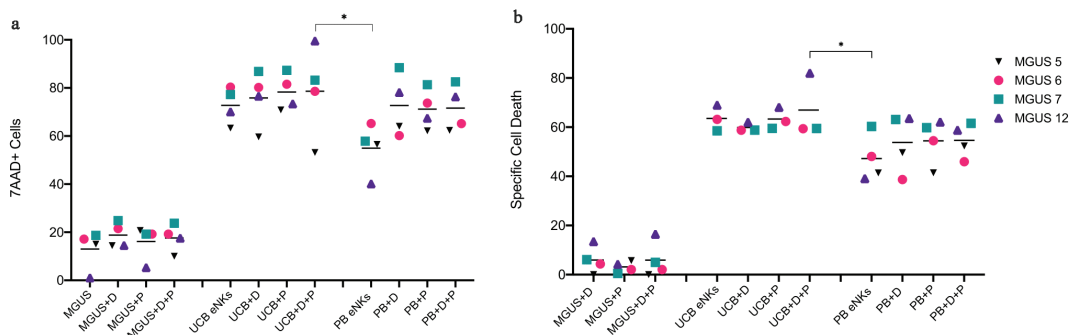


Figure 5. eNK cytotoxicity assays on samples from MGUS patients. Cells from MGUS patients were left untreated (MGUS), or they were treated with daratumumab at 5 $\mu\text{g}/\text{ml}$ (d), with pembrolizumab at 10 $\mu\text{g}/\text{ml}$ (p), or with their combination (D + P), or incubated with PD-1-negative UCB or PB eNKs for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. After the incubations, cell death was estimated by 7-AAD labeling in gated target cells (a), as indicated in Material and Methods. In (b) results are shown as the percentage of specific cell death induced, after subtracting basal cell death in each sample. Horizontal lines indicate the mean cell death in each experimental condition. One way ANOVA, ANOVA post hoc Tukey's analysis were applied for multiple comparisons among various groups. * indicates p value less than 0.05 and considered to be significant.

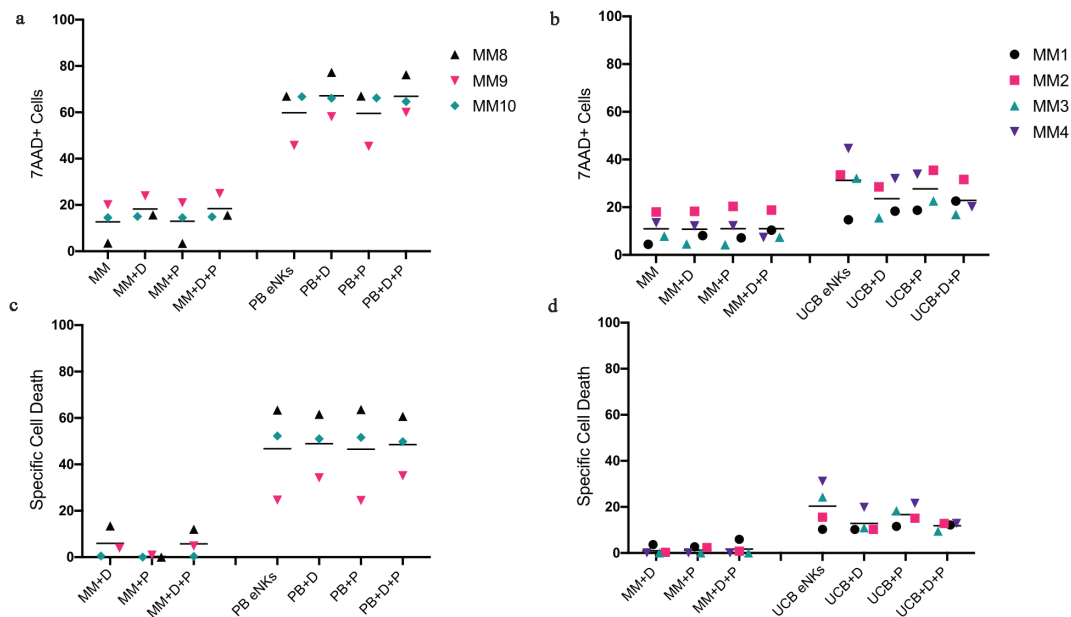


Figure 6. Cytotoxicity of eNK cells that contained a PD-1⁺ population. (a, c) Cells from the three indicated MM patients were left untreated (MM), or they were treated with daratumumab at 5 $\mu\text{g}/\text{ml}$ (d), with pembrolizumab at 10 $\mu\text{g}/\text{ml}$ (p), or with their combination (D + P), or incubated with eNK cells from PB2 for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. (b, d) Cells from the four indicated MM patients were left untreated (MM), or they were treated with daratumumab at 5 $\mu\text{g}/\text{ml}$ (D), with pembrolizumab at 10 $\mu\text{g}/\text{ml}$ (P), or with their combination (D + P), or incubated with eNK cells from UCB1 for 4 h, as indicated, in the absence of in the presence of the indicated concentrations of daratumumab, pembrolizumab, or their combination. After the incubations, cell death was estimated by 7-AAD labeling in gated target cells (a, b), as indicated in Material and Methods. In (c, d) results are shown as the percentage of specific cell death induced, after subtracting basal cell death in each sample. Horizontal lines indicate the mean cell death in each experimental condition.

expression was high. This decrease in cytotoxicity was not due to the inhibitory effect of PD-1 because pembrolizumab did not increase cytotoxicity on any of the four patients tested. Interestingly, UCB1 was the only expansion that stopped proliferating at d 15 (see Suppl. Fig 2).

To complete the information on the cytotoxicity experiments, we include as Supplementary Figure 4 the individual cytotoxicity data obtained from the 22 patient samples, each treated with the eNK cells delineated in Table 1(b,c).

2.6. Pattern of PD-L1 expression in MM cells

In order to better understand the possible role of the PD-1/PD-L1 axis in the regulation of eNK cytotoxicity, we analyzed the PD-L1 levels of each MM sample (Figure 7(a)). In most of the samples, a population of PD-L1⁺ cells could be detected among MM cells, 40% being the average value. Only MM3 could be considered negative for PD-L1 expression. Thus, we divided MM samples as PD-L1^{high} or PD-L1^{low} depending on whether

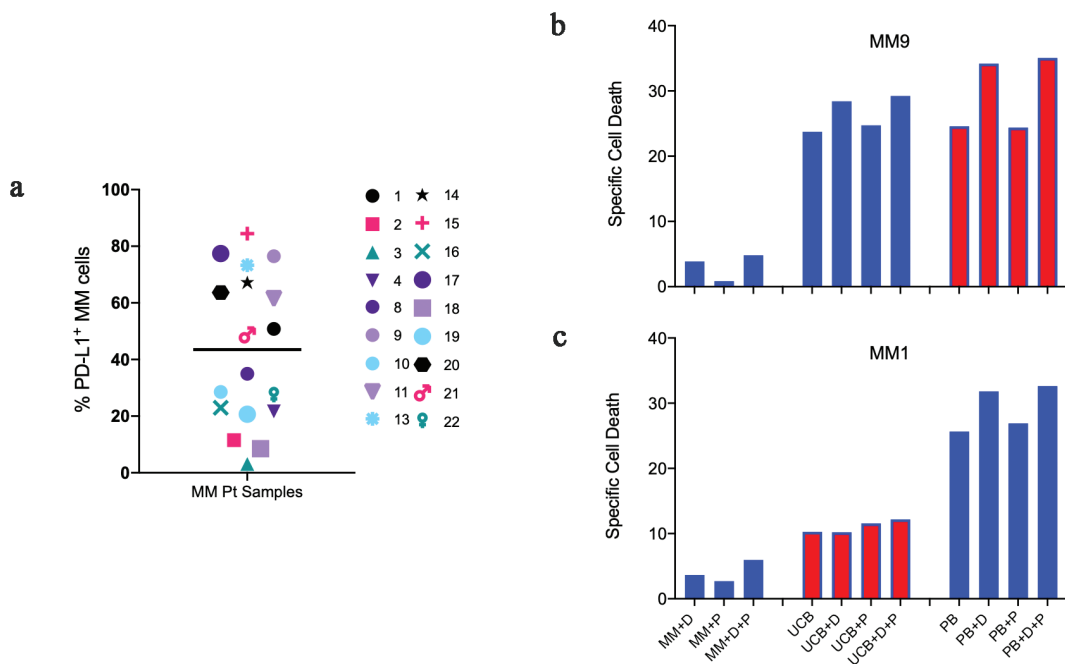


Figure 7. (a) Expression of PDL-1 on the surface of MM samples. The black line corresponds to the average value and is used to divide samples as PD-L1^{high} and PD-L1^{low}. Only cells from MM3 can be considered negative for PD-L1 expression. (b, c) Cytotoxicity assays using eNK cells that contained a PD-1⁺ population on PD-L1^{high} MM samples. B, MM9, which is PD-L1^{high}, was treated with PB2 eNK cells (red bars) or with PD-1 negative UCB2 eNK (blue bars). C, MM1, which is PD-L1^{high}, was treated with UCB1 eNKs (red bars) or with PD-1 negative PB1 (blue bars). Cell death was tested by 7-AAD labeling, as indicated in the legends of Figures 4, 5 and 6. Results are shown as the percentage of specific cell death induced, after subtracting basal cell death, which was never higher than 15%.

this population accounted for more or less than 40%, respectively. Nine patients fell into each category, although within each group there was high variability in expression levels. The average MFI value in the patients considered PD-L1^{high} was 10819, while in the patients considered PD-L1^{low} was 5720. The PD-L1 labeling for each individual MM and MGUS patient is shown in Suppl. Fig 5. We confirm previous data indicating that MGUS cells are negative for PD-L1 expression, while most MM samples showed PD-L1 expression to some degree.³⁵

Taking into account that only few NK cell expansions contained a significant PD-1⁺ population (PB2 and UCB1), we were able to study the effect of pairing these eNK cells with PD-L1^{high} MM cells only in one patient for each UCB and PB eNKs. Patient 9 (MM9), in which 79% of the cells expressed PD-L1, was one of the patients treated with the PB2 eNKs. The cytotoxicity of PB2 eNKs on MM9 cells was intermediate (Figure 7(b), red bars). Of the MM samples treated with these eNKs, MM9 exhibited one of the lowest levels of cytotoxicity (see Figure 6(a,b)). In this respect, the interaction of PD-1 on eNKs with PD-L1 on MM cells correlates with rather low cytotoxicity levels. A slight increase in cytotoxicity was observed when PB2 eNKs were combined with daratumumab. However, the expected increase in cytotoxicity due to PD-1 blocking by pembrolizumab was not observed. MM9 cells were also treated with eNKs from the PD-1 negative UCB2. A similar level of cytotoxicity was observed, slightly increased by the combination with daratumumab and unaffected by incubation with pembrolizumab (Figure 7(b), blue bars).

Patient 1 (MM1) showed 54% of cells expressing PD-L1, and was one of the patients treated with the UCB1 eNKs. In

agreement with results obtained from the other patients treated with this UCB, cytotoxicity was markedly low with no more than 10% of specific cell death induction (compare Figure 7(c), red bars, with Figure 6(c,d)). Neither daratumumab nor PD-1 blocking with pembrolizumab increased the cytotoxicity of UCB1 eNKs on cells from this patient. Of note, the same result was observed using the same UCB eNKs against MM cells from patients 2, 3 and 4 (Figure 6(c,d)) that were PD-L1^{low} or negative (see Figure 7(a)). MM1 cells did not exhibit an intrinsic resistance to NK cell cytotoxicity, since when treated with eNKs from PB1, that were PD-1 negative, showed an intermediate level of cytotoxicity, somewhat increased by combination with daratumumab (Figure 7(c), blue bars).

Pembrolizumab was active in the experimental conditions used in this study. When used in combination with two PB eNKs that exhibited PD-1 expression in a significant percentage of cells, pembrolizumab was able to increase cytotoxicity on the PD-L1⁺ B-CLL cell line Mec-1 (Supplemental Fig. 6). When this assay was performed using another 5 PB eNKs that did not exhibit PD-1 expression, pembrolizumab was without effect (data not shown).

3. Discussion

The present work demonstrates how the activation and expansion of allogeneic NK cells generates eNK cells that are active against MM cells. The activation and expansion were achieved in UCB and PB Nks by using LCL lymphoblasts as feeders in combination with IL-2 and IL-15. In both cases, the final eNK product exhibited CD16 expression on an important fraction of their population (more than 80%), which would allow for

their combination with a variety of therapeutic mAbs directed against tumor antigens.⁷ This increase in cytotoxicity could be associated with increased expression of activation markers,⁷ and of the activating receptor Nkp44.⁴⁵ Moreover, in a similar study using a 5-d activation protocol, it was shown that the main change justifying the increase in cytotoxicity was the net increase in the level of granzyme B expression in activated NK cells.⁴⁰

We observed how UCB eNKs showed a higher average cytotoxicity on MM cells than PB eNKs. The combination of PB eNKs with the anti-CD38 mAb daratumumab, which is already an effective treatment in a fraction of MM patients, increased their cytotoxicity to the levels observed for UCB eNKs.

This is especially relevant as allogeneic NK cell activation-based therapy, alone or in combination with antibodies, is yet to be approved for treatment of MM. While CAR T cell technology is at the forefront of current studies, it is expensive and has detrimental side effects, such as humoral immunity inhibition that needs to be treated over long periods of time. eNK cell treatments do not generate a durable memory response and would not have this side effect. Given the wide variety of previous treatments undergone by the patients in this study, relapsed and refractory patients would benefit from and respond to eNK cell therapy.

MM patients present an immunosuppressive tumor micro-environment, in which almost all arms of the immune system are subverted.^{22–24} Specifically, NK cell activity in MM patients is compromised and patient NK cells are ineffective against the development of the disease.²⁵ However, clinical data indicate that NK cells could be a therapeutic strategy in MM. In a pioneering work, Shi used haplo-identical mismatched NK cells treated with IL-2 for 2 d (no expansion) resulting in positive outcomes in 5 out of 10 relapsed MM patients.²⁹ Another study used expanded NK cells from patients as a therapy, with response in 2 out of 7 patients.³⁰ A similar study used expanded patient NK cells in combination with anti-myeloma drugs in five relapsed MM patients, showing partial but durable responses.²⁶ Nevertheless, no studies have been performed using allogeneic expanded NK cells obtained from healthy donors. More recently, an increase in cytotoxicity has been demonstrated on *ex-vivo* cells obtained from MM patients by combining expanded NK cells with carfilzomib²⁷ and also by combining daratumumab with the transformed NK cell line KHYG1 transiently expressing CD16.³¹ Additionally, preclinical studies have demonstrated the efficiency of anti-CS1 (SLAMF7, CD319) NK-CAR cells on MM cell lines.⁴⁷ A recent study using a humanized mice model demonstrated a positive effect of combining NK cells with the anti-CD137 mAb urelumab, but not with daratumumab.⁴⁸

PD-1 is an inhibitory receptor present on NK cells and activated CD4⁺ and CD8⁺ T lymphocytes. It is involved in immunosuppression by binding to its ligands PD-L1 and PD-L2, the former showing a broader expression.⁹ MM is characterized by augmented PD-L1 expression^{49,50} and some reports indicate that PD-L1 expression correlates with disease progression from the MGUS stage.^{36,37} Because of that, several phase III clinical trials were conducted with checkpoint inhibitors in combination with established MM treatments:⁵¹ pembrolizumab plus lenalidomide and dexamethasone (Dex) (KEYNOTE-185, NCT02579863); pembrolizumab plus pomalidomide (Poma)

and dexamethasone (KEYNOTE-183, NCT02576977); and another study testing three different combination regimens (Poma and Dex vs. nivolumab, Poma and Dex vs. nivolumab, elotuzumab, Poma and Dex; CheckMate 602, NCT02726581). However, these studies were discontinued due to an increase in deaths in the pembrolizumab group as well as no objective responses. Indeed, the FDA has put on hold another clinical trial combining the anti-PD-L1 mAb atezolizumab with daratumumab for similar reasons (NCT02431208, see³⁸).

Our data indicate that most eNK cells obtained using the protocol described showed a small population of PD-1⁺ cells, suggesting that this inhibitory pathway would have a low relevance in the treatment of MM patients by these eNKs. However, in a minority of the cases, eNK cells showed a significant population of PD-1⁺ cells. Particularly in the case of UCB eNKs that contained this PD-1⁺ population, cell death induction on MM cells decreased dramatically and addition of pembrolizumab did not restore cytotoxicity. This result was observed on MM cells positive or negative for PD-L1 expression. This suggests that these eNK cells from UCB are intrinsically nonfunctional, independent of the PD-1 signaling pathway. This result indicates that the status of PD-1 expression in the final cellular UCB eNK product should be determined as a marker of lower cytotoxic activity.

The present data could be supported by studies in 3D cultures or in *in vivo* MM models. However, 3D cultures require relatively long-term periods *in vitro*. During this time, tumor cells express ligands that were not expressed *ex vivo* and down-modulate the expression of other molecules. Moreover, there is a selection bias of certain clones that grow better in *in vitro* culture. In contrast, we examined tumor cell sensitivity to eNK cells directly *ex vivo*, without culturing them *in vitro*. We chose to test tumor cell sensitivity to eNK cells quickly after obtaining the cells from the patient, avoiding culturing the tumor cells, a process that can affect their sensitivity to allogeneic NK cells. Likewise, similar problems can be envisaged in human MM cell engraftment in NSG mice, as the human microenvironment is not reproduced.

UCB, rather than PB, contains different NK cell progenitor populations with the capacity to differentiate into NK cells⁵² and this could originate NK cells with higher cytolytic activity against primary MM cells. Interestingly, UCB NK cells, compared to PB NK, have a higher expression of the bone marrow homing receptor CXCR4⁵² and somehow this could help to recognize primary MM cells that niche in the bone marrow.

The present data indicate that UCB eNKs are to be preferentially used against MM in the absence of daratumumab while PB eNKs have significant cytotoxic advantage when combined with this mAb. Our present data, obtained with MM patients ranging from newly diagnosed to relapsed/refractory, reinforce the feasibility of using allogeneic eNK cells in the treatment of MM, mainly in view of their high cytotoxicity against tumor cells in combination with daratumumab.

4. Materials and Methods

4.1. Ethical statement

The use of human specimens for scientific purposes was approved by the French National Ethics Committee. All

methods were carried out in accordance with the approved guidelines and regulations of this committee. Written informed consent was obtained from each patient or donor prior to surgery.

4.2. Bone marrow aspirates from multiple myeloma patients

Data and samples from patients were collected at the Clinical Hematology Department of the CHU Montpellier, France, after patient's written consent and following French regulations. Patients were enrolled in the HEMODIAG_2020 (ID-RCB: 2011-A00924-37) clinical program approved by the "Comités de Protection des Personnes Sud Méditerranée I" with the reference 1324. Samples were collected at diagnosis and kept by the CHU Montpellier. MM and MGUS samples were processed to isolate the mononuclear cell fraction from the bone marrow aspirate by density gradient centrifugation. Cells were then washed and frozen in a 10% DMSO solution in liquid nitrogen until use.

4.3. Feeder cell lines

721.221 and PLH are EBV+ transformed, human B-cell lymphoblastoid cell lines. Both were cultured in RPMI (Invitrogen) with 10% FBS at 37°C. Before use in the expansion protocol, cells were inactivated with γ -irradiation. PCR was carried out on cell lines periodically to test for mycoplasma contamination.

4.4. NK cell isolation and expansion

Peripheral blood was obtained from five individual donors of the "Etablissement Français du Sang (EFS)". This work also benefited from umbilical cord blood units (UCBs) and the expertise of Prof. John De Vos, in charge of the Biological Resource Center Collection of the University Hospital of Montpellier – <http://www.chu-montpellier.fr/en/platforms> (BIOBANQUES Identifier – BB-0033-00031).

PBMCs and UCBMCs were isolated through density gradient centrifugation using Histopaque-1077 (Sigma). Blood samples were diluted at 1:1 ratio with RPMI then layered above 10 mL Histopaque in a 50 mL conical tube. Once centrifuged for 30 minutes at 400xg, the white layers of mononuclear cells (MCs) were collected and washed.

Using EasySep™ Human CD3 Positive Isolation kit (StemCell Technologies), the CD3⁺ cell fraction (T and NKT cells) of the MCs was depleted in each sample to better culture the NK cells. Once depletion was verified through flow cytometry, cells were cultured for 20 d. UCB NKs were cultured with γ -irradiated PLH at a 1:4 (NK cell: feeder cell) ratio. PB NKs were cultured with γ -irradiated 721.221 cells at a 5:1 (NK cell: feeder cell) ratio. The same concentration of IL-2 (100IU/mL) and IL-15 (5 ng/mL) were added to both NKs. Feeder cells and cytokines were refreshed every 3–4 d for UCB NKs and every 5 d for PB NKs. To monitor expansions, NK cells were stained with APC-labeled anti-CD3 mAb and PE or Vio770-labeled anti-CD56 mAb (both from BD Biosciences) and the percentage of CD3⁺CD56⁺ cells estimated at each time point. At d 20,

NK cell purity was 95% as average (see Suppl. Table 1). Culture viability was determined at regular intervals through flow cytometry analysis.

4.5. FACS analysis

The expression of CD16 during the expansions was also estimated by flow cytometry using VioBlue or PerCP. Cy5.5-labeled anti-CD16 mAb (BD Biosciences). The expression of PD-1 in the final eNK product was analyzed with PE-labeled anti-PD-1 mAb (BD Biosciences). PD-L1 expression in gated MM cells from patients (positive for CD38) was tested with an APC-labeled anti-PD-L1 mAb (BD Biosciences). Flow cytometry analysis was performed with Beckman Coulter Gallios and data analyzed using BC Kaluza Analysis Software.

4.6. Cytotoxicity assays of patient samples with eNKs and mAbs

The cytotoxic potential of eNKs and mAbs against target cells was determined through a flow cytometry cytotoxicity assay. Patient samples were removed from liquid nitrogen storage, washed and incubated at 37°C for 30 minutes with relevant antibodies (daratumumab at 5 μ g/ml, and pembrolizumab at 10 μ g/ml) prior to use in the assays. eNK cells were co-cultured with target cells at a 5:1 effector:target ratio in the presence or absence of the indicated mAbs and incubated for 4 hours at 37°C. Subsequently, cells were marked with an APC-labeled anti-CD38 mAb or with a PE-labeled anti-CD138 mAb (all from Beckman) and with 7-AAD. NK cells were excluded from the gating by their smaller size and lower CD38 expression than MM cells, or by the absence of CD138 expression, respectively. MM cells present in the sample were gated using the CD38 or CD138 labeling and cell death estimated in the gated population by 7AAD positivity. In the samples where daratumumab is used, there is a competition between the therapeutic mAb and the anti-CD38 mAb used for flow cytometry, so only a CD138-based gating was used. Basal cell death was calculated from control samples and values were subtracted from those obtained for treated cells to obtain specific cell death. It is possible that this flow cytometry method would underestimate cytotoxicity, since cell debris coming from dead cells cannot be included in the analysis.

4.7. Statistical analysis

Statistical data were obtained using GraphPad Prism (5.0). The statistical relevance of each study was first evaluated via ANOVA, analysis variance. For statistical significance ($p < .05$), Post-hoc Tukey's analysis was employed.

5. Conclusions

NK cells are a promising source for cancer immunotherapy against hematological malignancies. Both UCB and PB are potential sources of NK cells. Our data present the optimal conditions for each eNK to produce maximal cytotoxicity against all types of MM – new, relapsed/refractory and MGUS. UCB eNKs were highly cytotoxic when used as monotherapy,

while PB eNKs combined with daratumumab had the greatest cytotoxic effect. These results indicate a potent future for eNK cell adoptive immunotherapy against MM and a need to tailor each eNK cell treatment based on treatment regimen.

Conflicts of interest

The authors report no conflict of interest.

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