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Novel treatment strategy with autologous activated and expanded natural killer cells plus anti-myeloma drugs for multiple myeloma

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ABSTRACT

This proof-of-concept single-arm open-label phase I clinical trial (NCT02481934) studied the safety and efficacy of multiple infusions of activated and expanded natural killer (NKAE) cells in combination with anti-myeloma drugs in multiple myeloma patients. It included five patients with relapsed or refractory MM who had received two to seven prior lines of therapy; NK cells were expanded for 3 weeks with K562-mb15-41BBL cells. Patients received four cycles of pharmacological treatment with two infusions of 7.5×10^6 NKAEs/kg per cycle. NKAE generation, expansion, and NK monitoring was assessed using flow cytometry. Eighteen clinical-grade NKAE cell GMP-grade products were generated to obtain 627×10^6 NKAEs (range: $315–919 \times 10^6$) for the first infusion and 943×10^6 (range: $471–1481 \times 10^6$) for the second infusion with 90% (±7%) purity. Neutropenia grade II occurred in two patients and was related to chemotherapy. Of the five patients, four showed disease stabilization before the end of NKAE treatment, and two showed a 50% reduction in bone marrow infiltration and a long-term (>1 y) response. The NKAE cells had a highly cytotoxic phenotype and high cytotoxicity *in vitro*. Infused NKAE cells were detected in bone marrow and peripheral blood after infusions. *Ex vivo* expansion of autologous NK cells is feasible, NKAE cells are clinically active and the multiple infusions are well tolerated in patients with relapsed or refractory myeloma.

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Introduction

Immune-based therapies represent a new weapon in the fight against cancer. Natural killer (NK) cells comprise approximately 15% of lymphocytes, and their main function is to destroy cells that are virally infected, damaged, or transformed.[1,2] Although NK function is relatively well preserved in multiple myeloma (MM), the disease is generally associated with intense immune dysregulation.

It has been suggested that enhanced NK cell cytotoxicity might be part of the mechanism of action of effective anti-myeloma drugs such as lenalidomide (LEN) and bortezomib (BOR).[3-6] We found that NK cells effectively kill clonogenic MM cells in methylcellulose cultures.[7] The clinical experience with NK cell infusions in MM is limited. Two studies showed modest results in the setting of autologous stem cell transplantation in MM patients with very bad prognosis; notably, the toxicity was very low.[8,9]

There are several approaches to the *in vitro* activation of NK cells, but none are optimal for meeting clinical requirements.[10,11] Co-culture with the genetically modified cell line K562-mb15-41BBL makes it possible to expand *ex vivo* large numbers of activated NK cells from MM patients under treatment. This cell line specifically activates NK cells even when only a small number of NK cells are available.[12-14]

There are several questions regarding NK cell therapy that must be resolved in order for this therapy to be clinically useful. (i) Can NK cells be used out of the transplantation setting? (ii) Can NK cells be used in combination with other anti-myeloma drugs? (iii) Can NK cells be infused and expanded several times? (iv) Are NK cells effective in this clinical setting? To answer these questions, we designed a phase I clinical trial that uses for the first time multiple infusions of autologous activated and expanded NK cells (NKAEs) in combination with the anti-myeloma drugs BOR or LEN in MM patients.

Results

Clinical results

NKAE generation, activation, and expansion

Eighteen clinical GMP-grade products were generated for infusion. The five patients received a total of 36 NKAE infusions: 8 infusions in 4 patients, and 4 infusions in 1 patient (due to an

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unrelated complication). We obtained a mean of 20.82 × 10^6 (range: 3.6–47 × 10^6) starting NK cells from 200 mL of peripheral blood (PB)/patient with no need for apheresis; this represented 17.4% (range: 6.5%–23.6%) of the total PBMCs. After the first week, the number of $CD56^+CD3^-$ NKAEs increased 13-fold (mean of 277.53 × 10^6 NKAEs; range: 162.6–403·8.1 × 10^6). After the second week, NKAEs had increased 30-fold (mean of 626.8 × 10^6 NKAEs; range: 314.6–919.25 × 10^6). We collected 550 × 10^6 (± 50 × 10^6) NKAEs from culture for the first infusion and left 281 × 10^6 (range: 153–439 × 10^6) growing in culture for the next infusion. At the time of harvest in the third week, the median number of NKAEs was 942.6 × 10^6 (range: 470.8–1480.8 × 10^6), and the cells were 91.7% (± 4.7%) pure (Fig. 1). At harvest, the cells had expanded 45-fold. The mean purity of the $CD3^-CD56^+$ NKAEs the third week was 90% (range: 80.1%–99.2%). The purity was greater than 75% at all times, except for one patient who needed two expansion procedures for the second cycle of treatment because the first one did not meet our purity requirements. Overall, the median viability was 92.28% (range: 40.05%–99.05%).

T cell contamination

CD3 depletion was not necessary as we used autologous products, and the NKAE cells were produced using low concentrations of IL-2 to reduce potential T-cell proliferation (100 IU/mL). Even so, T cells represented 52% (range: 44.6%–66.3%) of peripheral blood mononuclear cells (PBMC) after PB collection, but this percentage had decreased to 4% (range: 0.0%–11.6%) at the time the NKAE cells were harvested (Fig. 1).

Safety

C-Myc and telomerase (TERT) expression were not altered in the NKAE end products compared to the starting cell population. Two patients showed increases in cMyc and TERT expression during the second week of expansion, but the expression levels were back to baseline during the third week. Even so, no patient had any complications or secondary neoplasia. BCR-ABL expression was undetectable after the first week of culture and was undetectable in PB cells after NKAE therapy (Fig. S1). This indicates that the feeder cell line was eradicated from the cultures prior to infusion.

Efficacy and toxicity

Just prior to starting NKAE infusion therapy, patient 01 was being treated with LEN and showed asymptomatic progression (AP) with 70% bone marrow infiltration after 40 cycles. After NKAE infusion, the patient achieved a partial response (PR), the bone marrow infiltration had decreased to 30%, and M spike had decreased from 1.13 g/dL to 0.59 g/dL. PR was maintained for 13 mo with no other treatment after NKAE cell infusion than LEN + dexamethasone (Fig. 2 and Table 1), the same treatment used before NK infusion. There was no serious toxicity attributable to NKAE infusion, but this patient had grade II neutropenia that was related to the use of LEN before the clinical trial and did not require dose adjustment (Fig. S2).

Patient 02 was also being treated with 12 cycles of LEN and began receiving NKAE infusions while in relapse. She achieved stable disease that was maintained for 9 mo before disease progression, and she did not need further treatment for 15 mo. Notably, bone marrow infiltration by MM cells decreased from 16% to 7% at the end of NKAE treatment in this patient (Fig. 2). Grade II neutropenia was observed, but like patient 01, patient 02 did not need dose adjustment.

Patient 03 achieved a very good PR (VGPR) after 14 cycles with BOR + bendamustine (BEN) treatment before enrolling in the clinical trial. This patient had no bone marrow infiltration and was monitored by serum lambda chain levels during follow-up. At follow-up, her serum lambda levels were lower than her initial levels during the first cycle of NKAE treatment (from 21.8 mg/mL to 10 mg/mL), but she had disease progression 2 mo after stopping treatment due to unrelated toxicity. There was no other toxicity related to NKAE therapy.

Patient 04 achieved PR with BOR+BEN (five cycles) before joining the clinical trial and showed 7% plasma cells in his bone marrow. He achieved disease stabilization 4 mo after the first NKAE infusion with no need for additional treatment (Table 1). After that, he received the same treatment he was receiving before NKAE cell infusions (BOR+BEN).

Patient 05 showed biological progression under LEN treatment (nine cycles) and 53% plasma cell infiltration into her bone marrow before NKAE cell treatment. Once she finished NKAE treatment, similar to patient 04, she showed disease stabilization 4 mo after the first NKAE infusion with no need for additional treatment (Table 1). This was maintained for 2 mo after the end of treatment. Afterward, she continued with the same treatment scheme with LEN she was receiving before NKAE infusions.

Effect of treatment on NKAEs

Patients 03 and 04 were under treatment with BOR, and patients 01, 02, and 05 were under treatment with LEN. The patients did not show any differences in NK cell *ex vivo* expansion due to the anti-myeloma treatment they were receiving in that PBMCs obtained from patients under LEN treatment showed a proliferation capacity that was equal to that of PBMCs obtained from patients under BOR treatment. However, patients under BOR treatment showed better *in vivo* expansion. These patients had 15.15% NKs from PB (range: 8.8%–21.5%) before the first infusion, and this percentage increased to 26.9% (range: 24.9%–28.9%) before the next infusion. In contrast, patients treated with LEN showed only a 0.43% increase, from 24.86% (range: 17.22%–32%) to 25.29% (range: 23.5%–27.1%) (Fig. S3).

Biological studies

NKAE cell immunophenotype

After activation and expansion, the NKAE cells overexpressed activating receptors (Fig. 1) such as NKG2D ($p = 0.07$), NKp44, NKp46, NKp30 DNAM-1, CD25 ($p = 0.07$), and CD69 ($p = 0.07$). TRAIL and FasL expression by NKAE cells also increased. Additionally, NKAE cells exhibited very high CD56 expression, whereas the expression level of CD16 (FcγRIIIa) was maintained. We evaluated the expression levels of the inhibitory protein NKG2A this tended to show higher expression levels in NKAE cells, but the increase was not significant ($p = 0.317$).

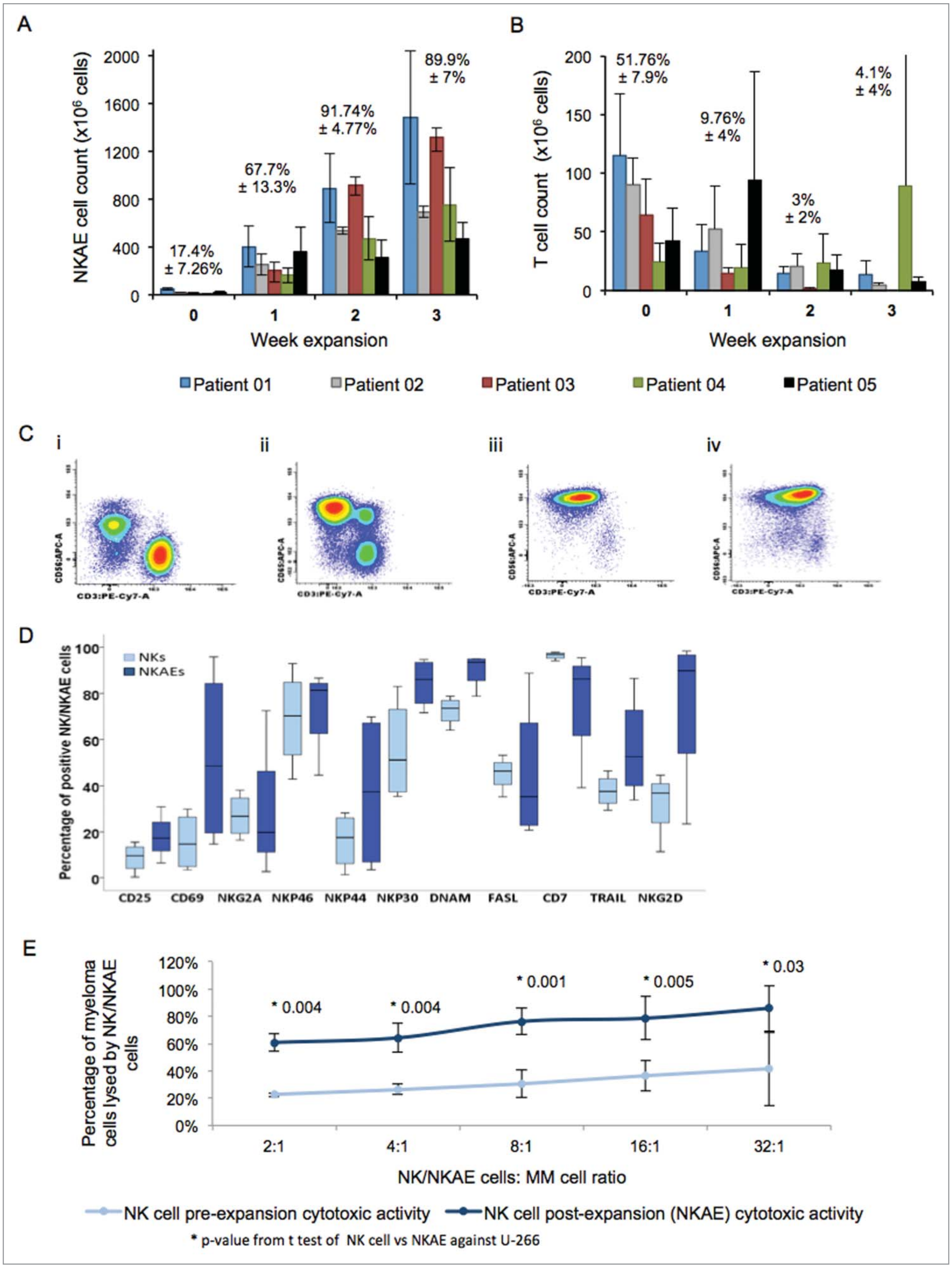

Figure 1. Characteristics of the activated and expanded natural killer cells (NKAEs). The characteristics of NKAEs expanded from the multiple myeloma patients in the NCT02481934 clinical trial were monitored every week by flow cytometry, and cell counts were also performed weekly. The results are reported as the mean value $\pm$ standard deviation of four independent expansion procedures from each patient. (A) The NKAE cell counts and (B) The reduction in T cell counts in the NKAE end products for the 18 expansion procedures. NKAE cell purity and T cell contamination percentages from each week are shown above its corresponding chart (as mean value $\pm$ standard deviation). (C) Representative dot plots of flow cytometry analyses of NKAEs during the expansion process. Each dot plot corresponds to each week of expansion (i: day 1, ii: day, 7, iii: day 14, and iv: day 21). (D) NKAEs overexpressed activating receptors and apoptosis ligands relative to NK cells before expansion. The data are reported as the median with interquartile range (IQR). (E) NKAEs had significantly better cytotoxic activity than NK cells against U-266 myeloma cells. The data are reported as the mean value $\pm$ standard deviation ($n > 3$). The p-values are from t-tests comparing the percentage of U-266 cells lysed by the NK cells pre-expansion with those lysed by NKAEs (NK cells post-expansion).

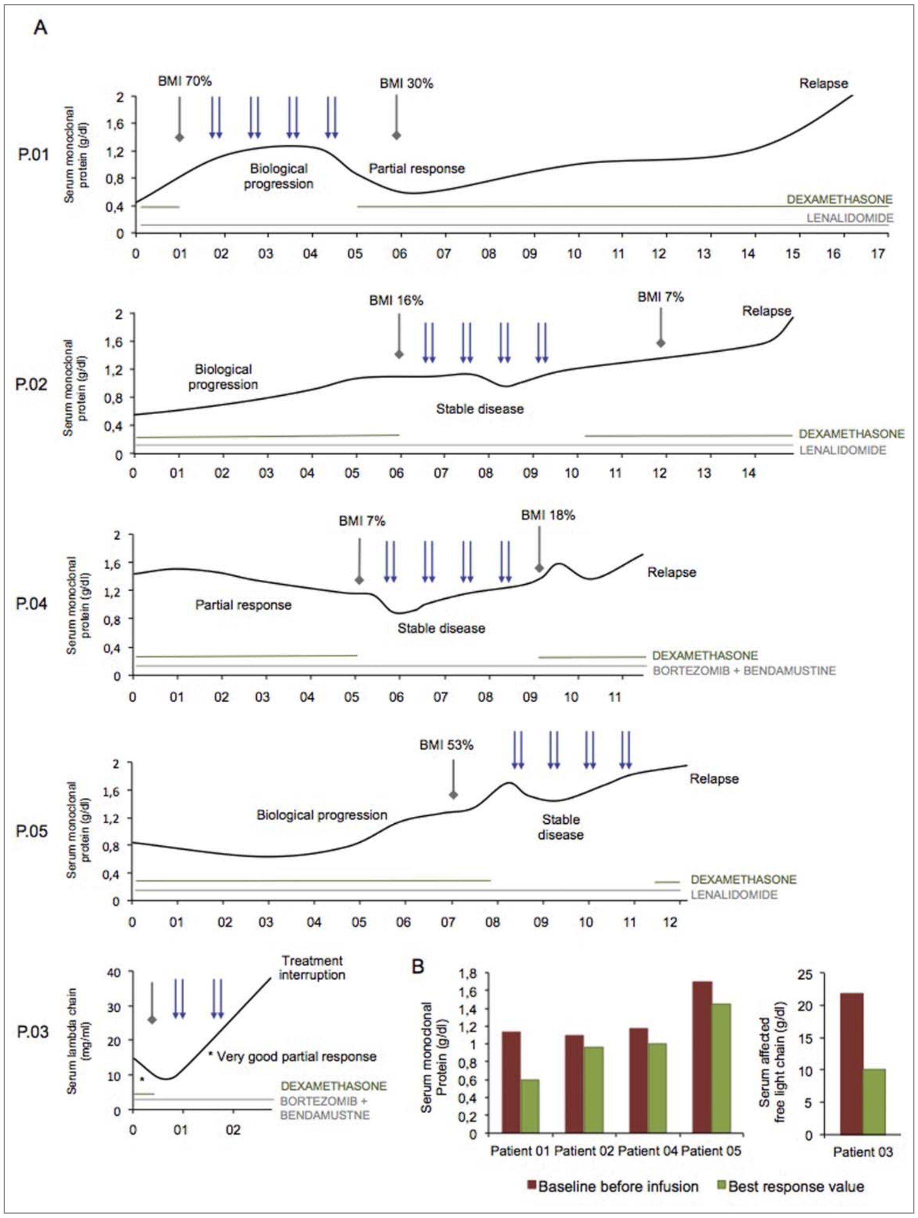

Figure 2. Assessment of the response of multiple myeloma patients to the infusion of activated and expanded natural killer cell (NKAEs). (A) The response to NKAE infusion was determined by monitoring the peripheral blood monoclonal protein levels (g/dL) until relapse and the bone marrow plasma cell infiltration (BMI) before and after NKAE cell therapy. The scale on the *x*-axis is months. Blue arrows indicate NKAE cell infusions, and gray arrows indicate when bone marrow aspirations were performed to determine BMI percentages. (B) The serum monoclonal protein levels and serum lambda chain levels (mg/mL) in patient 5 were compared before treatment and at the time of the best response.

Cytotoxic activity of NKAE cells

The *in vitro* cytotoxic activity of freshly prepared NKAEs against the U-266 MM cell line was always higher than the NK cell activity before expansion. Compared with NK cells before expansion, the activity from NKAE cells was at least 2-fold higher at the same ratio ($p < 0.03$) in a ratio range of 2:1 to 32:1 (Fig. 1).

Table 1. Summary of the results of the NCT02481934 clinical trial.

Situation before inclusion	Complete treatment	Response to NKAEs	Response duration (months)	Time to next treatment (months)	Hematologic toxicity
Biological progression	YES	Partial response	13	15	GII
Biological progression	YES	Stable disease	9	12	GII
Very good partial response	NO (TWO CYCLES)	Not evaluable (NE)	NE	5	NO
Partial response	YES	Stable disease	7	10	NO
Biological progression	YES	Stable disease	6	NE	NO

*Patient 3 withdraws the clinical trial before cycle 3 by an unrelated complication.

NKAE and immune monitoring

NKAE cells were detected in PB. After NKAE cell treatment, activating receptors such as NKG2D and NKp30 and the NK cell apoptosis receptors TRAIL and FasL were overexpressed in NK cells (Fig. 3). Moreover, NKAE cells were detected in the PB after each infusion. Examination of PB smears showed 3.2 ± 2 activated circulating lymphocytes before infusion and a 2.6-fold increase of activated circulating lymphocytes ($p < 0.05$) just after NKAE infusion (8.5 ± 3, $p < 0.05$). This increase was maintained 1 h after the infusion (7.7 ± 3.7). One day after the infusion, we observed a 2.01-fold increase (6.7 ± 3) in activated circulating lymphocytes (Fig. 3).

NKAE cells were detected in patients 1 week after the first NKAE infusion, just before the second NKAE infusion (Fig. 3). The patients did not receive any cytokine infusions, such as IL-2 infusions, in order to avoid collateral effects. The median NK

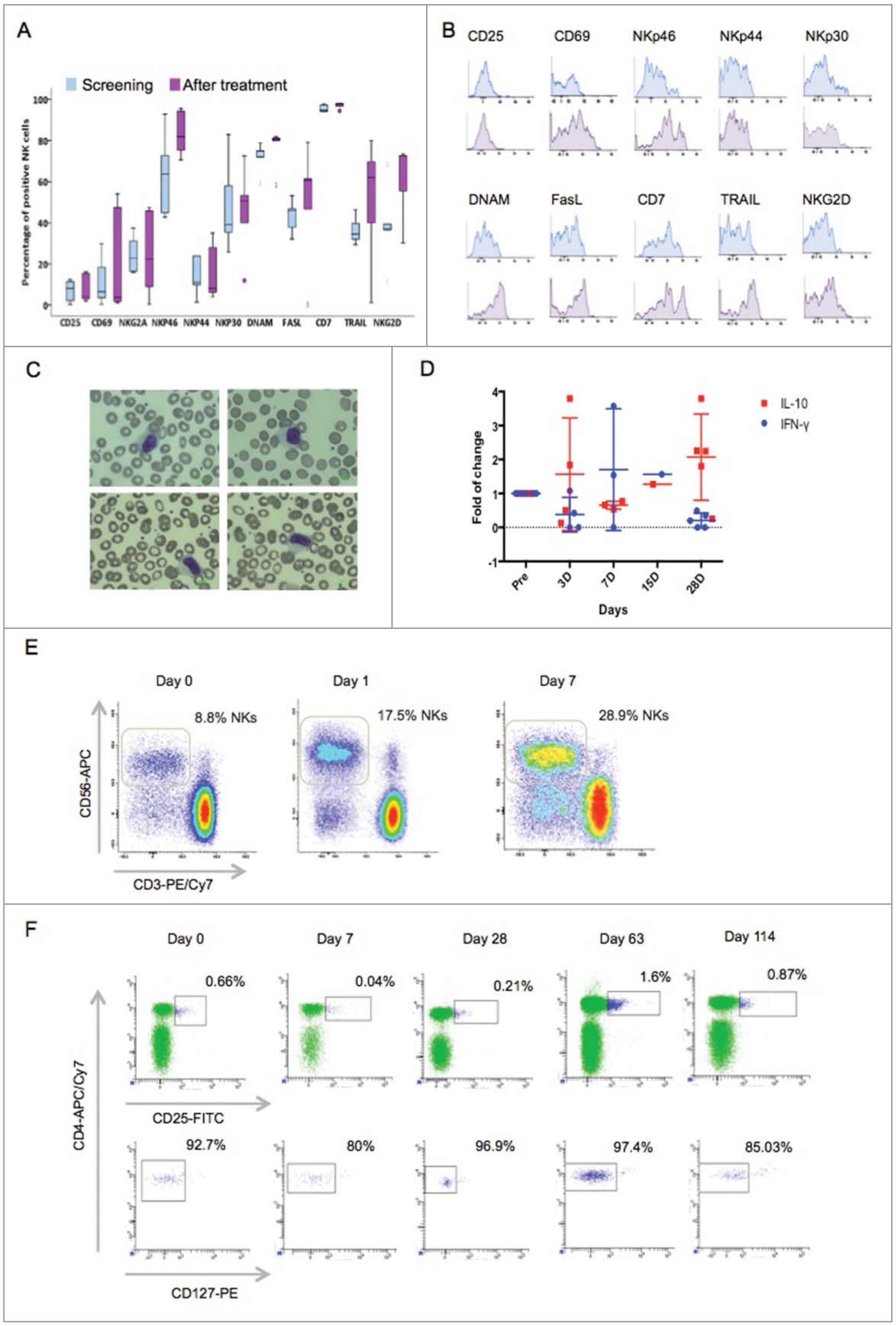

Figure 3. Monitoring activated and expanded natural killer (NKAE). NKAE cells were detected in peripheral blood samples by flow cytometry. Peripheral blood was obtained from the patients each cycle before each infusion, then after infusion, and then 1 h, 1 d, and 3 d after infusion, as possible. (A) The expression of NK cell surface receptors before and after treatment with NKAE cells. The results are reported as the median with interquartile range for five independent procedures. (B) Representative histograms showing the expression of NK cell receptors from a single patient before and after NKAE cell treatment. (C) NK cells were detected on peripheral blood Wright stained smears. (D) Cytokine serum levels were analyzed before treatment and on days 1, 3, 7, 15, and 28 of NKAE cell treatment. The IL-10 and IFNγ levels in five patients are shown as the fold changes compared to the screening (pre) cytokine concentrations. (E) Representative flow cytometry data of lymphocyte populations from a single patient during the first cycle. (F) The percentage of peripheral blood Treg CD4$^+$CD25$^+$CD127$^-$ cells during treatment with NKAEs.

cell percentage in PBMCs at screening was 21.5% (range: 8.8%–32%), while 7 d after the first infusion (before the second infusion) the median NK cell percentage was 25.3% (range: 23.5%–27.1%).

In all cases, the patients had higher absolute NK cell counts 7 d after the first infusion. When they were screened before the first infusion, the patients had a median absolute NK cell count of 99.4 NK cells/μL (range: 61.6–384 NK cells/μL), while before the second infusion they had 188 NK cells/μL (range: 74.7–406.5 NK cells/μL). This shows that NKAE cells persist in PB with no need for extrinsic stimulus.

Circulating cytokine levels

Circulating cytokines levels were analyzed in serum samples from the five patients. The patients showed increases in IFNγ during the first 7 d after the infusion of NKAE cells, from 2.232 pg/mL (range: 0–3.25 pg/mL) to a maximum of 3.987 pg/mL (range: 2.34–8.82 pg/mL). Subsequently, the IFNγ concentration decreased to levels lower than the basal serum concentration (0.599 pg/mL, range: 0–1.34 pg/mL) at the end of cycle (day 28), and this coincided with increases in IL-10 from 1.49 pg/mL (range: 0.69–8.1 pg/mL) to 2.424 pg/mL (range 1.67–2.04 pg/mL) (Fig. 3). Further increases in the IFNγ levels might be greater than described due to the sampling interval.

Mechanisms of NK cell activity suppression

T cells and regulatory T cells (Treg cells) were not upregulated after NKAE cell infusion. We found that $CD3^+CD4^+CD25^+$ $CD127^-$ Treg lymphocytes were not overexpressed during the course of the NKAE infusions. In addition, the percentage of $CD3^+$ T cells was unchanged (Fig. 3).

Discussion

Immunotherapy is increasingly important in cancer treatment. Some very interesting approaches based on immune therapies are emerging for the treatment of MM, including monoclonal antibodies that target immune checkpoints and CAR T cell infusion.[15,16]

This is the first study to combine NK cell therapy and anticancer drugs in a refractory MM patient population. One key finding was that it was feasible to perform several PB extractions from patients with MM, while they were receiving pharmacological treatment with no relevant adverse events. This is the first study to show that multiple infusions of NK cells can performed without any relevant toxic effects.

Our data demonstrated that *ex vivo* expansion of autologous NK cells is feasible in patients with MM under treatment with the anti-myeloma agents LEN, BOR and even during the last phases of the disease. Our results showed that the anti-myeloma treatment of choice will not affect the success of the expansion procedure, so patients receiving immunomodulatory drugs as well as proteasome inhibitors, may be eligible for this kind of treatment. Multiple blood collections and cell expansions can be performed without difficulty. Blood collection is inexpensive relative to leukapheresis, which is also more complex.[8] Using PB collection, one can obtain enough cells for several repeated expansions, and the infusion of moderate doses of NKAE cells is painless.

The design of this study allowed us to confirm that NKAE infusions have clear clinical efficacy in this population of patients with a bad prognosis and relapsed refractory MM. One patient who had relapsed after six lines of therapy achieved PR and subsequent disease stabilization. Another patient also achieved controlled MM after therapy with NKAE cells. This shows that two of the five patients had clinical benefits from the treatment; given the serious conditions of these patients, this is an excellent result.[17] We found that the combination of NKAEs plus LEN was the most clinically efficacious, since one patient achieved PR and the other achieved long-term disease stabilization; with BOR, one of two patients progressed. LEN and BOR are known to promote NK cell activity. In agreement with reports from other groups, our patients who were receiving BOR prior to and during NKAE cell treatment had better *in vivo* proliferation of NKAEs.[18,19]

The functionality of the NKAE cells was tested, and in all cases these cells showed high cytolytic activity against U-266 cells. In addition, the NKAE cells had a highly cytolytic phenotype, with high expression levels of activating receptors and apoptosis ligands. Notably, this correlated with their potent cytolytic activity *in vitro*.

The presence and increase in the number of NKAEs after infusion were described previously by Szmania et al.[8] Our study demonstrated that autologous NKAE cells could persist in PB between infusions without any noticeable toxicity. These data suggest that it is possible to perform regular NKAE infusions rather than just one infusion at the beginning of pharmacological treatment and that there is no need for cytokine infusions.

The autologous NKAEs were well tolerated. Although the patients did not receive dexamethasone during their treatment, we observed no relevant mechanisms of regulation of NKAE cells activity. The presence of Treg cells was considered important, as these cells are responsible for NK and T cell suppression.[20,21] The absence of IL-2 administration might favor the lack of mechanisms of regulation, since IL-2 administration increases the number of suppressive Treg cells in cancer patients.[22] The Treg cell level was unchanged during the course of this study.

Only the increase in IL-10 at the end of the cycle, which corresponded to a reduction in IFNγ levels, was notable. Interestingly, the IL-10 levels were always inversely related to the IFNγ levels. It is known that IL-10 is associated with regulation mechanisms,[23,24] but its role in myeloma pathogenesis still unknown, and its increase could be a way to compensate for the increase in IFNγ. Patients enrolled in this clinical trial did not exhibit any symptoms related to the upregulation of mechanisms of suppression, and IL-10 levels had no direct impact on patient prognosis.

In summary, we found that the activation and expansion of NK cells using the K562-mb15-41BBL cell line allowed multiple infusions of NK cells in MM patients. NKAEs plus LEN showed clinical efficacy in MM. Further clinical trials are warranted to assess the efficacy of NKAE in different settings.

Methods

Study design and eligibility

This single-arm open-label phase I trial (NCT02481934) tested the tolerability of the combination of multiple infusions of

NKAEs plus LEN or BOR in patients with relapsed refractory myeloma. Patients with MM with more than two relapses were eligible. Patients had to show a partial or stable response or AP to their most recent treatment, which was the treatment of choice in the clinical trial. The presence of measurable disease was defined as serum M protein 0.1 g/dL; urine M spike 200 mg/dL; or abnormal free light chain ratio, a measurable plasmacytoma, or >10% plasma cells in the bone marrow aspirate. Additional eligibility criteria are shown in Table S1.

All patients provided written informed consent as per the Declaration of Helsinki, and the study was approved by the *Hospital Universitario 12 de Octubre* Institutional Review Board. The study was conducted using funding from a Spanish Health program for clinical trials without support from the pharmaceutical industry. Common terminology criteria for adverse events version 3.0 were used to define and grade adverse events.

Objectives of the study

The main objective of this trial was to assess the toxicity and feasibility of NKAES in combination with the anti-myeloma drugs LEN or BOR. A secondary objective was to evaluate the efficacy of NKAE therapy. Eligible patients had to maintain the same rescue treatment (LEN or BOR) that had elicited their earlier response. This way, any change in the patient's status during the clinical trial would be attributable to NKAE cell infusion or to MM.

Cell lines

K562-mb15-41BBL GMP grade cells were kindly provided by Dario Campana,[14,25] former researcher from St. Jude Children's Research Hospital (Memphis, TN) and U-266 myeloma cells were purchased from DSMZ (Catalog number ACC-9) that performs cell line characterizations by short tandem repeat DNA typing and were used within 6 mo after resuscitation. In addition, cells were tested for mycoplasma contamination. Cells were incubated in RPMI medium (Biowest, catalog number L0498-500) with 10% FCS (Hyclone, catalog number SV30160) in a humidified 5% CO2 chamber at 37°C.

Patients

Five eligible patients with relapsed refractory MM (2–7 prior lines of therapy) were included in this study. The patient characteristics are shown in Table 2. Notably, three patients showed AP during the last treatment line just prior to the inclusion in the clinical trial and two showed PR after progression treatment. Three were treated with LEN-based regimens and two with BOR-based regimens.

Treatment and monitoring schema

After screening and registration, patients underwent four blood extractions of 200 mL of PB every 28 d to activate and expand the NK cells. The blood was collected in conventional bags for blood donation with 15 IU/mL of sodium heparin. Each of the four blood extractions was performed before each of the four scheduled treatment cycles (Fig. 4). PBMCs cells were isolated by centrifugation over a density gradient (Ficoll-Paque PLUS; GE Healthcare, catalog number 17-1440-02). The PBMCs were then activated and expanded for 3 weeks to achieve NKAE expansion. PBMCs were co-culture with the K562-mb15-41BBL feeder cell line plus 100 IU/mL of IL-2 under good manufacturing practice (GMP) conditions as described previously.[12-14], [25] NKAE cells were harvested on day 14 and 21 for infusions. Four cycles of pharmacological treatment and two infusions of 7.5×10^6 autologous NKAEs/kg were performed on days 1 and 8 of each cycle (Fig. 4).

Patients were treated with 4-week cycles of LEN or BOR, whichever they had received previously with no pause prior study inclusion and during the clinical trial; corticoids (dexamethasone) were withdrawn 15 d before starting the NK cell expansion. After finishing the NKAE infusions, the same previous treatment (LEN or BOR) with dexamethasone was performed until relapse.

Evaluation the safety of NKAE cell infusion

The safety and the lack of oncogenic effects of the NKAE end products were verified using real-time PCR to detect c-MYC and TERT expression in NKAE cells cDNA in the second and third weeks of expansion. Primers and probes were from Applied Biosystems (Life Technologies). Additionally, BCR-

Table 2. The characteristics of the five multiple myeloma patients prior to the infusion of activated and expanded natural killer cells (NKAEs).

Patient	Age	Sex	Cytogenetics by FISH in CD138$^+$ cells	Relapses (n) before the clinical trial	Previous ASCT	Previously treated with lenalidomide and bortezomib	Time from diagnosis of SMM and NKAES treatment (years)	Concomitant treatment with NKAEs	Situation before inclusion
1	53	M	1q amplification	5	2	Y	7	Len	Biological Progression
2	61	F	14q deletion	2	1	Y	2	Len	Biological Progression
3	73	F	1q amplification 17p deletion	4	0	Y	5	Bor+Ben	Very Good Partial Response
4	62	M	1q amplification 17p deletion	6	2	Y	10	Bor+Ben	Partial Response
5	72	F	1q amplification17p deletion	7	2	Y	13	Len	Biological Progression

Notes: SMM: symptomatic multiple myeloma, ASCT: autologous haematopoietic stem cell transplantation.

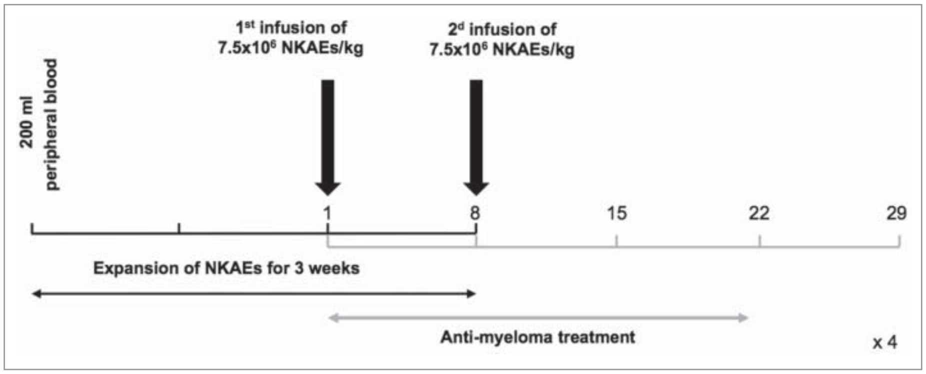

Figure 4. Treatment procedures and timeline. Two weeks before each cycle of chemotherapy, 200 mL of peripheral blood was extracted from each of the five multiple myeloma patients to produce activated and expanded natural killer cells (NKAEs). The NKAEs were infused in the second and third weeks of expansion on days 1 and 8 of each of the four cycles of lenalidomide- or bortezomib-based treatment. The scale in the *x*-axis is days.

ABL qPCR studies were carried out using cDNA from NKAE cultures and from patient PB samples after treatment to verify the absence of K562-mb15-41BBL cells in NKAE end products with rimers and probes from Tip Molbiol (Eresburgstrasse). Real-time PCR studies were performed as described previously using an ABI PRISM® 7900HT Sequence Detection System (Life Technologies).[26]

Immune monitoring and MM response assessment

Immune monitoring was carried out using PB samples. Blood samples for immune monitoring were obtained before treatment and on days 1, 3, 7, 14, and 28 of the first cycle; on days 1, 7, and 28 of the subsequent cycles; and 30 d after completion of the entire therapeutic regimen. The tumor response was evaluated every 4 weeks and 30 d after all protocol therapy was completed, including bone marrow assessment.

NK and T cell monitoring

Flow cytometry analysis of NK cells

The number and presence of NK and NKAE cells ($CD3^{-}CD56^{+}$) were analyzed by flow cytometry on a FACSCanto II™ cytometer (BD Biosciences) based on the percentage of the NK cell population and on the expression of the activating receptors NKG2D and NKp30. The gating strategy was based on dead/live cells and doublet discrimination. The antigen expression profiles of NK and NKAE cells were evaluated using the following fluorescently-conjugated antibodies: CD69, CD25, CD31, DNAM-1, FasL, CD7, TRAIL, NKG2D, NKp46, NKp30, NKp44, and NKG2A (Table S2 lists all of the antibodies used in this study). The data were analyzed with FACSDiva™ software (BD Biosciences).

Peripheral blood smears

Wright stained smears of peripheral blood from patients were also performed before infusion, immediately after infusion, 1 h after infusion and 1 d after infusion. Images were acquired on a Nikon 80i light microscope supplied with a Nikon DS-Fi1 camera and a PLAN 100 XA/ NA 1.25 Oil objective (Nikon).

Flow cytometry analysis of Treg $CD4^{+}CD25^{+}CD127^{-}$

The regulatory $CD4^{+}CD25^{+}CD127^{-}$ T-lymphocyte responses of three patients were analyzed by flow cytometry of fresh PB samples based on the percentage of cells.

Eu-TDA release assay

The cytotoxicity of PBMC and NKAE cells was assessed using Eu-TDA release assays following the manufacturer's instructions. U-266 myeloma cells were used as target cells and were incubated for 2 h with effector cells (NKAEs) at the indicated effector: target (E:T) ratio. The specific lysis percentage was calculated as described previously.[13]

Cytokine expression profile analysis

The cytokine expression profile was analyzed by flow cytometry using the BD™ Cytometric Bead Array from BD Biosciences following the manufacturer's protocol. IL-2, IL-4, IL-6, IL-10, TNF, IFNγ, and IL-17A were monitored in serum samples from the five patients on days 0, 3, 7, and 30 after infusion.

Statistical analysis

Assay data were compared using the Student's *t*-test or the Wilcoxon test. Significance was defined as $p < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

A.L., J.M.L., J.J.L., and A.P.M. contributed to the study conception and design. A.L., J.M.L., and J.J.L. were responsible for the protocol development. J.M.L., J.J.L., and M.J.B. contributed to the patient recruitment. A.L. and E.M.C. performed experiments. A.L., J.M.L., E.M.C., and L.F. contributed to the acquisition of data. A.L., L.F., A.P., and J.M.L. contributed to the data analysis and interpretation. All authors were responsible for the preparation and review of the final version of the manuscript.

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