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Haploidentical IL-15/41BBL activated and expanded natural killer cell infusion therapy after salvage chemotherapy in children with relapsed and refractory leukemia


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ABSTRACT

Primary refractory or relapsed pediatric leukemia yield significant morbidity and mortality, with long-term survival rates < 40%. Here we present a post-hoc analysis assessing safety and efficacy of infusing activated and expanded Natural Killer cells (NKAE) from haploidentical donors in patients from 2 clinical trials. In total, 18 children, adolescents and young adults with relapse or refractory acute leukemia were treated with two cycles of rescue chemotherapy followed by fresh NKAE cells infusions and low doses of IL-2. The overall response rate, complete remission achievement at the end of the study, was 72% (13 of 18). We infused 52 NKAE cell products containing a median of 6.76x10^6 NK cells/kg (0.7-34.16) and 0.49x10^6 T cells/kg (0-11). All infusions were well tolerated with no graft versus host disease nor other serious adverse events. Among the 14 patients who completed treatment, 4 of them are alive and leukemia-free more than 750 days post-transplant. We conclude that infusion of fresh NKAE cell therapy is feasible and safe in heavily pretreated pediatric population, and should be further investigated in advanced-phase clinical trials as well as a consolidation therapy to decrease relapse in patients with high-risk leukemia.

Running title: Activated NK cell therapy in pediatric refractory leukemia

Key words: Natural killer cells, Hematopoietic stem cell transplantation, Immunotherapy, Acute myeloid leukemia, Lymphoblastic leukemia, Myeloblastic leukemia

Trials registration: Registered at www.clinicaltrials.gov as NCT01944982 and NCT02074657.
Haploidentical IL-15/41BBL activated and expanded natural killer cell infusion therapy after salvage chemotherapy in children with relapsed and refractory leukemia

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ABSTRACT

Primary refractory or relapsed pediatric leukemia yield significant morbidity and mortality, with long-term survival rates < 40%. Here we present a post-hoc analysis assessing safety and efficacy of infusing activated and expanded Natural Killer cells (NKAЕ) from haploidentical donors in patients from 2 clinical trials. In total, 18 children, adolescents and young adults with relapse or refractory acute leukemia were treated with two cycles of rescue chemotherapy followed by fresh NKAE cells infusions and low doses of IL-2. The overall response rate, complete remission achievement at the end of the study, was 72% (13 of 18). We infused 52 NKAE cell products containing a median of 6.76x10^6 NK cells/kg (0.7-34.16) and 0.49x10^6 T cells/kg (0-11). All infusions were well tolerated with no graft versus host disease nor other serious adverse events. Among the 14 patients who completed treatment, 4 of them are alive and leukemia-free more than 750 days post-transplant. We conclude that infusion of fresh NKAE cell therapy is feasible and safe in heavily pretreated pediatric population, and should be further investigated in advanced-phase clinical trials as well as a consolidation therapy to decrease relapse in patients with high-risk leukemia.

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1. INTRODUCTION

The incorporation of multidisciplinary treatment strategies over the last 20 years has resulted in major advances in the treatment of pediatric cancers, especially hematologic malignancies. Nearly 80% of children diagnosed with acute lymphoblastic leukemia (ALL) and 60% diagnosed with acute myeloid leukemia (AML) are cured with current multiagent regimens and enjoy a relatively normal quality of life and subsequent development\(^1,2\). Despite well-recognized improvements in outcomes, primary refractory or relapsed pediatric leukemia still yield significant morbidity and mortality, with poor long-term survival rates — less than 40\(^3,4\). Therefore, new therapeutic approaches that bypass the cellular mechanisms of drug resistance are urgently needed, particularly for those patients with relapsed and refractory leukemia.

Natural Killer (NK) cells are lymphocytes from the innate immunity characterized by the expression of CD56 and or CD16 and the lack of CD3. They have a critical role in immune control of tumor development and growth. Their antitumor activity relies on the balance of activating and suppressive signals including those delivered by killer immunoglobulin-like receptors (KIRs) that recognize specific Major Histocompatibility Complex (MHC) class I alleles\(^5,6\). To ensure ‘self-tolerance’, NK cells are ‘educated’ or ‘licensed’ during their development\(^7\). They gain functional competence through a maturation process involving interactions between KIR receptors and their respective HLA ligands. Licensed NK cells that express, as their only inhibitory receptor, a KIR whose ligand is an HLA class I group that is absent on allogeneic targets, mediate alloreactions. Preclinical and clinical data from the haploidentical T cell–depleted transplantation setting have shown that haploidentical KIR ligand–mismatched NK cells play a main role as anti-leukemia effector cells\(^8-12\).

Preclinical studies report how NK cells can target and kill leukemia cells without prior exposure to those cells and the magnitude of NK cell cytotoxicity is directly proportional to
the ratio between the number of NK cells and target cells\textsuperscript{13,14}. For this reason, in order to achieve clinical benefits, large numbers of highly activated and cytotoxic NK cells are needed. As NK cells only represent 5–20\% of peripheral blood mononuclear cells (PBMCs)\textsuperscript{15}, various NK cells production protocols have been explored. NK-cell expansion in the presence of antigen presenting cells shows comparable \textit{in vivo} survival with IL-2-mediated activation and can result in increased anti-tumor cytotoxicity\textsuperscript{16,17}. Different cell types have been used to expand NK cells \textit{in vitro}, including irradiated PBMCs, Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCL), and the K562 leukemia cell line genetically modified to express membrane-bound interleukin (IL)-15 and the 41BB ligand (K562-mb15-41BBL)\textsuperscript{18,19}.

The present analysis compiles data from two clinical trials where relapsed or refractory childhood leukemia/lymphoma patients were infused IL-15/41BBL activated and expanded NK (NKAES) cells from haploidentical donors after rescue chemotherapy. Two NKAES infusion schemes were followed. In the first clinical trial (HNJ-NKAES-2012) one NKAES cell product per expansion was infused, while in the second one (LYDIA) two NKAES cell products from each expansion were infused. The primary endpoint of both clinical trials was to assess the safety of infusing NKAES cells from haploidentical donors in combination with rescue chemotherapy.
2. PATIENTS AND METHODS

Patients
The population included in this analysis came from two open-label phase I/II trials: HNJ-NKAES-2012 (NCT01944982; EudraCT: 2012-000054-63); and LYDIA (NCT02074657; EudraCT: 2012-005146-38). Inclusion criteria were first or subsequent relapse of refractory T cell acute lymphoblastic leukemia/lymphoma (ALL-T) or AML and second or subsequent relapse of B cell lymphoblastic leukemia (ALL-B). The relatives of minor patients and those > 18 years provided written informed consent before entering the trials in accordance with the Declaration of Helsinki, and all research was approved by the ethical committee of the Hospital Infantil Universitario La Paz.

Treatment plan
Based in the good tolerance of NK cell therapy and low doses of IL-2 previously described by Rubnitz et al.\textsuperscript{20}, we established the following treatment plans:

Patients enrolled in HNJ-NKAES-2012 trial received two cycles of chemotherapy and one infusion of a maximum of $5 \times 10^7$ allogeneic NKA cells/kg on day 0 of the first cycle and another infusion of a maximum of $1 \times 10^8$ allogeneic NKA cells/kg on day 0 of the second cycle. They received intrathecal triple therapy (methotrexate, cytarabine, and hydrocortisone sodium succinate) before chemotherapy. Rescue chemotherapy consisted of nelarabine (Ara-G), etoposide (VP) and cyclophosphamide (Cy) according to Commander LA et al.\textsuperscript{21}. On alternate days, $1 \times 10^6$ U/m\textsuperscript{2} of IL-2 were administered subcutaneously for 6 doses starting on day 0 to activate and expand circulating donor NKA cells (Fig. 1A).

The patients enrolled in LYDIA trial received two cycles of chemotherapy and two infusions of a maximum of $2.5 \times 10^7$ allogeneic NKA cells/kg on days 0 and 7 of the first cycle and two
infusions of a maximum of $5 \times 10^7$/kg on days 0 and 7 of the second cycle. Three major pharmacological regimens were administered: CLOVE (clofarabine, etoposide and cyclophosphamide)\textsuperscript{22,23}; FLAG-Ida (fludarabine, idarubicin, cytarabine and G-CSF)\textsuperscript{24}, and FluCy (fludarabine plus cyclophosphamide)\textsuperscript{20} before NKAE cell infusion. As in the HNJ-NKAES-2012 trial, $1 \times 10^6$/U/m$^2$ of IL-2 were administered on alternate days starting on day 0 (Fig. 1B).

The chemotherapy regimen and the interval between cycles were decided by the investigators based on previous chemotherapy regimen and toxicities-recovery after first cycle, ranging from 3 to 8 weeks, ideally 4 weeks. The persistence of the disease after the first cycle was not an exclusion criterion.

**HLA and KIR typing**

*HLA typing by PCR*

Patients’ and donors’ DNA samples were HLA typed at the intermediate-resolution level for alleles at HLA-A, -B, -C, -DRB1 and -DQB1 loci by PCR amplification and oligonucleotide hybridization. Additionally, the KIR ligand HLA-C (C1 and C2) and the HLA-B (Bw4 and Bw6) allotypes were determined using high-resolution PCR-sequence-based typing commercial kits from Thermo Fisher, ELPHA and/or One Lambda.

*Donor KIR B content score by PCR*

Donors’ fifteen human KIR genes and two pseudogenes were analyzed by PCR with a KIR typing kit (Miltenyi Biotec). We determined the KIR B content score for all the donors according to the system proposed by Cooley et al\textsuperscript{25} (www.ebi.ac.uk/ipd/kir/donor_b_content.html).
**KIR mismatch analysis by flow cytometry**

KIR receptors expression was studied in donors’ NK cells by multiparameter flow cytometry. PBMCs were obtained from peripheral blood (PB) samples by centrifugation over a density gradient (Ficoll-Paque; GE Healthcare). NK population was identified gating CD56⁺/CD3⁻ cells. Frequencies of NK cells binding HLA-C1 (KIR2DL2⁺ or KIR2DL3⁺), HLA-C2 (KIR2DL1⁺) or HLA-Bw4 (KIR3DL1⁺) were measured using a Navios flow cytometer (Beckman Coulter). Antibodies used are listed in Suppl. Table 1.

**Donor selection**

The haploidentical donor was selected according to the following order of preference, as previously described²⁶: (i) donor inhibitory KIR receptors and patient HLA-I ligand mismatch model; (ii) in the case of KIR-HLA match setting, KIR B haplotype donors were sought; (iii) if (i) and (ii) were not conclusive, we chose the haploidentical donor whose NK cells showed the highest *in vitro* cytotoxic activity against leukemia blast cells. In an absence of blasts, highest *in vitro* cytotoxicity against the K562 cell line was considered.

**Immunomagnetic isolation of donor-derived NK cells for adoptive immunotherapy**

Selected donors underwent one blood extraction of 250 ml of PB before each scheduled treatment cycle. The blood was collected in conventional bags with 15 IU/ml of sodium heparin in a facility complying good manufacturing practice (GMP). PBMCs were isolated by centrifugation over a density gradient (Ficoll-Paque; GE Healthcare). The GMP genetically modified K562-mbIL15-41BBL cell line, kindly provided by Professor D. Campana (National University of Singapore), was irradiated with 100 Gy. NKAESs were obtained by co-culturing donor’s PBMCs with K562-mb15-41BBL cells in a 1:1.5 ratio plus 100 U/ml of IL-2 over 14 days (LYDIA trial first infusion) or 21 days (HNJ-NKAES-2012 trial and LYDIA
trial second infusion). In HNJ-NKAES-2012 trial, the final infused cell products contained > 80% NK phenotype cells (CD56+/CD3−) and < 1x10^6/kg of T phenotype cells (CD56/CD3+). If T cells were present at higher numbers, CD3 cells were depleted from NKAE products using anti-CD3 antibody-coated beads and a CliniMACS device (Miltenyi Biotec). In LYDIA trial, this purity limits for NK and CD3 cells were not a discard criterion.

The safety and the lack of oncogenic effects of the NKAE end products were verified using real-time quantitative PCR (RQ-PCR) to detect c-MYC and telomerase reverse transcriptase (TERT) expression. Additionally, BCR-ABL RQ-PCR studies were performed using cDNA from NKAE cultures and from patient’s PB after treatment in order to verify the absence of K562-mb15-41BBL cells in NKAE end products.

**Toxicity register criteria**

The toxicity was classified according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0.

**Response Assessment**

Patients with acute leukemia were assessed by bone marrow response after hematomal recovery or, if there was no recovery, after a 6-week cycle. Complete remission (CR) was defined as trilineage hematopoietic recovery with less than 5% blasts in the marrow by morphologic assessment. Minimal residual disease (MRD) was defined as > 0.1% blasts and was studied by flow cytometry as previously described. The evaluation of the mass for mediastinal lymphomas was assessed by CT, PET-CT or X-Ray as follows: CR was defined as the disappearance of clinical symptoms and radiological findings. Partial response was defined as decrease in target lesion diameter sum > 25%. No response was defined as decrease in target lesion diameter sum < 25%.
Chimerism

Percentage of donor chimerism in the different blood cell populations was assessed using semi quantitative PCR–based analyses of polymorphic Short Tandem Repeat (STR) microsatellite regions as previously described (quantifiable limit 1-3%)\textsuperscript{32,33}. For that purpose, eight STR and amelogenin polymorphisms were employed: D21S11, TPOX, D18S51, Penta E, VWA, D22S689, D7S820, Penta D.

When lineage-specific analysis was performed, different cell populations were collected using monoclonal antibodies against CD56 conjugated with ferromagnetic particles (Miltenyi Biotech). After incubation, positive and negative cellular fractions were collected using an autoMACS\textsuperscript{®} device (Miltenyi Biotech).

Statistics

Descriptive statistics were used to characterize the population. Overall survival was calculated from the date of inclusion in the clinical trial until the date of death. Survival curves were constructed by the Kaplan–Meier method. The analyses were performed using GraphPad Prism version 5 (GraphPad Software).
3. RESULTS

Patients and donors

Patients characteristics are shown in Tables 1 and 2. Twenty eligible patients (7 in the HNJ-NKAES trial and 13 in the LYDIA trial) with relapsed or refractory ALL-B, ALL-T, AML, or biphenotypic acute leukemia (BAL) were enrolled. The mean age was 12 years (range 1–23). Ten of them had previously received a hematopoietic stem cell transplantation (HSCT).

All patients included in HNJ-NKAES trial received Ara-G/VP/Cy (n = 7); while patients included in LYDIA trial received CLOVE (n = 6), FLAG-ida (n = 3) or FluCy (n = 4) rescue chemotherapy.

Eighteen patients received at least one NKAE cell infusion. Two patients died of progressive disease (PD) before they could receive any NKAE infusion.

All NK cell donors were haploidentical related donors. PB extraction was performed without complications in all cases. Eighteen donors were parents of the patients and 2 were siblings. NK donor-recipient KIR mismatch and KIR haplotype score are shown in Suppl Table 2.

NKAE cell products

We obtained 52 NKAE products with a mean NK cells proportion of 79.16% (± 20.53%). One product was discarded due to bacterial contamination. Two products needed additional T cell depletion before infusion. The characteristics of the infused products are shown in Table 3. More detailed information regarding NKAE and T cells total dose infused per patient is available in Suppl. Table 3”.

c-Myc and TERT expression were not altered in the NKAE cell end products compared to the starting cell population. BCR-ABL expression was undetectable after the first week of culture and was undetectable in patients’ PBMCs after NKAE therapy, indicating no residual K562-mb15-411BBL cells presence in the infused products.
Safety of NK cell therapy

Adverse effects registered during HNJ-NKAES-2012 and LYDIA clinical trials are shown in Table 4. We observed fever related to NKAE infusion in 3 patients (15%). Two patients had low-grade fever and one patient a local reaction in relation to the administration of IL-2 injections.

The chemotherapy caused a total of 22 episodes of febrile neutropenia (100% of patients that received CLOVE or FLAG-Ida, 67% of patients that received AraG-based chemotherapy and 0% of patients that received FluCy).

Bacterial infection was observed in 8 bacteremia episodes (4 S. epidermidis, 1 E. coli, 1 K. pneumonia, 1 P. pnomenusa, 1 C. sputigena) and 1 C. difficile colitis. Viral infections were observed in 7 episodes (1 EBV reactivation, 1 HHV-6 viremia, 1 CMV encephalitis, 2 mucosal HSV-I herpes, 1 cystitis by BK virus and 1 influenza B pneumonia). Fungal infection was diagnosed in 5 episodes, 4 aspergillosis (3 probable and 1 proven) and 1 hepatosplenic candidiasis. Additionally, one patient developed Cryptosporidium colitis.

During the trials 4 patients died due to chemotherapy-related toxicity: typhlitis (patient #12); pericardial tamponade probably related to clofarabine treatment (patient #8); severe somnolence related to nelarabine treatment in a patient who had previously received total body irradiation as conditioning regimen for a mismatch unrelated transplantation (patient #1); and a multi-drug resistant K. pneumoniae–mediated sepsis (patient #16).

Chimerism

Donor cell chimerism analysis was performed on 15 of the 18 patients who ultimately received an NKAE infusion. The maximum peak reached in each patient sample is shown in Table 2. Cell donor chimerism ranged from 1 to 3%, except for patient #16, who reached 95%
of donor chimerism after a second cycle of 20%-reduced CLOVE, reflecting a severe myelosuppression state.

Cell donor chimerism was observed in 7 of the analyzed patients (46.7%). Of them, four achieved negative MRD at the end of the trial (57.1%). In 8 patients no cell donor chimerism was detected (53.3%). Three of these patients achieved negative MRD at the end of the trial (37.5%). Lymphocyte reconstitution in chimerism-detected and undetected patients showed no significant differences and no correlation between patients’ outcome and donor chimerism was observed (Fig. 2).

**Response**

Representative clinical responses after therapy and evolution of percentage of blasts throughout the trials are shown in Fig. 3 and Suppl. Fig. 1.

The flow chart of the trial is shown in Fig. 4. A total of 18 patients were evaluated after the first cycle: 10 achieved CR/MRD-, 6 achieved CR/MRD+ and 2 patients, one from each trial, showed no remission.

At the end of the trial, 15 of 20 patients were alive. The evaluation of bone marrow response showed that 6 patients achieved CR/MRD-, 7 patients achieved CR/MRD+ and in 2 patients no remission was observed.

Following the trials a total of 10 patients proceeded to HSCT, one patient received palliative care and one patient was enrolled in another clinical trial. Of the HSCT-receiving patients, 6 finally died, 4 due to PD and 2 due to transplant-related toxicity (TRM). In September 2017, 4 patients were alive (with no evidence of disease, median of follow-up of 750 days [range, 613-1001]). Both clinical trials data and overall survival are summarized in Table 4 and Fig. 5, respectively.
4. DISCUSSION

Treatment of hematological malignant disorders has improved over the last years, but high relapse rate, mainly attributable to the presence of MRD, still persists. Therefore, it is of great interest to explore novel therapeutic strategies to obtain long-term remission in heavily pre-treated patients. Cell therapy is a promising alternative to harsh chemotherapy and radiation. Particularly, the ability of NK cells to eradicate leukemic blasts is well documented and represents a promising arm of therapy for chemotherapy-resistant disease. In this regard, it is necessary to build a solid foundation establishing the safety and feasibility of infusing these cells in patients with cancer prior to its implementation in the clinical practice. To the best of our knowledge, this is the largest report to date of pediatric patients treated with ex vivo expanded NK cells.

There is some literature about adoptively transferred haploidentical NK cells in clinical trials, mainly using resting NK cells or IL-2–cultured NK cells. In most cases, systemic IL-2 was administered following NK infusion. The use of activated and expanded NK cells co-cultured with human derived antigen presenting cells is an emerging alternative.

Preclinical studies have demonstrated that expansion using the K562 leukemia cell line genetically modified to express membrane-bound IL-15 and the 41BB ligand upregulates NK activating receptors expression and augments NK killing capacity. However, their potential adverse effects should be carefully evaluated. A recent study from Shah et. al reported acute GVHD in 5 out of 9 patients receiving IL-15/41BBL activated NK cells following HLA-matched, T-cell–depleted HSCT. In contrast to Shah et al., we have observed neither GVHD nor other serious adverse reactions attributed to NKAЕ therapy in any of the patients studied, despite up to 11x10⁶ haploidentical T cells/kg infused. Our studies are out of
the TPH setting, include low dose IL-2 administration, which may have a protective effect on GVHD because of expansion of regulatory populations\textsuperscript{44,45} and infused NKAЕ end products include no stimulatory K562-mb15-41BBL cells that potentially could trigger a GVHD response. Our results indicate that therapy with IL-15/41BBL NKAЕ cells is well tolerated in this setting. We have observed a single case of GVHD reactivation after the first HNJ-NKAES-2012 treatment cycle. The patient had previously received a mismatched unrelated donor transplantation (MMuD). Our plausible explanation is that combined chemotherapy and NKAЕ cell infusion decreased the leukemia burden and re-established MMuD chimerism, reactivating previous GVHD. The serious adverse effects observed in our trials were not related to NKAЕ or IL-2 infusions, but were, as expected, infection episodes as well as other severe toxicities derived of intensive chemotherapy treatment (typhlitis, pericardial tamponade, severe somnolence).

NK cell therapy can be derived from autologous or from allogeneic sources. Leukemia and lymphoma patients’ autologous NK cells might have functional defects\textsuperscript{46}. In contrast, allogeneic NK cells from non-HLA identical related healthy donors, as proposed in this study, not only have normal function but can also enhance the graft versus leukemia effect via KIR-HLA-I mismatch\textsuperscript{47,48}. The data obtained in our trials, however, shows no differences in overall survival among KIR matched and KIR mismatched allogeneic NK treated patients. Infused NKAЕ have an extremely activated phenotype\textsuperscript{42,43}, so KIR mismatch presumably does not augments its already exacerbated cytotoxic capacity.

One of the critical drawbacks in NK cell immunotherapy has been the lack of a large-scale clinical grade NK cell expansion method. NK cells comprise only a limited number of PBMCs and their isolation requires a costly selection process. In our report, NKAЕ cell
infusions were performed after 14-21 days of GMP procedure, which represents a reasonable time frame in patients that require immediate treatment options, confirming the feasibility of this therapy. In the HNJ-NKAES-2012 trial the designed schedule allowed a single infusion but of larger cell numbers. In LYDIA trial an additional NKAES product was infused, but the amount of NKAES cells available for that infusion was lower than expected. In our short cohort-studies we cannot conclude which schedule is better in terms of bone marrow response or donor chimerism peak. Randomized studies are needed to determine the optimal therapeutic regimen. Major challenges, such as the best method to obtain the large number of NK cells needed to make cell-based therapy cost-effective and to enhance effector cells activity, remain open.

Biological features, such as number of NKAES cells infused and its immunophenotype, were evaluated in an effort to correlate these data with patients’ outcome. Although not statistically significative, on account of the limited sample size, a correlation between the number of infused NKAES and CR achievement was observed (Suppl. Fig. 2A). As described, donor T lymphocytes could induce a graft versus leukemia effect. Donor lymphocyte infusion is commonly used to treat leukemia relapse following HSCT\(^9\). In our trials, we have observed a correlation between the number of T lymphocytes infused and better outcome (Suppl. Fig. 2B). NKAES expression level of KIR2DL1 and KIR2DL2 receptors was also associated with a better response (Suppl. Fig. 3). The leukemic blasts of the patients were also evaluated. ULBP1, ULBP2 and ULBP4 expression was apparently correlated with a worse response to treatment (Suppl. Fig. 4). These data might suggest additional immunoescape mechanisms developed by these blasts theoretically good responders to NK cell therapy\(^{50,51}\). In addition, we assayed \textit{in vitro} donors’ NK cytotoxicity against patients’ blasts. Higher \textit{in vitro} NK cytotoxicity against patients’ blasts showed a trend towards a better outcome (Suppl. Fig. 5).
The setting proposed in this study suggests clinical efficacy of combining chemotherapy and haploidentical IL-15/41BBL activated and expanded NK cell therapy. Among the 18 heavily pretreated patients who received chemotherapy and NK cells, 10 proceeded to HSCT and 4 are alive at the end of the trial, which represent 55.5% and 22.2% of the studied population, respectively. We propose NKAE cell therapy as a complement to chemotherapy in order to eliminate chemotherapy-resistant leukemic cells, responsible of relapse and refractoriness\textsuperscript{52,53}. NK cell responses are short in time, so this cell therapy constitutes a bridge treatment to a consolidation procedure such as an allogeneic HSCT. Indeed, in our trials, all patients currently alive received an HSCT after trial completion (n = 4).

A limitation of our study is that the specific contribution to patients’ outcome of the NK cell infusions alone cannot be determined. It is remarkable that all patients that achieved a HSCT after the trial had received high-intensity rescue chemotherapy, while no patients who received FluCy achieved it. 100% of patients who received FLAG-Ida (3 out of 3), 71.4% of patients who received AraG/VP/Cy (5 out of 7), and 33.3% of patients who received CLOVE (2 out of 6) got a transplantation. CLOVE, on the other hand, showed the highest toxicity rate (50% of CLOVE-treated patients died of chemotherapy-related toxicity). Previous clinical trials to treat relapsed/refractory leukemia/lymphoma using CLOVE already reported febrile neutropenia and high rate of infections as common adverse effects\textsuperscript{22,23,54}. Hijiya et al.\textsuperscript{23}, in a study with a cohort of 25 relapsed/refractory ALL patients, reported 7 cases of death related to CLOVE treatment: veno-occlusive disease (2), septic shock (2), acute renal failure (1), pulmonary edema (1), and infection (lung/fungal) (1). In this study, the overall survival after 217 days was 24%. Tavil et al.\textsuperscript{24}, in a study with a cohort of 25 poor prognosis AML/ALL patients treated with FlagIda reported 20% overall survival (1200 days). Finally, Commander
et al.\textsuperscript{21}, in a 7 patients relapsed/refractory T-ALL/lymphoma treated with Arag/VP/Cy chemotherapy described a 14\% overall survival (450 days). Our trials combining different chemotherapeutic strategies and NKAЕ and IL-2 infusions led to a 20\% overall survival after 750 days. The balance between efficacy and acceptable toxicity, according to our data would be FLAG-Ida or AraG/VP/Cy regimens. The adverse effects observed in those patients in our study, including febrile neutropenia, mucositis, infections and AraG-related neurotoxicity, correspond in frequency and severity with that found in the literature\textsuperscript{20,21,23}.

The present study shows that the activation and expansion of NK cells using the K562-mb15-41BBL cell line is feasible and allows secure and effective infusions of NK cells in relapsed/refractory leukemia/lymphoma. Our results, together with previous reports, have encouraged us to investigate NK cell therapy not only for patients with relapsed/refractory cancer, but also as a consolidation therapy in AML patients with cytological remission (ongoing clinical trial NCT02763475). Further clinical trials are therefore warranted to assess the efficacy of NKAЕ in the management of cancer patients in various settings.
5. LIST OF ABBREVIATIONS

ALL-B: B cell lymphoblastic leukemia
ALL-T: T cell acute lymphoblastic leukemia/lymphoma
AML: Acute myeloid leukemia
Ara-G: Nelarabine
BAL: Biphenotypic acute leukemia
CLOVE: Clofarabine, etoposide and cyclophosphamide
CR: Complete remission
CTCAE: Common terminology criteria for adverse events
Cy: Cyclophosphamide
EBV: Epstein-Barr virus
FLAG-Ida: Fludarabine, idarubicin, cytarabine and G-CSF
FluCy: Fludarabine and cyclophosphamide
GMP: Good manufacturing practice
GVHD: Graft versus host disease
HSCT: Hematopoietic stem cell transplantation
IL: Interleukin
ITT: Intrathecal triple therapy
KIR: Killer immunoglobulin-like receptor
MMuD: Mmismatched unrelated donor transplantation
MRD: Minimal residual disease
NKAE: Activated and expanded Natural Killer cells
PBMCs: Peripheral blood mononuclear cells
PD: Progressive disease
TRM: Transplant-related toxicity
VP: Etoposide
6. DECLARATIONS

Ethics approval and consent to participate

The relatives of minor patients and those > 18 years provided written informed consent before entering the trials in accordance with the Declaration of Helsinki, and all research was approved by the ethical committee of the Hospital Infantil Universitario La Paz (Madrid, Spain).

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

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Author’s contributions

All authors contributed to data collection and interpretation.

MV was major contributor in writing the manuscript.

AP-M supervised the studies.

Acknowledgements

We are indebted to all the patients who agreed to participate in this trial, as well as to the personnel of the hospital involved in the study for their support and for sample collection.
7. REFERENCES


### Table 1. Patients demographics

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<tr>
<td>Gender, n (%)</td>
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<td>BAL</td>
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M: male; F: female; ALL-T: T cell acute lymphoblastic leukemia; ALL-B: B cell acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; BAL: biphenotypic acute leukemia
Table 2. Patients disease characteristics, treatment and current status

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<th>NKAE infusions</th>
<th>Mean infused NKAE cells (10^6 xkg)</th>
<th>Mean infused T cells (10^6 xkg)</th>
<th>Peak donor chimerism (%)</th>
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Table 3. Characterization of NKAE infusions

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<td>Cells /kg (x10⁶)</td>
<td>8.5 (1.2-48.8)</td>
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<tr>
<td>NK cells (x10⁶)</td>
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<td>NK cells (%)</td>
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<td>NK cells/kg (x10⁶)</td>
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<tr>
<td>T cells (x10⁶)</td>
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<td>NKT cells (x10⁶)</td>
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<td>NKT cells (%)</td>
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<tr>
<td>NKT cells/kg (x10⁶)</td>
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NK cell: Natural Killer cell, NKT cell: Natural Killer T cell.
Table 4. Adverse effects related to chemotherapy, to IL-2 and to NKAIE infusion

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<td><strong>N</strong></td>
<td><strong>%</strong></td>
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<td>%</td>
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**NKAЕ INFUSSION-RELATED ADVERSE EFFECTS**

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SOC: System Organ Class; CTCAE: Common Terminology Criteria for Adverse Events; N: Number of cases of the indicated event registered; %: Percentage of patients suffering the indicated event.
Table 5. End of trials (HNJ-NKAES-2012 and LYDIA) summary

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<td>1 (5)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Bone marrow response, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Non complete remission</td>
<td>2 (10)</td>
</tr>
<tr>
<td>Complete remission/MRD+</td>
<td>7 (35)</td>
</tr>
<tr>
<td>Complete remission/MRD-</td>
<td>6 (30)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Survival, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>End of the trial survival</td>
<td>14 (70)</td>
</tr>
<tr>
<td>Follow up survival</td>
<td>4 (20)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Donor chimerism detected, n (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor chimerism = 0%</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Donor chimerism 0.1-5%</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Donor chimerism &gt; 5%</td>
<td>1 (7)</td>
</tr>
</tbody>
</table>

MRD: Minimal Residual Disease.
FIGURE LEGENDS

Figure 1. Clinical trials treatment schedules.
Clinical trial HNJ-NKAES-2012 (A) and LYDIA (B) treatment plans. In both cases, two cycles of rescue chemotherapy followed by NKAE and IL-2 infusions were administered. IL-2: Interleukin-2 ($10^6$ U/m$^2$); ITT: Intrathecal Triple Therapy (methotrexate, cytarabine, and hydrocortisone sodium succinate; VP-16: Etoposide (100 mg/m$^2$); CPM: Cyclophosphamide (440 mg/m$^2$); Ara-G: Nelarabine (650 mg/m$^2$).

Figure 2. Lymphocyte reconstitution and outcome in chimerism-detected and undetected patients.
A: Mean and SEM of lymphocyte counts of patients after the first cycle of chemotherapy followed by NKAE cells infusion. B: Peak donor chimerism versus outcome in patients that presented progressive disease (PD), complete remission with positive minimal residual disease (CR/MRD+) or complete remission with no minimal residual disease (CR/MRD-) at the end of the trials.

Figure 3. Bone marrow response to treatment.
Percentage of blasts of each patient at trial inclusion, after first treatment cycle and at the end of the study are indicated. CR: Complete remission.

Figure 4. Flow chart of the subjects who participated in the clinical trials.
Treatment responses were evaluated after cycle I, at the end of the study and during the follow-up period (up to 1000 days). Patients that received a HSCT are indicated in
orange boxes, patients derived to other clinical trials in red boxes, patients entering palliative care programs in blue boxes, and those who died in white boxes.

**Figure 5.** Overall survival of patients enrolled in the clinical trials.

Kaplan-Meyer survival curves of patients enrolled in HNJ-NKAES-2012 and LYDIA trials at the end of the study (A) and after the follow-up period (B).
A

Figure 1

CYCLE I

NKAΕ < 5x10^7 cells/kg

-12 -6 0 11

ITT/VP and Cy Ara-G

RESCUE CHEMOTHERAPY

IL-2

CYCLE II

NKAΕ < 1x10^6 cells/kg

-12 -6 0 11

ITT/VP and Cy Ara-G

RESCUE CHEMOTHERAPY

IL-2

B

CYCLE I

NKAΕ < 2.5x10^7 cells/kg

-12 -2 0 7 11

CLOVE, FLAG-Ida or FluCy

RESCUE CHEMOTHERAPY

IL-2

CYCLE II

NKAΕ < 5x10^6 cells/kg

-12 -2 0 7 11

CLOVE, FLAG-Ida or FluCy

RESCUE CHEMOTHERAPY

IL-2
Figure 2

A

- Donor chimerism detected
- Donor chimerism undetected

B

Donor peak chimerism vs outcome

0
10
20
30
40
50
60
70
80
90
100

Day

Lympocytes/μl

OUTCOME

PD
CR/MRD+
CR/MRD−

Peck donor chimerism (%)
Figure 3
### CYCLE I

**n = 20**

- **HNJS-NKAES-2012**
- **LYDIA**
- **n = 7**
- **n = 13**

1. **1st evaluation**
   - **Exitus**
     - **Complete Remission/MRD+**
     - **Complete Remission/MRD-**
     - **Total 18**

2. **HSCT**
   - **n = 1**
   - **Complete Remission/MRD+**
   - **[#10]**

### CYCLE II

**n = 16**

- **HNJS-NKAES-2012**
- **LYDIA**
- **n = 6**
- **n = 10**

1. **End of the study**
   - **Exitus**
     - **Complete Remission/MRD-**
     - **Complete Remission/MRD+**
     - **Total 15**

   - **End of the study**
     - **Exitus**
     - **Pericardial tamponade**
     - **Sepsis**
     - **n = 2**
     - **[#40]**
     - **[#31]**

   - **HSCT**
     - **n = 9**
     - **Complete Remission/MRD-**
     - **Complete Remission/MRD+**
     - **[#12, #5, #7, #11, #19]**
     - **[#9, #6, #10, #20]**

   - **Other Clinical Trial**
     - **n = 1**
   - **Palliative care**
     - **n = 1**
   - **Exitus**
     - **n = 2**
     - **Progressive Disease**
     - **[#14, #15]**
     - **[#13, #17]**

### Follow-up

**n**

- **Complete Remission/MRD-**
  - **n = 4**
- **Total 4**

### Exitus

- **Progressive Disease**
  - **n = 6**
  - **[#4, #5, #10, #18]**
  - **[#8, #16]**

- **Other Clinical Trial**
  - **n = 1**
  - **Progressive Disease**
  - **[#31]**

- **Palliative care**
  - **n = 1**

- **Exitus**
  - **n = 2**
  - **Progressive Disease**
  - **[#13, #17]**

- **Exitus**
  - **n = 1**
  - **Progressive Disease**
  - **[#14]**

---

**Figure 4**
HIGHLIGHTS

- The NK cell immunotherapy described is clinically feasible.

- Pediatric refractory or relapsed leukemia patients could benefit from this therapy.

- The NK cell therapy in combination with rescue chemotherapy described showed a response rate of 72%.

- No severe adverse effects due to NK cell therapy were registered.