bjh research paper

Adoptive immunotherapy with double-bright (CD56^{bright}/CD16^{bright}) expanded natural killer cells in patients with relapsed or refractory acute myeloid leukaemia: a proof-of-concept study

Lucia Silla,[1,2] Vanessa Valim,[2] Annelise Pezzi,[2] Maria da Silva,[2] Ianae Wilke,[1] Juliana Nobrega,[2] Alini Vargas,[2] Bruna Amorin,[2] Bruna Correa,[1] Bruna Zambonato,[2] Fernanda Scherer,[2] Joice Merzoni,[2] Leo Sekine,[1,2] Helen Huls,[3] Laurence J. Cooper,[3] Alessandra Paz[2] and Dean A. Lee[4]

[1]Universidade Federal do Rio Grande do Sul, [2]Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil, [3]University of Texas MD Anderson Cancer Center, Houston, TX, and [4]Nationwide Children's Hospital, Columbus, OH, USA

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Correspondence: Lucia Silla, Rua Ramiro Barcelos #2350, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil 90035-903.
E-mail: dralucia.silla@gmail.com

Summary

Patients with acute myeloid leukaemia (AML) have a five-year survival rate of 28·7%. Natural killer (NK)-cell have anti-leukaemic activity. Here, we report on a series of 13 patients with high-risk R/R AML, treated with repeated infusions of double-bright (CD56^{bright}/CD16^{bright}) expanded NK cells at an academic centre in Brazil. NK cells from HLA-haploidentical donors were expanded using K562 feeder cells, modified to express membrane-bound interleukin-21. Patients received FLAG, after which cryopreserved NK cells were thawed and infused thrice weekly for six infusions in three dose cohorts (10^6–10^7 cells/kg/infusion). Primary objectives were safety and feasibility. Secondary endpoints included overall response (OR) and complete response (CR) rates at 28–30 days after the first infusion. Patients received a median of five prior lines of therapy, seven with intermediate or adverse cytogenetics, three with concurrent central nervous system (CNS) leukaemia, and one with concurrent CNS mycetoma. No dose-limiting toxicities, infusion-related fever, or cytokine release syndrome were observed. An OR of 78·6% and CR of 50·0% were observed, including responses in three patients with CNS disease and clearance of a CNS mycetoma. Multiple infusions of expanded, cryopreserved NK cells were safely administered after intensive chemotherapy in high-risk patients with R/R AML and demonstrated encouraging outcomes.

Keywords: R/R AML, NK cell, adoptive immunotherapy, CNS leukaemia.

Introduction

Surveillance, Epidemiology, and End Results (SEER) data from 2020 show a five-year survival rate for acute myeloid leukaemia (AML) of 28·7%.[1] Intensive salvage chemotherapy for patients with relapsed or refractory AML (R/R AML) has changed very little over the past 25 years with overall survival (OS) and disease-free survival (DFS) rates rarely above 12 months..[2,3] Clinical trials of targeted agents in association with standard chemotherapy report a superior OS and DFS for younger patients[4] and overall response rates (ORRs) applicable only to select subsets of AML.[5] Therefore, AML remains a fatal disease for most patients and novel therapeutic alternatives are needed.

Natural killer (NK) lymphocytes are innate immune system cells that express an array of receptors capable of recognising and killing virus-infected or malignant cells without the need for prior priming or exposure.[6] The anti-leukaemic role of NK cells has been most demonstrable in T-cell-depleted haploidentical SCT,[7] although NK cells also play a central role in the alloSCT graft-versus-leukaemia effect,[8] and its early recovery following alloSCT is associated with fewer relapses and improved survival.[9]

Haploidentical NK-cell infusions in patients with R/R AML have been shown to be well tolerated, with remission reported in five of 19 patients,[10] five of nine patients[11] when given after cyclophosphamide and fludarabine and in four of six patients when administered after intensive chemotherapy.[12] We developed an *ex vivo* NK-cell expansion platform based on genetically modified feeder cells expressing membrane-bound interleukin-21 (mbIL21) and 4-1BBL which facilitates the expansion of highly active Double-Bright

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(both CD56^{bright} and CD16^{bright}) NK cells.[13] DB-NK cells generated using this strategy have been safely delivered in combination with alloSCT in a phase 1 study.[14]

Here, we report the results from a proof-of-concept study investigating the safety and feasibility of multiple infusions of DB-NK cells in patients with high-risk R/R AML after standard induction chemotherapy.

Methods

Study design and participants

This series of patients was of an open-label, phase 1/2 study for patients with R/R AML at a single academic centre in Brazil. The primary objective was to determine the safety and feasibility of DB-NK cells following standard induction chemotherapy.

Full eligibility and exclusion criteria are detailed in the supplemental materials (p. 3). Patients with relapsed AML following alloSCT were eligible if they had no active graft-versus-host disease (GVHD) and were not receiving immunosuppressive agents. For this series of patients, the Eastern Cooperative Oncology Group Performance Status (ECOG PS) was not an exclusion criterion.

Donors were haploidentical relatives selected for best NK alloreactivity according to (1) missing-ligand model or (2) killer-cell immunoglobulin-like receptor (KIR)-B haplotype, where possible. Further details regarding donor selection, KIR and haplotyping are described in the supplemental materials (p. 4).

The study was approved by the institutional review board of the Hospital de Clínicas de Porto Alegre (IRB/HCPA 00000921; CAAE: 44444214·7.0000·5327) and was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization for Good Clinical Practice. Donors and recipients or their legal guardians gave written informed consent.

Study procedures

NK cells were expanded using K562-based feeder cells as previously described[13,14] (supplemental materials p. 6). For this proof-of-concept study dose escalation was performed at the following dose levels: 10^6, 5×10^6, and 10^7 cells/kg; expansion cultures were maintained for 8–21 days as needed to generate the number of cells required, based on previous data supporting stable proliferation, phenotype, and function for up to six weeks.[15,16] The maximum T-cell dose allowed was 10^5 cells/kg/infusion.

A summary schema of the treatment is summarised in Fig 1. Briefly, one unit of peripheral blood (300–400 ml) was drawn from donors and NK-cell expansion was performed with feeder cells for 21 or more days. Patients were treated with FLAG (fludarabine, high-dose cytarabine, and granulocyte colony-stimulating factor) beginning seven or more days prior to the first DB-NK-cell infusion. DB-NK cells were administered three times per week for a total of six infusions starting on day 0.

Study assessments and outcomes

Patient physical wellbeing and complete blood counts were routinely evaluated, and disease assessments were performed after neutrophil recovery or by day 28, whichever occurred

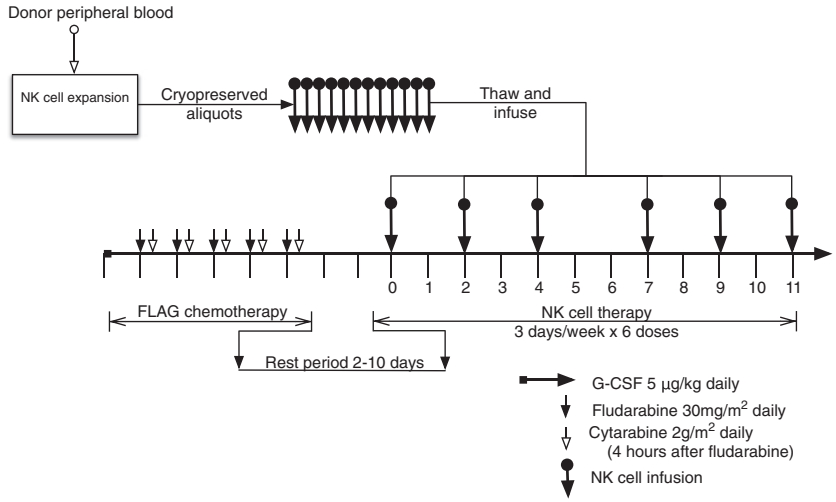

Fig 1. Treatment schema. Approximately one unit (300–400 ml) of peripheral blood was drawn from donors, and NK-cell expansion was performed with weekly stimulation with feeder cells for up to 21 days. Patients were treated with standard FLAG chemotherapy beginning at least seven days prior to the first NK-cell infusion (day 0). G-CSF (5 µg/kg IV daily) was administered beginning one day before fludarabine/cytarabine and until the post-nadir ANC was equal to or more than 1000. Fludarabine IV 30 mg/m^2 and cytarabine IV 2 g/m^2 (approximately 4 h after fludarabine) were administered daily for five days. A rest period of 2–10 days was allowed until NK cells were ready for infusion. NK-cell infusions were administered three times per week for a total of six infusions starting on day 0. Lymphocyte subsets were assessed prior to, and 2 h following, each NK-cell infusion. Anti-inflammatory corticosteroids were avoided where possible.

first (supplemental materials p. 7). Cytogenetic risk was determined according to European LeukemiaNet.[17]

Safety and feasibility endpoints were defined as being able to generate and infuse NK cells without exceeding toxicity limits in at least seven of ten patients. Rapid cell dose escalation was allowed when very low rates of adverse events (AEs) were observed. AEs were reported from day 0 up to day 56 and were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4·0 (supplemental materials p. 7). DLTs were defined as equal to or greater than a grade 3 reaction related to NK-cell infusion; equal to or greater than a grade 3 acute GVHD that did not resolve with treatment, equal to or greater than grade 1 within one week; or equal to or greater than grade 3 unexpected toxicity related to NK-cell infusion. Grade 3 toxicities that resolved within 72 h were not reported as a DLT.

Secondary endpoints included: persistence of adoptively transferred expanded NK cells (as inferred by flow cytometry); ORR and CR rate at day 28–30 following infusion.

Response was assessed according to International Working Group 2003 criteria.[18] CR was defined as disappearance of measurable disease, CRi as CR with incomplete haematological recovery, and partial response (PR) as equal to or more than a 50% decrease in blasts. Responses in patients with only central nervous system (CNS) leukaemia were assessed using the Response Assessment in Neuro-Oncology (RANO) criteria.[19] ORR was defined as CR + CRi + PR. *Post hoc* efficacy endpoints included duration of response, time to recovery of absolute neutrophil count to more than 500/μl and more than 1,000/μl and platelets to more than 50,000/μl, OS, and DFS.

Immune recovery assessment

Immune recovery was analysed before and after each cell infusion and on approximately day 28. Samples (5 ml) were collected in citrate and were treated according to the Bulk Lysis™ EuroFlow™ standard operating protocol,[20] adapted here to improve the detection of rare lymphocyte subsets. PBMC were stained and analysed using BD Pharmingen™ fluorochrome-conjugated antibodies: anti-CD56 Pe-Cy7, anti-CD14 BB515, anti-CD3 PERCP, anti-CD16 APCH-7, anti-CD45 PE, anti-CD19 APC, anti-CD3 FITC, anti-CD8 APC-H7, anti-CD4 PE. A total of 100,000 events were acquired using the FACS Canto II equipment through the BD FACS Diva™ acquisition software (BD Biosciences). Infinicyt™ version 1·8 software (Cytognos S.L.) was used for data analysis.

Statistical considerations

Statistical analyses were performed using GraphPad Prism 8 for macOS version 8·0.2. Descriptive statistics were used unless otherwise stated. For efficacy endpoints, point estimates and interquartile range (IQR) are provided. Time-to-event endpoints were summarised using the Kaplan–Meier method and reported as median (95% confidence interval [CI]). OS and DFS analyses were also performed in those who responded (CR+CRi+PR) or achieved remission (CR+CRi), respectively. Survival outcomes were censored as of 26 March 2020, and DFS was also censored at time of relapse or consolidation therapy with alloSCT. Survival probabilities were calculated using the competing-risk model, with initiation of additional therapy (including haploidentical stem cell transplantation) as a competing outcome. Recovery of T and NK cells was compared across time points and between responders versus non-responders using Student's *t*-test, corrected for false discovery (Q = 1%).[21]

The original study is registered at ClinicalTrials.gov (NCT02809092).

Results

Patient characteristics

Between May 2017 and July 2019, 16 patients were enrolled. Three patients had cells manufactured but did not receive the infusions: one was determined ineligible (achieved first CR during NK cell production) and the other two died before initiating FLAG. Patient and disease characteristics for the 13 patients are shown in Tables I and II. Treated patients had a median age of 22 years (IQR 11–47), were predominantly male (62%), and 69% had intermediate- or adverse-risk cytogenetics. Five patients had primary refractory and eight had relapsed disease. The median number of prior lines of therapy was five (IQR 4–6). Median bone marrow blasts at enrolment was 38% (IQR 4–80). Three patients had concurrent CNS leukaemia (bone and nerve root disease; uncus/brain stem disease; and chloromas), two of whom were in bone marrow remission, and three had severe infections including one with presumed CNS mycetoma, identified after treatment was initiated.

NK cell donors and product characteristics

Donor KIR ligand mismatch and KIR-Bx/KIR-A haplotype are shown in Table SI. Adequate numbers of clinical-grade DB-NK cells were produced from all donors. Supplemental Table SII shows manufacturing and product characteristics. Median NK-cell purity was 96% (IQR 92–99), median T-cell content was 0·3% (IQR 0·2–0·5), and median post-thaw cell viability was 96% (IQR 92–98). The predominant cell phenotype was $CD56^{bright}/CD16^{bright}$ (Figure S1). All products were free of microorganisms and endotoxins, except for one infusion sample (positive for *Staphylococcus*, which was discarded). Median lysis of K562 (E:T ratio 50:1) by ten donor products tested was 55% (IQR 40–70).

One patient received 10^6, four 5×10^6, and eight 10^7 DB-NK cells/kg. Ten patients completed six cell infusions, including patient 1 who was treated twice (the first course comprised six infusions at 10^6 cells/kg, while aplastic after

Table I. Baseline characteristics of patients treated with DB-NK.

Characteristics	Patients treated with DB-NK (n = 13)*
Median age, years (IQR)	22 (11–46·5)
Sex, *n* (%)	
Female	5 (38)
Male	8 (62)
French-American-British classification, *n* (%)	
M0	2 (15)
M1	3 (23)
M2	2 (15)
M4	3 (23)
M5	1 (8)
M6	1 (8)
M7	1 (8)
ECOG performance status score, *n* (%)	
2	2 (15)
3	7 (54)
4	4 (31)
Cytogenetic risk group,†,‡ *n* (%)	
Favourable	3 (23)
Intermediate	4 (31)
Adverse	5 (38)
Missing	1 (8)
FLT3 internal tandem duplication mutation status,‡ *n* (%)	
Mutated	3 (23)
Wild type	7 (54)
Missing	3 (23)
% bone marrow blasts, median (IQR)	38 (4–80)
Primary refractory disease, *n* (%)	5 (38)
Relapsed disease, *n* (%)	8 (62)
Median number of relapses (IQR)§	3 (2–5)
Median number of prior treatments (IQR)	5 (4–6)
Patients with prior SCT, *n* (%)	9 (69)
Matched related donor, *n*	3‖
Matched unrelated donor, *n*	1
Haploidentical, *n*	3¶
Autologous, *n*	2

CNS, central nervous system; ECOG, Eastern Cooperative Oncology Group; FLAG, fludarabine, high-dose cytarabine, and granulocyte colony-stimulating factor; IQR, interquartile range; SCT, stem cell transplantation; SUS, Sistema Único de Saúde.

*Three patients were treated under compassionate-use waivers for eligibility: one, aged one year and seven months, had concurrent CNS disease and was younger than the minimal age defined in the protocol (patient 6); one patient had concurrent CNS disease and chronic graft-versus-host disease (patient 5; skin score 3, eye score 1, liver score 1, and gastrointestinal tract score 1); and one patient had a low ECOG performance status score 4 and was ineligible due to multidrug-resistant *E. coli* septicaemia that was clinically controlled just before starting FLAG (patient 11). Upon enrolment, one patient was colonised with vancomycin-resistant *Enterococcus* (patient 1) and one presented with a residual subdural hematoma secondary to previous treatment (patient 12);

†Cytogenetic risk was determined according to European LeukemiaNet guidelines;[35]

‡Six patients were referred for treatment on this protocol from other Brazilian states or institutions. Eight patients were treated within the public health system, SUS. In most centres (particularly at public health institutions in SUS and at our institution), only molecular analysis of *FLT3* mutations was available; its mutation, irrespective of cytogenetic risk, was considered adverse;

§Median calculated from eight patients;

‖One patient had two allogeneic SCTs, from different donors;

¶One patient had two haploidentical SCTs, different donors.

prior chemotherapy; the second course comprised five infusions at $1{\cdot}76 \times 10^6$ cells/kg following FLAG).

Adverse events

Adverse events (AEs) observed during study treatment not considered to be related to DB-NK-cell infusions included: febrile neutropenia in all patients (grade 4); thigh abscess (grade 1); typhlitis (grade 4); and hyperphosphataemia (grade 1) (Table III).

No infusion-related toxicities or cytokine release syndrome were observed. Treatment-emergent AEs arising during treatment were: pulmonary signs and symptoms (both grade 3) in two patients with unsuspected pre-existing pulmonary tuberculosis and invasive pulmonary aspergillosis; probable CNS aspergillosis (grade 2) in one patient; transient CNS hypertension secondary to CNS inflammation in one patient (Patient #5, grade 4, which resolved with high-dose dexamethasone); rectal mass bleeding and worsening of jaundice (both grade 2); skin rash in one patient (grade 1); anaemia in one patient (grade 4), which resolved with high-dose dexamethasone; and pneumonitis (grade 1) in one patient, which resolved with low-dose oral steroids. Although some pre-existing conditions (such as underlying infections or CNS leukaemia) were initially exacerbated with DB-NK treatment, all eventually resolved without sequela. On final review, these were deemed unlikely or unrelated to the NK cells except for the grade 4 CNS toxicity, which represented an on-target anti-leukaemic immune response.

Dose modifications

DB-NK infusions were discontinued after the third infusion in three patients due to high *in vivo* expansion of NK cells (patient 9; Fig 2), worsening jaundice and *in vivo* expansion of NK cells (patient 11; Fig 2), and grade 1 infection/ulceration of the glans penis with central necrosis and oedema (patient 13). Despite withholding the remaining three DB-NK-cell infusions, patient 13 achieved CR.

Graft-versus-host-disease

Graft-versus-host-disease (GVHD) occurred in one patient who had received a prior alloSCT and developed diarrhoea two days after the sixth DB-NK infusion (patient 5). A biopsy demonstrated grade 1 gastrointestinal acute GVHD.

Table II. Individual patient and disease characteristics at baseline in treated patients.

Patient ID*	Age, years	Sex	FAB classification	Cytogenetics	Cytogenetic risk†'‡	Molecular analysis‡	Refractory/ relapse (*n*)	Concurrent CNS disease	Prior SCT (*n*)	Prior treatments (*n*)#	% BM blasts	ECOG PS score
1	48	Female	M4	inv(16)(p13;q22)	Favourable	*FLT3* Wt	Relapse (4)	Mycetoma§	Auto	6	12	3
1'	48	Female	M4	inv(16)(p13;q22)	Favourable	*FLT3* Wt	Relapse (5)	No	Auto	7	8	2
2	11	Male	M1	Normal	Adverse	*FLT3*-ITD Mt	Refractory	No	No	4	88	4
3	45	Female	M2	Normal	Intermediate	*FLT-3* Wt	Relapse (3)	No	Allo (×2)	6	43	3
4	31	Male	M0	t(8;21)(q22;q22)	Favourable	*FLT3* Wt	Relapse (2)	Bone and nerve root	Allo	4	50	3
5‖	22	Male	M6	t(6;9)(p22;q34) DEK/NUP214	Adverse	*FLT3* Wt and *NPM1* Wt¶	Relapse (3, 2 CNS)	Uncus/brain stem	Allo	7	0·08	4
6‖	1·6	Female	M4	Normal	Intermediate	*FLT3* Wt	Refractory	Chloromas	Haplo	5	3	4
7	61	Female	M1	t(15;17)(q24q21)	Favourable	*FLT3* Wt	Relapse (4)	No	Auto	4	80	3
8	11	Male	M5	Normal	Intermediate	*FLT3* ND	1	No	Haplo	5	20	3
9	59	Male	M0	(Kt:46,XY,der(3)t(3;11;15)(p21;p11·2;q22)t(3;12)(q25;p11·2)[19]/46,XY[1])	Adverse	*FLT3*-ITD Mt**	Refractory	No	Haplo (×2)	9	34	2
10	45	Male	M4	Normal	Intermediate	*FLT3* ND	Refractory	No	No	4	73	2
11‖	2	Male	M7	Missing	Missing	*FLT3* ND	Refractory	No	Allo	3	38	4
12	16	Female	M1	Normal	Adverse	*FLT3*-ITD Mt	Relapse (1)	No	No	2	97	3
13	13	Male	M2	46-y e t(8;21)	Adverse	*FLT3* Wt	Relapse (5)	No	No	6	4	3

Allo, allogeneic; auto, autologous; BM, bone marrow; CNS, central nervous system; ECOG, Eastern Cooperative Oncology Group; FAB, French-American-British; FLAG, fludarabine, high-dose cytarabine, and granulocyte colony-stimulating factor; haplo, haploidentical; ID, identifier; ITD, internal tandem duplication; Mt, mutated; ND, not determined; PS, performance status; SCT, stem cell transplantation; Wt, wild-type.

*Patient 1 completed two DB-NK treatments: the first treatment comprised six infusions of DB-NK cells only; the second (noted as patient 1') comprised five infusions of DB-NK cells following FLAG chemotherapy (the 6th infusion was discarded owing to contamination of the infusion sample);

†Cytogenetic risk was determined according to European LeukemiaNet guidelines;

‡Six patients were referred for treatment on this protocol from other Brazilian states or institutions. Eight patients were treated within the public health system Sistema Único de Saúde (SUS). In most centres (particularly at public health institutions in SUS and at our institution), only molecular analysis of *FLT3* mutations was available; its mutation, irrespective of cytogenetic risk, was considered adverse. Comprehensive genomic profiling (Foundation One assay) was only available for patients 5 and 11;

#Prior treatments, with discrete regimens separated by (;): 1- 7 + 3 induction, HD-cytarabine x3; Ida-FLAG x2; BuCy autologous HSCT; azacytidine x8; MAC; mitoxantrone/cladribine/cytarabine. 1'- as above, plus FLAG+NK cells. 2- BFM-AML2004 intermediate; BFM-AML2004 high-risk; Ida-FLAG; Ida-FLAG 3- 7 + 3 induction, HD-cytarabine; 7 + 3 re-induction, HD-cytarabine; allogeneic HSCT; Ida-FLAG; 2nd allogeneic HSCT; azacytidine x2. 4- 7 + 3 induction x2/HD-cytarabine x2; Ida-FLAG; allogeneic HSCT; azacytidine x3·5- cladribine/cytarabine; cytarabine/etoposide/daunorubicin; HD cytarabine; allogeneic HSCT; azacytidine/DLI; FLAG/MADIT/dasatinib/CNS radiation; gemtuzumab/MADIT. This patient had cutaneous sclerotic chronic GVHD at enrolment, tacrolimus was withdrawn just before starting DB-NK-cell infusions. He was treated under compassionate use. 6- BFM-AML2004 (IAE); BFM-AML2004 (HAM); FLAG+daunorubicin x 2; haplo HSCT; MADIT/CNS radiation/DLI. 7- 7 + 3, HD-cytarabine x2; Ida-FLAG; autologous HSCT; HD-cytarabine. 8- NOPHO; Ida-FLAG; HAM x3; HD-cytarabine x3; haplo HSCT; azacytidine x2. 9- 7 + 3; Ida-FLAG; azacytidine/sorafenib; Flu/TBI haplo HSCT; mylotarg/DLI; decitabine, DLI x4; venetoclax; BuFlu halpo HSCT; azacytidine/venetoclax/hydroxycarbamide x2. 10- 7 + 3; Ida-FLAG; ALFA98; FLAG. 11- BFM-AML2004 x4; haplo HSCT; FLAG x3. 12- BFM-AML2004; Ida-FLAG/IT MADIT. 13- Complete course unknown, but included BFM-AML2004, FLAG, and MADIT.

§Patient diagnosed with unsuspected mycetoma during treatment;

‖Treated under compassionate use;

¶Positive for *DEK-NUP214*;

**Positive for *TP53* E11Q, *BRCA2* K467, *TBL1XR1*, *PDGFRB* mutations.

Table III. Adverse events.

Adverse events observed during the study	Grade	N	Related to NK cells
Infection			
Febrile neutropenia	4	13	No
Abscess	1	1	No
Typhlitis	4	1	No
Tuberculosis (pre-existing)	3	1	No
Pulmonary aspergillus (pre-existing)	3	1	No
CNS aspergillus (pre-existing)	3	1	No
Hepatic			
Elevated bilirubin	3	1	Unlikely
Renal			
Hyperphosphataemia (grade 1)	1	1	Unlikely
Neurological			
Increased intracranial pressure	4	1	Probable
	2	1	Possible
Hematological			No
Bleeding	2	1	No
Anaemia	4	1	Probable
GI			
Jaundice	2	1	Unlikely
Dermatological			
Rash	1	1	Unlikely
Pulmonary			
Pneumonitis	1	1	Possible
Signs/symptoms (cough and chest pain)	3	2	Unlikely

Corticosteroids and re-introduction of tacrolimus were effective. Immunosuppression was tapered and the patient recovered and lived for 505 days without any further occurrence of acute or chronic GVHD.

Responses

Given that one patient was treated twice (patient 1/1'), 14 courses of treatment were considered for response evaluation. The ORR was 78·6%, comprising CR in seven courses (50·0%), CRi in one course, and PR in three courses (including two patients with primary refractory disease) (Fig 3); patient 1 achieved PR with the first course and CR with the second course. Of 14 treatment courses, eight (57·1%) resulted in a sufficient response and performance status for patients to be considered for transplant. A total of five (38·5%) of 13 patients underwent subsequent haploidentical stem cell transplantation. Median time to transplant was 87 days (95% CI 53–305).

For 13 evaluable courses, median time to recovery of neutrophils to more than 500 cells/μl and more than 1,000 cells/μl was 28 days (95% CI 24–126) and 33 days (95% CI 25–173), respectively, and median time to recovery of platelets to more than 50,000 cells/μl was 81 days (95% CI 32–158) (Fig 4A). To correct for continued marrow failure by ongoing disease in non-responders and to better assess the therapeutic toxicity of the regimen, we recalculated median neutrophil (more than 500 and more than 100 cells/μl) and platelet recovery in all partial and complete responders, which was 27·5 (95% CI 23–28), 28·5 (95% CI 24–173), and 40·5 (95% CI x–28-126) days, respectively (Table SIII). Median OS was 254 days (95% CI 158–440) for all 14 treatment courses and 344 days (95% CI 166–505) for the 11 courses resulting in an objective response (Fig 4B). Median DFS was 132 days (95% CI 0–220) among all 14 treatment courses and 199 days (95% CI 56–304) for the eight courses that resulted in CR or CRi (Fig 4C). Using a competing-risk model to account for the effect of alloSCT, the six-month OS and DFS following DB-NK treatment were estimated at 54 ± 12% and 67 ± 14%, respectively, and estimated one-year OS was 35 ± 15% (Fig 4D).

There were ten deaths: seven owing to relapse in patients who had been discharged, two occurred following alloSCT (patients 2 and 10), and one from refractory leukaemia while hospitalised (patient 11). At the censoring date, three patients were alive and in remission following alloSCT.

Activity observed across the blood-brain barrier, possible antimicrobial activity, and genes associated with NK cell homing

CNS responses, as indicated by local inflammatory responses or lesion improvement, were observed by magnetic resonance imaging (MRI) in all four patients with concurrent CNS disease (including one patient with probable aspergilloma) (Fig 5). Given the peri-lesional inflammation observed on imaging, the CNS adverse events were considered on-target responses of NK cell-mediated inflammation, and not the neurotoxicity syndrome (ICANS) that has been reported with CD19-CAR-T-cell therapy.

Putative antimicrobial activity was suspected in three patients following cell infusions: pulmonary and CNS aspergillosis (patient 1), pulmonary tuberculosis (patient 3), and ascending cholangitis (patient 11) (supplemental materials p. 9).

RNA sequencing was performed to assess gene expression in NK cells before and after expansion, and we investigated gene expression profiles that might be associated with CNS trafficking. Among these, 28 addressins involved in lymphocyte homing were significantly altered during expansion (Figure S2). The top expressed addressins include those involved in vascular extravasation and peripheral tissue homing (ITGB2/CD18 and CXCR6), and specific migration toward inflammation (CCR1, CCR2, CCR5, CXCR3, and SELPLG/CD162), liver (CXCR3), bone marrow (CXCR4), CNS (ITGA4/CD49D and NCAM1/CD56), and gut (ITGA1/CD49A, ITGA4/CD49D, and ITGB7).[22,23]

Immune reconstitution and evidence of persistence

Chimerism studies were not available to assess persistence, but flow cytometry for recovery of immune cell subsets was

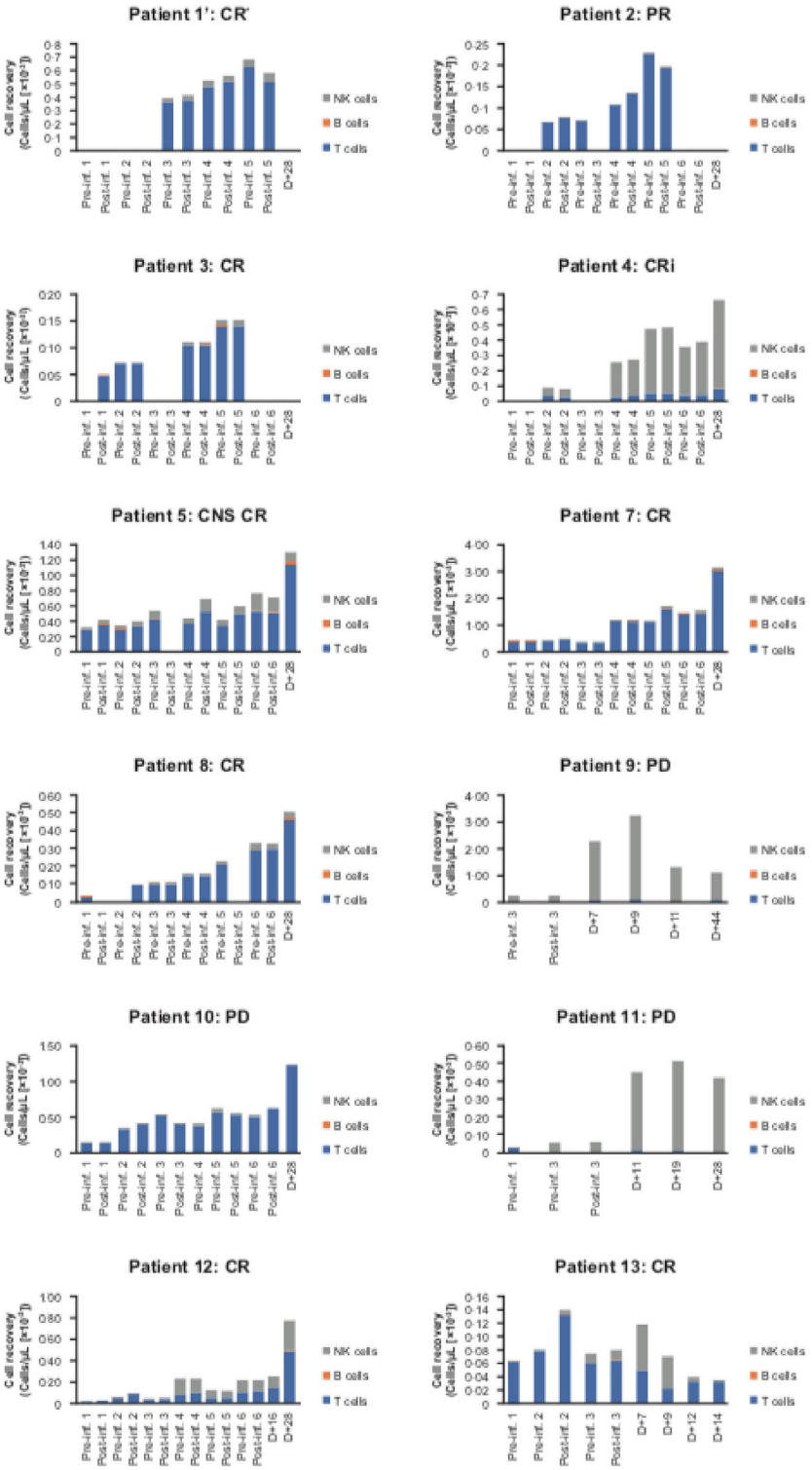

Fig 2. Persistence, expansion, and immune recovery following DB-NK infusions. Individual patient data for immune recovery assessed in 12 patients treated with double-bright ($CD56^{bright}/CD16^{bright}$) natural-killer-cell adoptive immunotherapy (one patient treated as compassionate use was too ill to have frequent blood draws). NK-cell, T-cell, and B-cell recovery before and after each NK-cell infusion, and two weeks after the last infusion, are shown. *Patient 1 completed two DB-NK treatments: the first treatment comprised six infusions of DB-NK cells only (during prolonged aplasia induced by prior therapy); the second comprised five infusions of DB-NK cells following FLAG chemotherapy (the sixth infusion was discarded owing to contamination of the infusion sample). Data for patient 1 are from the second course. Note that patient 11 received only three DB-NK-cell infusions and these cells persisted afterwards. [Colour figure can be viewed at wileyonlinelibrary.com]

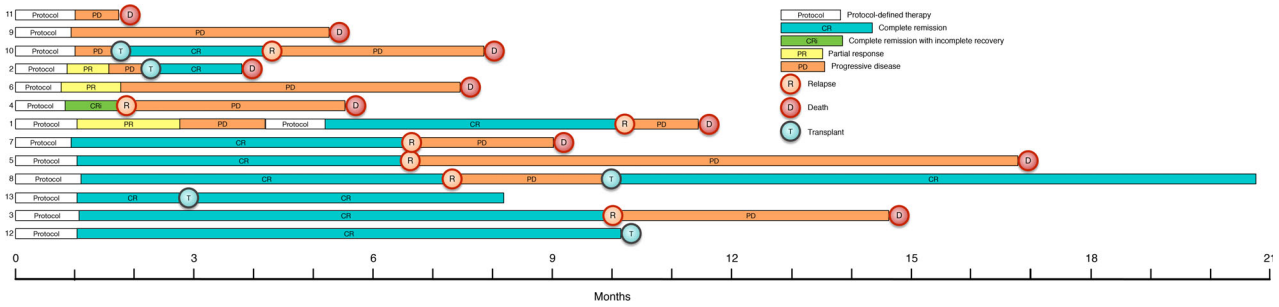

Fig 3. Clinical outcome. Swimmer plot of individual patient courses, indicating duration of protocol therapy, treatment response including complete response (CR), complete response with incomplete haematological recovery (CRi), partial response (PR), progressive disease (PD), relapse (R), stem cell transplantation (T), and death (D) (ordered by response, response duration, and survival). [Colour figure can be viewed at wileyonlinelibrary.com]

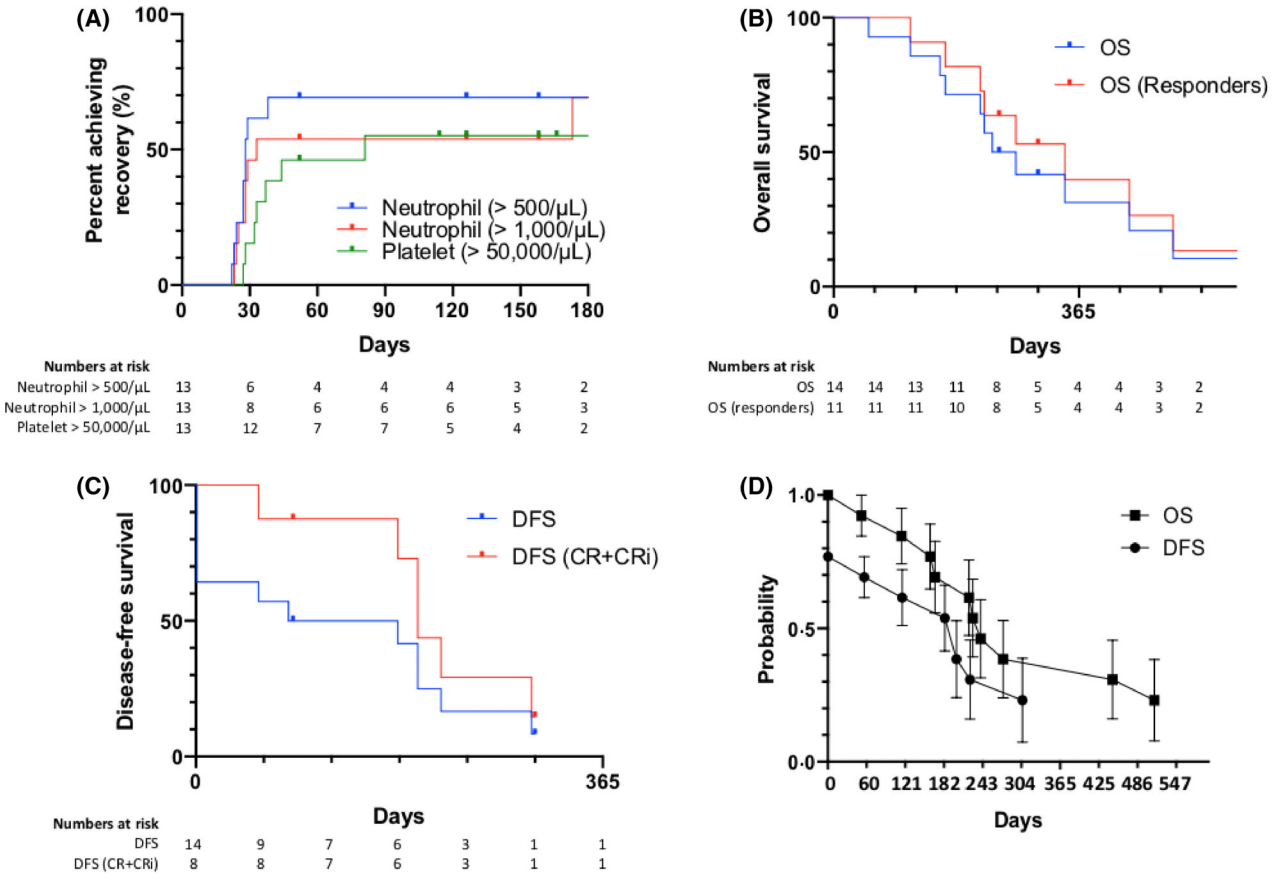

Fig 4. Haematopoietic recovery and clinical outcomes. (A) Recovery of absolute neutrophil count (ANC) to more than 500/µl and more than 1,000/µl and platelets to more than 50,000/µl. All responders (CR+CRi+PR) recovered ANC to 500/µl between day 22 and 29, except for one patient who recovered at day 38. (B) Overall survival (OS) in all patients treated with DB-NK cells and in responders (CR+CRi+PR). (C) Disease-free survival (DFS) in all patients treated with DB-NK cells and in those who achieved remission (CR+CRi). OS and DFS were measured from the first day of chemotherapy (or first dose of DB-NK cells when delivered without chemotherapy) and analysed based on 14 treatment courses. (D) OS and DFS using competing-risk model accounting for haploidentical stem cell transplantation as a competing event (± standard error of the mean). Data were censored at death or initiation of additional treatment including haploidentical stem cell transplantation. Patient 1 completed two DB-NK treatments: the first treatment comprised six infusions of DB-NK cells only (during prolonged aplasia induced by prior therapy); the second comprised five infusions of DB-NK cells following FLAG (fludarabine, high-dose cytarabine, and granulocyte colony-stimulating factor) chemotherapy (the sixth infusion was discarded owing to contamination of the infusion sample). [Colour figure can be viewed at wileyonlinelibrary.com]

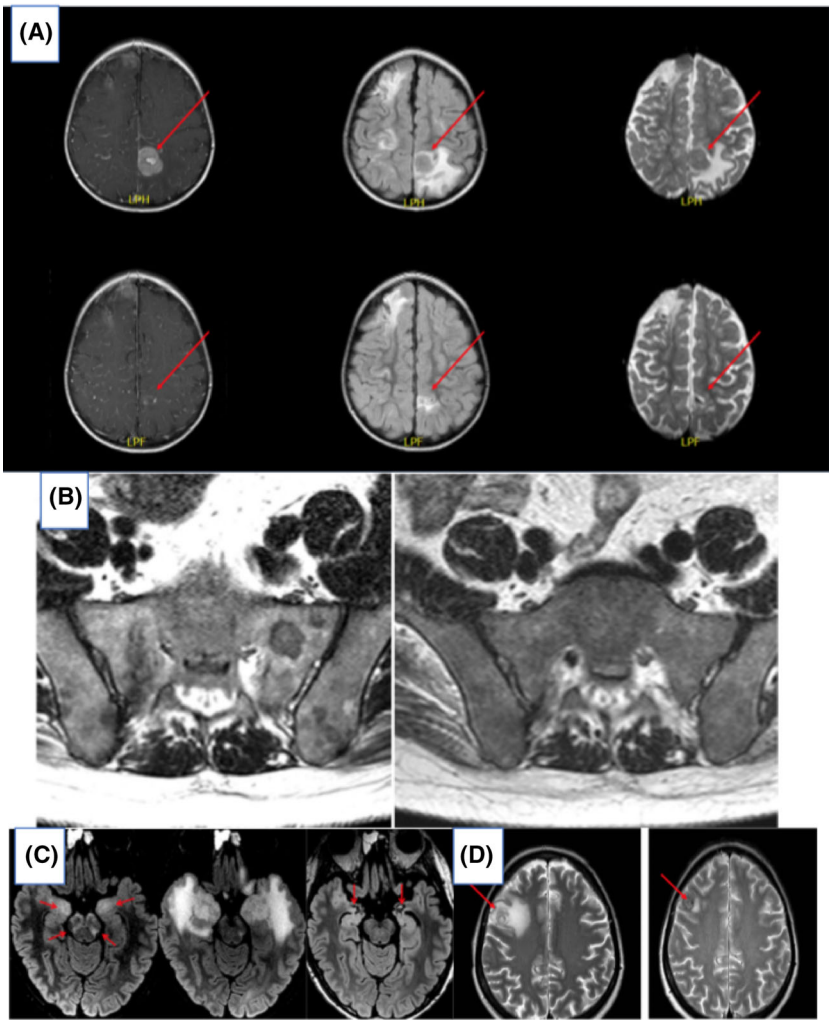

Fig 5. CNS responses in patients treated with DB-NK. (A) T1, axial, and diffusion-weighted head MRI of patient #6 obtained prior to starting study treatment, showing chloromas non-responsive to prior treatment (top row) and two months after treatment showing a 50% reduction of CNS chloromas (bottom row). (B) Spine MRI of patient #4 obtained prior to study treatment, showing sacral leukaemic infiltration with soft tissue and probable *cauda equina* involvement (left) and almost complete resolution at day 28 (right). (C) Head MRI axial FLAIR of patient #5 obtained one week prior to starting study treatment, showing signal intensity consistent with refractory AML in both unci and brainstem (far left); five days after the last DB-NK-cell infusion, showing a large extension of the signal to both anteromedial temporal lobes (that became more tumefactive) and optic chiasm, optic nerves, and ventral striatum (not shown) consistent with inflammatory response (middle); and six months after treatment, showing normalisation of the high signal in both unci with evolution to cavitary lesions, bordered by low signal rim (consistent with hemosiderin; far right). (D) Head MRI of patient #1 obtained after the fifth NK-cell infusion to investigate headaches and rising galactomannan in serum and present in bronchial lavage, showing parenchymal mycetoma (left) and at day 28 (right). [Colour figure can be viewed at wile yonlinelibrary.com]

performed before and after each infusion and for several weeks afterwards. Among the 12 evaluable patients, T cells predominated during immune recovery except in patients 4, 9, and 11 (Fig 2). Overall, T cells rose slowly from the post-chemotherapy nadir through day 28 (Fig 6A). In contrast, NK-cell numbers rose dramatically (79-fold) and significantly by day 7 and was maintained through day 28 ($P = 0{\cdot}04$; Figs 6A,B). Surprisingly, NK-cell numbers were higher in non-responders than in responders (Fig 6C), but no differences were seen in T-cell numbers between responders and non-responders (Fig 6D).

Discussion

Multiple infusions of DB-NK cells were feasible in patients with R/R AML when administered following FLAG chemotherapy, and generally well tolerated with no treatment-related cytokine storm or tumour lysis and no treatment-related deaths. AEs were manageable, being related to disease status or expected NK-cell activity or secondary to FLAG-related neutropenia.

Clinical outcomes in this cohort were encouraging and suggested activity of DB-NK cells, given that all patients were

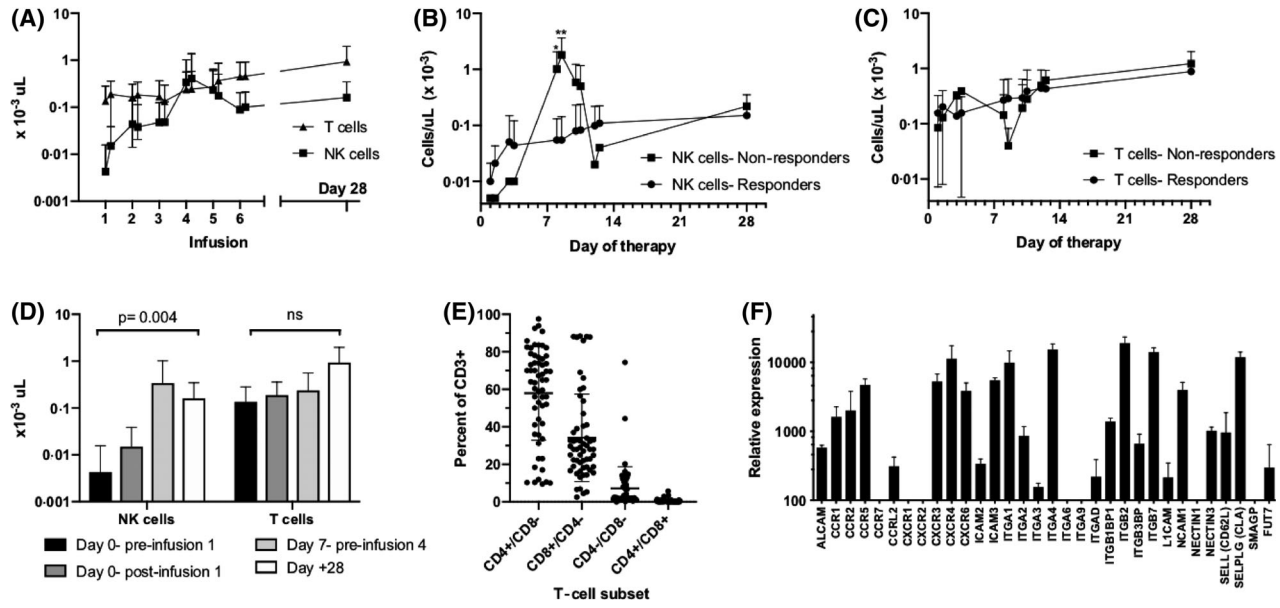

Fig 6. Immune recovery following DB-NK. (A) NK-cell and T-cell counts prior to infusions, after infusion 1, and prior to infusion 4. (B) NK-cell immune recovery grouped by clinical response. (C) T-cell immune recovery grouped by clinical response. (D) Statistical comparison of NK- and T-cell recoveries at day 0, 7 and 28. (E) CD4- and CD8-T-cell subsets in patients following treatment. (F) Normalised gene expression of homing and adhesion receptors in the expanded NK cells (n = 4). Immune recovery was assessed in 12 patients treated with DB-NK-cell adoptive immunotherapy. One infant patient treated as compassionate use was too ill and small for frequent blood draws. *P = 0·00002; **P < 0·000001. All figures show mean ± standard deviation.

R/R to prior FLAG. The ORR was 78·6%, considering all 14 treatment courses, with CR in 50% and prolonged DFS in patients achieving CR or CRi (median 199 days). These clinical outcomes are also encouraging in view of the frailty of patients in this cohort and in the context of results from early clinical trials of new agents in patients with R/R AML,[24] including studies on NK-AI followed by administration of interleukin-2 or interleukin-15.[25,26]

Unprecedented CNS responses were detected in all four patients with concurrent CNS disease. These patients are usually excluded from clinical trials because of concerns that the CNS is inaccessible to the therapy due to the restrictive nature of the blood–brain barrier. Nevertheless, there is emerging evidence of a permissive or disrupted blood–brain barrier and understanding of the CNS lymphatic system in patients with CNS pathologies.[27,28] In a murine *in vivo* model, injected NK cells suppressed medulloblastoma growth associated with NK-cell migration through the CNS.[29] We believe the resolution of CNS disease may provide evidence of DB-NK cells trafficking and homing to the brain.

NK cells are known to have antimicrobial activity, and despite transient, manageable symptoms, DB-NK cells seemed to have potent anti-infective effects against infections that may normally be fatal in this patient population. Promising antimicrobial activity was documented in three patients. Activated NK cells seem to be required for the generation of antibacterial specific adaptive immunity through inducing maturation of dendritic cells.[30,31] Furthermore, the anti-tuberculosis activity of NK cells seems to vary with KIR haplotypes, suggesting that KIR variation may be related to tuberculosis susceptibility.[32]

Immune reconstitution was predominantly T cells, although three patients demonstrated NK-cell-dominant reconstitution. Notably, CRs were not associated with NK-cell recovery. This may suggest both an NK-cell modulation effect and an anti-leukaemic effect from infused DB-NK. It has also been proposed that NK cells can influence the quality of T-cell responses via cytokine secretion or direct killing of target cells in which antigen release and presentation to T cells increases T-cell response.[33,34] Prior *in vitro* and *in vivo* studies have shown that DB-NK cells are both cytotoxic and cytokine producing.[13,14]

The median age of patients treated in this study was 22 years (IQR 11–46·5). However, AML is most commonly diagnosed in elderly patients (median age 67 years), many of whom have comorbidities and for whom intensive chemotherapy and alloSCT are unlikely options. Although younger in age, our patients had a median ECOG performance status score of 3 at enrolment, three patients had severe infections, and four had concurrent CNS disease. Side effects were manageable and promising anti-tumour and antimicrobial responses were documented.

Though the data from this study are encouraging, the findings should be treated with caution; the main limitation is the small number of patients which also precluded any conclusions on KIR phenotyping and anti-tumour effect, and

the fact that, besides reasonable DFS after DB-NK-cell immunotherapy, among this high-risk and highly refractory group of AML patients, a small proportion might have responded to FLAG therapy alone.

In summary, multiple infusions of cryopreserved DB-NK cells were safely administered and provided encouraging responses, including CNS responses, in this heavily pre-treated and fragile population. A future multi-centre clinical study is planned to investigate this treatment protocol in less compromised patients with R/R AML or frail patients with newly diagnosed AML.

Author contributors

DAL designed the study, with minor amendments and translation by LS for use at her institution. LS oversaw all treatments and DAL was involved in donor selection. All authors were involved in data collection. LS, VV, HH, LJC, AP, and DAL analysed the data. LS and DAL were involved in manuscript preparation. All authors contributed to data interpretation; provided critical revision of the manuscript and final approval to submit; and were accountable for the accuracy and integrity of the publication.

Conflicts of interest

Kiadis Pharma commissioned medical writing services to support drafting of the report. The funders had no role in the study design, data collection, analysis or interpretation, writing or approval of the report, or decision to submit the paper. The corresponding author had full access to all study data and had final responsibility for the decision to submit for publication. All authors provided final approval to submit.

LS, VV, AP, MdS, IW, JN, AV, BC, BZ, FS, JM, LSe, HH, LJC and AP declare no conflicts. DAL holds stocks and options in Courier Therapeutics, Caribou Biosciences, and Kiadis Pharma; received consulting fees (personal) from Kiadis Pharma; received research grants from Kiadis Pharma; and has intellectual property licensed to Kiadis Pharma from which royalties are received.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Phenotype of $CD56^{bright}/CD16^{bright}$ DB-NK-cell product. After NK-cell expansion on feeder cells, NK cells were phenotyped by flow cytometry. (A) Uniform $CD56^{bright}$ population with intermediate granularity typical of NK cells. (B) predominantly $CD56^{bright}/CD16^{bright}$ population.

Fig. S2. Change in normalised mRNA expression of genes involved in lymphocyte migration and adhesion.

Table SI. Individual patient/donor KIR ligand mismatch, donor KIR-Bx haplotype status, and tumour response in treated patients.

Table SII. Product characteristics.

Table SIII. Response by patient.

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