Blood First Edition Paper, prepublished online August 23, 2017; DOI 10.1182/blood-2017-05-785659

Phase 1 Clinical Trial using mbIL21 *Ex-vivo* Expanded Donor-derived NK Cells after Haploidentical Transplantation

Stefan O. Ciurea[1], Jolie R. Schafer[2], Roland Bassett[3], Cecele J. Denman[4], Kai Cao[5], Dana Willis[5], Gabriela Rondon[1], Julianne Chen[1], Doris Soebbing[1], Indreshpal Kaur[6], Alison Gulbis[7], Sairah Ahmed[1], Katayoun Rezvani[1], Elizabeth J. Shpall[1], Dean A. Lee[8*], Richard E. Champlin[1*]

* Equal contribution

Departments of [1]Stem Cell Transplantation and Cellular Therapy, [2]Pediatrics, [3]Biostatistics, [4]Immunology, [5]Laboratory Medicine, [6]GMP Laboratory, [7]Pharmacy, The University of Texas MD Anderson Cancer Center, Houston, TX; [8]Division of Hematology, Oncology, and Bone Marrow Transplantation, Department of Pediatrics, Nationwide Children's Hospital, Columbus, OH.

Correspondence to: Stefan O. Ciurea, M.D., Associate Professor, Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Unit 423, Houston, Texas 77030. Phone: 713-745-0146, fax: 713-794-4902, e-mail: sciurea@mdanderson.org

Presented in part as oral presentation at the American Society of Hematology Meeting in 2015 (abstract #102) and 2016 (abstract #500).

Word count: Manuscript – 4,519; Abstract – 241

References: 40

Tables and Figures: 8

Supplementary Tables and Figures: 3

Short title: Infusion of mbIL-21 NK cells post haplotransplant

Funding: This study was funded in part by the M.D. Anderson Cancer Center Support Grant (P30 CA016672), P01 (CA49639), Leukemia and Lymphoma Society Translational Research Program Award (6149-14), Cancer Prevention Research Institute of Texas (RP110553), The University of Texas MD Anderson Cancer Center AML Moonshot Program and High Impact Clinical Research Support Program, and from the McKee Family Foundation and the Taylor Trudeau Cycle for Life Charitable Foundation.

ABSTRACT

Relapse has emerged as the most important cause of treatment failure after allogeneic hematopoietic stem cell transplantation (HSCT). To test the hypothesis that NK cells can decrease the risk of leukemia relapse we initiated a phase-1 dose-escalation study of expanded donor NK cells infused before and after haploidentical HSCT for high-risk myeloid malignancies. The goals were to determine the safety, feasibility and maximum tolerated dose (MTD). Patients received a melphalan-based reduced-intensity conditioning regimen and post-transplant cyclophosphamide-based GVHD prophylaxis. NK cells were infused on Days -2, +7 and +28 post-transplant. All NK expansions achieved the required cell number, and eleven of thirteen patients enrolled received all three planned NK cell doses ($1x10^5$ to $1x10^8$/kg/dose). No infusional reactions or dose-limiting toxicities occurred. All patients engrafted with donor cells. Seven patients (54%) developed grade 1-2 aGVHD, none developed grade 3-4 aGVHD or cGVHD, and a low incidence of viral complications was observed. One patient died of non-relapse mortality and one patient relapsed. All others were alive and in remission at last follow-up (median 14.7 months). NK cell reconstitution was quantitatively, phenotypically, and functionally superior compared to a similar group of patients not receiving NK cells. In conclusion, this trial demonstrated production feasibility and safety of infusing high doses of *ex vivo* expanded NK cells after haploidentical HSCT without adverse effects, increased GVHD or higher mortality, and was associated with significantly improved NK cell number and function, lower viral infections, and low relapse rate post-transplant.

KEY POINTS

- High doses of haploidentical NK cells expanded ex vivo with mbIL21-expressing feeder cells can be safely infused post-transplant
- Infusion of NK cells was associated with improved NK cell function, low relapse and incidence of viral infections

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is effective treatment for patients with advanced hematological malignancies.[1] After progressive improvements in treatment-related mortality,[2] disease relapse emerged as the most important cause of treatment failure.[3] Hence there is urgent need for novel therapies to reduce the risk of relapse post-transplant.

Natural killer (NK) cells have the capability to eliminate leukemic or virally infected cells.[4,5] In mice, NK cells have been shown to improve engraftment and decrease graft-versus-host diseases (GVHD) after transplantation.[6,7] Higher absolute NK cell numbers in the early post-transplant period was associated with lower relapse and improved survival.[8,9] Moreover, NK cell alloreactivity was reported to decrease relapse rate after haploidentical transplantation (haploHSCT).[10]

Several studies have used NK cells from the peripheral blood (PB) of the donor collected by steady state apheresis, with typical doses ranging 1-3x10^7/kg.[11-15] Most studies showed no major toxicities, except in one report, in which infusion of IL-15/4-1BBL activated NK cells was associated with a high incidence of acute graft-versus-host disease (GVHD).[16]

Obtaining sufficient numbers of NK cells to achieve a therapeutic effect has been a major limitation.[17] Attempts to expand NK cells have typically used IL-2 and/or IL-15.[18-24] Our group developed a method to expand NK cells *ex vivo* using K562 feeder cells expressing membrane-bound IL-21 (mbIL21).[25] This approach expands NK cells up to 35,000 fold in 3 weeks and produces highly functional NK cells.[25]

NK cells are the first cells to recover after transplant; however, their function is significantly impaired.[26-28] We also observed that absolute NK cell numbers were low in the first month following T-cell replete haploHSCT with post-transplant cyclophosphamide, and had immature phenotype and markedly decreased function (Figure 1).[29] Therefore, we hypothesized that multiple infusions of high numbers of mature, fully functional mbIL21-expanded NK cells before and after transplantation would improve anti-tumor activity for high-risk myeloid malignancies, and performed a phase 1 study to determine safety, feasibility and maximum tolerated dose (MTD) of this approach.

METHODS

Patients

Patients age 18-65 years with high-risk acute myeloid leukemia (AML), myelodysplastic syndromes (MDS) or chronic myeloid leukemia (CML) ($\leq$ 5% bone marrow blasts), adequate performance status and organ function were included. Patients with high-risk myeloid malignancies (AML with high-risk disease by refractoriness to induction chemotherapy, cytogenetics and/or molecular mutations, in morphologic remission ($\leq$ 5 % bone marrow blasts), MDS with intermediate or high-risk IPPS score or CML that failed treatment with tyrosine kinase inhibitors or progressed to accelerated or blast phase, with $\leq$ 5% bone marrow blasts) ages 18 to 65 year old with adequate performance status ($\geq$ 70% Karnofsky) and organ function (EF $\geq$ 40%, corrected DLCO $\geq$ 50%, Br < 1.5mg/dL, ALT/AST < 200IU/ml, creatinine clearance by Cockroft-Gault $\geq$ 50ml/min) were included in this study. Exclusion criteria included: active hepatitis B, C or HIV +, liver cirrhosis, uncontrolled infections, CNS involvement < 3 months, positive pregnancy test in a woman with childbearing potential.

Donors

Donors were required to have at least 16 years and must agree to 2 donations: approximately 1 unit (500 mls of peripheral blood) collected on Day -16 for NK cell production and bone marrow progenitor cells

obtained via a bone marrow harvest procedure with the goal of $3x10^8$ TNC/kg collected and infused on Day 0 of transplant.

Transplant conditioning regimen and stem cell infusion

All patients were treated with a conditioning regimen consisting of melphalan 140 mg/m^2 for 1 dose on Day-7, fludarabine 160 mg/m^2 divided in 4 daily doses on Days -7 to -4. Older patients (> 55 years) or with comorbidities could receive reduced doses of melphalan at 100mg/m^2 at the discretion of the treating physician, as previously described by us. All patients received one dose of 200 cGy total body irradiation (TBI) on Day -3 (Figure 2). A bone marrow graft was infused fresh on Day 0. The goal of the bone marrow harvest collection was $3x10^8$ TNC/kg. GVHD prophylaxis consisted of post-transplant cyclophosphamide at 50 mg/kg/day on Day +3 and +4, tacrolimus starting on Day +5 and continued for 6 months in the absence of GVHD and mycophenolate mofetil (MMF) starting on Day +5 and continued for 3 months, unless otherwise indicated. All patients received filgrastim at 5mcg/kg/day starting on Day +7 until the absolute neutrophil count (ANC) reached $1,500x10^9$/L. Standard antimicrobial prophylaxis was provided with voriconazole, pentamidine or trimethoprim-sulfamethoxazole, and acyclovir or valacyclovir for fungal, pneumocystis jiroveci and herpes simplex, respectively.

***Ex vivo* expanded NK cell product**

Leukocyte fraction ("buffy coat") of haploidentical blood collected on Day-16 was source of PB mononuclear cells (PBMC). T cells were magnetically separated with colloidal super-paramagnetic anti-CD3 mAb (Miltenyi Biotec, Auburn, CA). NK cells from the transplant donor were expanded *ex vivo* for 14 days from CD3-depleted peripheral blood mononuclear cells (PBMC) (see Supplemental Material) by adding irradiated K562 Clone9.mbIL21 feeder cells once weekly, as previously described[25], thereafter denoted mbIL21-NK cells. Fresh mbIL21-NK cells were infused on Day-2. The remaining mbIL21-NK cells were cryopreserved (40% Plasmalyte, 50% human AB Serum, 10% DMSO) and were thawed, washed, and infused on Day+7 and +28 (or later, up to Day +90) (Figure 2). The cryopreserved NK cells were prepared for infusion by thawing in 37°C water bath, washing once with infusion buffer (0.5% human serum albumin in Plasmalyte A) and resuspending the final cell dose in 100 mls of infusion buffer. Dose escalation was planned in cohorts of minimum 2 patients starting at $1x10^5$/kg/dose and escalating by ½-log increments up to $3x10^8$/kg/dose or until the MTD was reached. A "safe dose" level of $1x10^4$/kg/dose was employed if a patient needed treatment before the $1x10^5$/kg/dose level was deemed safe.

HLA typing and determination of KIR content/matching

KIR genotyping and alloreactivity were evaluated. NK alloreactivity in the graft-versus-host direction and/or KIR B genotype was preferred for donor selection, but not required.[31,32] KIR genotyping and alloreactivity was evaluated. NK alloreactivity in the GVHD direction and/or KIR B genotype was preferred for donor selection, but not required. All patients had HLA typing by intermediate and/or high-resolution typing. KIR genotyping was performed for selected NK cell donors with reverse SSO method utilizing fluorescently labeled beads conjugated to oligonucleotide probes and detected in a Luminex platform (One Lambda, Canoga Park, CA). KIR ligand-ligand mismatch was predicted using KIR Ligand Calculator (http://www.ebi.ac.uk/ipd/kir/ligand.html). KIR receptor-ligand mismatch was refined by ligand-ligand mismatch prediction based on presence of KIR receptor gene in the donor. KIR-B content was determined using B Content Calculator (http://www.ebi.ac.uk/ipd/kir/donor_b_content.html).

NK cell phenotype and function

PBMC from patients and aliquots of donor mbIL21-NK cells were cryopreserved for batched testing of function and phenotype. PB was obtained around Day+30 from patients receiving haploHSCT without NK cells (MDACC #2009-0266, clinicaltrials.gov NCT01010217; control group), and prior to the 3rd infusion of NK cells around Day+28 from patients receiving haploHSCT with NK cells (MDACC #2012-0708, clinicaltrials.gov NCT01904136), both referred to hereafter as Day+28 samples. PBMC from patients and aliquots of donor NK cell infusion products were cryopreserved for batched testing of function and phenotype. Cells were thawed and rested overnight in 100 IU/mL IL-2 prior to testing. The calcein-release assay was used to determine cytotoxicity as previously described.[33] Cytokine production was determined by stimulating effectors (adjusted for NK cell content) with targets at a 2:1 NK:Target ratio for 3 hours in the presence of Golgi-stop (BD Biosciences) and metal-conjugated anti-CD107a, followed by labeling with 5μM cisplatin and surface and intracellular staining with metal-conjugated antibodies. NK cells were identified as CD56+CD3- and data was acquired by CyTOF (DVS Sciences) as previously described.[34] The antibody clones and heavy metal labels are listed in Table 2S.

Analysis of mass cytometry data

To unbias differences in sample event number between samples, an equal number of events from each CD3-CD56+ gated sample were exported using FlowJo. Clustering analysis was then performed using spanning-tree progression analysis of density-normalized events (SPADE V3·0) on CytoBank, downsampled to 3,000 events per sample, and using the following markers used for clustering: CD3, CD4, CD8a, CD11a, CD16, CD56, CD57, CD62L, CD94, CD107a, CD134 (OX40), CD223 (LAG3), CD226 (DNAM-1), CD253 (TRAIL), CD272 (BTLA), CD314 (NKG2D), CD357 (GITR), NKp30, NKp44, NKp46, NKp80, NKG2A, NKG2C, and TIM3. For ViSNE analysis, the equalized samples were then concatenated for each group (peripheral blood from patients on 2009-0266 and 2012-0708, and NK cell infusion products), and an equal number of events per group was then exported for clustering in order to weight each group equally. ViSNE analysis of group data was then performed on CytoBank, using the same markers for clustering as for SPADE analysis.

For unsupervised clustering of mass cytometry data, rows were centered, and unit variance scaling was applied to rows. Both rows and columns were clustered using correlation distance and average linkage and ordered by tightest clusters first. For principle compnenets analysis, unit variance scaling was applied to rows and SVD with imputation was used to calculate principal components. Prediction ellipses were generated such that with probability 0.95, a new observation from the same group will fall inside the ellipse. Principle components analysis, heatmaps, and clustering were generated using the ClustVis webtool (http://biit.cs.ut.ee/clustvis/, accessed November 28, 2016).

Trial design

Primary objectives were to evaluate the safety of infusion and determine the MTD of 3 doses of haploidentical mbIL21-NK cells administered in conjunction with haploHSCT (Days -2, +7 and +28). Secondary objectives were to determine NK cell persistence after infusion, immunologic reconstitution, phenotype and function during immune reconstitution, estimate the proportion of patients with engraftment/graft failure, 100-day non-relapse mortality (NRM), cumulative incidence (CI) of acute grade 2-4 GVHD and chronic GVHD, relapse rate (RR), overall survival (OS) and disease-free survival (DFS). These measures were compared to a control group.

The trial was approved by Institutional Review Board of MD Anderson Cancer Center and conducted under Investigational New Drug application from Food and Drug Administration. All patients and donors provided written informed consent according to the Declaration of Helsinki.

For the purpose of dose-finding, "toxicity" was defined as any of the events (i) Death, (ii) Grade 3-4 infusion reaction, (iii) Grade 4 organ toxicity (not including mucositis or myelosuppression) (iv) Graft failure, or (v) severe (grade 3 or 4) Graft-versus-host disease (GVHD) occurring within 72 days of the first NK cell infusion on Day -2 or Day -1, that is, by Day +70 post SCT. Consequently, the time window for evaluating toxicity was 72 days, from the day of the first NK cell infusion to day +70. Patient outcome was from the time of occurrence of toxicity or, if toxicity has not yet occurred by an observation time prior to day +70 post-transplant, the outcome will be the patient's follow up time without toxicity. Denoting the time of follow-up or toxicity by T, and the right-censoring indicator C = 1 if T was a follow up time without toxicity and C = 0 if T was the time of observed toxicity, each patient's data consisted of the pair (T,C), with T no larger than 72 days. A patient's outcome (T,C) will be considered "fully evaluated" if either C = 0 (toxicity has occurred at some time up to day 70 post-transplant) or (T,C) = (72,1), which says that no toxicity has occurred by day +70.

Dose-finding was carried out using the time-to-event (TiTE) continual reassessment method (CRM) of Cheung and Chappell. Denoting exponentiation by "E," the seven NK cell per-administration doses (PADs) to be studied are number of NK cells/kg = 10^5, 10^6, 10^7, $3x10^7$, 10^8, and $3x10^8$. The TiTE CRM was applied with cohorts of size 2, starting at 10^5 (the second lowest NK cell PAD level), not skipping a dose level when escalating, target Pr(toxicity by day 70 post-transplant) = 0.50, and maximum sample size 30. To avoid aggressive/unsafe escalation early in the trial, the design was to proceed in two stages. In Stage 1, the minimum waiting time between dose cohorts is 49 days from the first NK infusion. For mb21-NK cell infusion, patient must have been off systemic corticosteroids for $\geq$ 72 hours (< 0.5mg/kg prednisone), have no active grade II-IV aGVHD, no uncontrolled infections or fever (> 38.5 °C), and for third infusion, no grade $\geq$ 3 non-hematologic organ toxicity. The first cohort of two patients will be enrolled at d = 10^5 NK cells. If no toxicity is observed, then two new patients (the second cohort) will be enrolled at d = 10^6 NK cells. If no toxicities are observed among the previously accrued patients, then escalation will proceed in cohorts of size 2 to subsequently higher doses. If a new patient arrives to be treated at a time when either 1 or 2 of the patients in the previous cohort have been treated but not yet fully evaluated, then the new patient will be treated at one dose level below the current recommended level, up to a maximum of 3 new patients. Stage 2 begins when the first toxicity occurs, and thereafter the cohort size will be 1 with the TiTE-CRM used to choose each new patient's dose, using the fixed target 0·50 for Pr(toxicity within 72 days | dose). The "optimal" dose was defined as that for which the posterior mean of Pr(toxicity within 72 days | dose) given the current data is closest to 0.50. Each subject will be followed for the entire 72-day period for toxicity evaluation. Once a DLT occurs at any time in the 72-day window for any subject, new subjects will be assigned to the optimal dose. Additional safety restrictions applied during the trial were (1) An untried dose may not be skipped when escalating and (2) Escalation may not be done immediately after a toxic outcome (i.e., incoherent escalation). The skeleton corresponding to dose levels 1-7 that is the basis for the assumed TiTE CRM model is (0.30, 0.35, 0.42, 0.50, 0.60, 0.68, 0.75).

Description of retrospective control group

The control group of patients (n = 45, median age 45 years) with AML/MDS/CML in first or second complete remission (CR1/CR2) or morphologic CR ($\leq$ 5% bone marrow blasts) was treated on a previous clinical trial with same conditioning regimen but without NK cells (MDACC protocol 2009-0266) (Table 2S). Patient characteristics were similar with those of mb21-NK cell treated group (Table 1S). All patients achieved primary engraftment, with median time to neutrophil and platelet engraftment of 18 and 40 days, respectively. Grade 2-4 aGVHD occurred in 14/44 (31.8%) patients, grade 3-4 aGVHD in 1/41 (2.3%)

patients, and cGVHD occurred in 7/41 (17%) patients. Nine patients (20%) died of TRM, 10 patients (22%) relapsed and 25 patients (55.5%) were alive at last follow-up (Figures 4A-E).

RESULTS

Patients and transplant characteristics

Thirteen patients (6 males, 7 females, median age 44 years, range 18-60 years) were enrolled and treated on this phase 1 clinical trial between 5/2014-1/2016. The majority of patients had high risk of disease relapse (Table 1).

Characteristics of the mbIL21-NK cells, toxicities and NK cell reconstitution post-transplant

mbIL21-NK cell characteristics and infusional toxicities

Median purity of the product was 98.98% (CD3-CD16/56+) with median viability of 97% and extremely low or undetectable T and B cell content (Table 2). Donor and recipient KIR profiles are presented in Table 3. Five patients had KIR mismatch with the donor based on the ligand-ligand model, 4 donors had high B content, 4 had "better", 1 "best" and 8 "neutral" KIR score, and 4 patients had a KIR2DS1 donor. Five patients had donors without any NK-cell good-prognosis predictors.

NK cell expansion met release criteria and dose requirements for all patients at all dose levels. All but 2 patients received all 3 planned mbIL21-NK cell infusions (Table 3). The patient treated at the -1 Dose level ($1x10^4$/kg/dose) developed secondary graft failure related to infection, and died of NRM unrelated to the NK cell infusion.

NK cell reconstitution post-transplant

We assessed quantitative, phenotypic, and functional NK reconstitution at Day+28 (prior to the third NK cell infusion) using *in vitro* cytotoxicity assays and mass cytometry, and compared this with NK cell reconstitution from similar patients in the control group and with the mbIL21-NK cell infusion product.[33]

Compared to a control group, NK cell activity in PBMC from patients treated with NK cells was significantly greater (Figure 3A). Although degranulation (measured by CD107a) was similar (Figure 3B), patients who received NK cells also had greater proportion of NK cells that secreted TNF-α and INF-γ in response to 721.221 targets (Figure 3B). CD62L cleavage from the NK cell surface was greater for patients that received NK cell infusions (Figure 3C) suggesting increased activation in response to targets, and a functional shift from cytokine production to cytotoxicity was significantly associated with increasing NK cell dose (Figure 3C).

Phenotypic analysis of all NK cells from the two studies showed no significant differences in either percentage or median metal intensity (MMI) for any marker that survived multiple comparisons correction (Figure 1S A, B).[33] We then restricted analysis to only the mature single-KIR+ cells for each donor in order to remove KIR genotype and licensing biases between the groups (Figure 1S C, D). Significant differences were then observed for the percent of single-KIR+ NK cells expressing CD16, CD244 (2B4), NKG2C, CD223 (LAG3), CD62L, CD272 (BTLA), and CD357 (GITR), the first four being higher and the latter three lower in patients who received NK cell infusions (Figure 3D). MMI differences for CD223, CD244, CD272, CD357, and NKG2C were also observed but did not remain significant after multiple comparison correction. Unsupervised clustering analysis on the basis of receptor expression was able to fully differentiate the NK cells in the infusion product from those obtained from peripheral blood (Figure 3E), but peripheral blood NK cells from the two different treatment groups (with and without NK cells) did not cluster discretely.

To further distinguish between PB-NK cells of the two groups, SPADE and ViSNE algorithms were used to identify and quantify NK cell phenotypic subsets.[33] SPADE analysis identified relatively few dominant phenotypes in each group of NK cell sources (Figure 3F). No dominant subsets were common to both the infusion product and the PB-NK cells from patients, whereas there was close overlap between dominant subsets in PB-NK cells obtained from the two treatment groups (Figure 3F). Despite statistical significance between individual NK cell markers of patients in the two studies (Figure 3D), unsupervised clustering analysis of the subpopulation proportions identified by SPADE for each cohort did not distinguish NK cells of patients between the two trials (Figure 3G). Likewise, a principle components analysis of the SPADE nodal data showed significant overlap between the two PB-NK cell groups, although these were again clearly distinct from mbIL21-NK cell infusion products (Figure 3H). Using ViSNE to broadly assess phenotypic variation, PB-NK cells from patients on the different studies aligned closely, with exception of a distinct cluster having high CD4 and CD11a and absent NKG2A expression in patients that received NK cell infusions (Figure 3I). As with SPADE, however, delineation between PB-NK cells from patients and the expanded NK cell product was clear.

Transplant outcomes and comparison with retrospective controls

Engraftment, non-relapse mortality, relapse rate and survival

Transplant outcomes for patients treated are summarized (Table 1). Median follow-up duration for survivors was 14.7 months (range 8-25.1 months). All patients achieved primary engraftment (100%), all but one with 100% donor cell chimerism at Day+28 post-transplant. The patient having mixed chimerism developed Parainfluenza pneumonia during the neutropenic period and subsequently died (Day +85). The median time to neutrophil engraftment was 19 days (range 15-27 days) and platelet engraftment 22 days (range 13-39 days). Seven patients (54%) developed grade 2 acute GVHD, controlled with topical steroids, budesonide and/or systemic steroids. Only 5 of these patients required systemic steroids for acute GVHD, which resolved rapidly. No grade 3-4 acute GVHD nor chronic GVHD were observed. Only 1 patient relapsed (7.7%) at Day+120 post-transplant. This patient was treated at the lowest investigated dose ($1x10^5$ NK cells/kg/dose) and was alive at last follow-up (Day+582). One-year OS and DFS of the study group were 92% and 85%, respectively (Table 1). There were no toxicities > 3 grade attributable to NK cell infusion.

Comparison with retrospective control group of patients

The control group of patients consisted of all 45 patients (median age 45 years) with AML/MDS and CML in morphologic CR (≤ 5% bone marrow blasts) treated on the previous clinical trial with the same conditioning regimen but without NK cells (MDACC protocol 2009-0266).[30] Characteristics of these patients are summarized in Supplementary Table 1S. All patients in this group achieved primary engraftment (100%), with a median time to neutrophil and platelet engraftment of 18 and 40 days, respectively. Grade 2-4 acute GVHD in this group occurred in 14/44 (31.8%) patients, grade 3-4 acute GVHD in 1/41 (2.3%) patients, and chronic GVHD occurred in 7/41 (17%) of evaluable patients. Nine patients (20%) died of NRM, 10 patients (22%) relapsed and 25 patients (55.5%) were alive at last follow-up. Comparative outcomes between the current study and the control group of patients are presented in Figure 4.

Incidence of viral reactivation

For patients treated with NK cell infusions, 4 patients (30.8%) had CMV reactivation (2 of which received treatment with systemic steroids), and only 1 patient (7.7%) had asymptomatic BKV viruria (grade 1). Incidence in the control group was significantly higher for CMV reactivation (70.4%, $p = 0.011$) and trended higher for BKV cystitis (31.8%, $p = 0.15$), with six patients having grade 1, 6 patients grade 2, and 2 patients grade 3/4 hemorrhagic cystitis, respectively (Figure 4 G, F). Median CMV

reactivation time was 28 days for patients treated without NK cells and 39 days for patients treated with NK cells (Figure 4G). [35]

Reconstitution of T cell subsets

Immunologic T cell reconstitution was evaluated around Day+28, +90 and +180 for patients in both groups (Figure 5). No significant differences were found in total WBC count, $CD3^+$ T cells, $CD25^+$ regulatory T cells, $CD45RA^+$ naïve T cells, CD45RO memory T cells or $CD19^+$ B cells (Figure 3). Though not statistically significant, patients treated with NK cells tended to have higher NK cell numbers at Day+28 (median 121.3 vs. 77.4, p = 0.47), Day+90 (median 248.1 vs. 159.0, p = 0.08) and Day+180 (median 287.7 vs. 185.2, p = 0.12). Although no significant differences in absolute $CD3^+CD4^+$ cell numbers were observed, $CD3^+CD8^+$ T cell numbers were significantly lower at Day+28 in patients treated with NK cells (median 0.15 vs. 20.5, p < 0·001) (Figure 5E). Later recovery of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets was similar for both treatment groups [Day+90 ($CD4^+$: 183.4 vs. 148.0, p = 0.99; and $CD8^+$: 378.1 vs. 291.3, p = 0.93) and Day+180 ($CD4^+$: 310.9 vs. 245.2, p = 0.29; and $CD8^+$: 673.2 vs. 525.0, p = 0.76].

DISCUSSION

We report results of a phase 1 clinical trial in which multiple high doses of *ex vivo* expanded NK cells were delivered peri-haploidentical transplantation, with the goal to enhance graft-versus-leukemia (GvL) activity and reduce risk of relapse.[36-38] Large NK cell doses were successfully generated and safely infused in this study with a promising improvement in relapse rate, immune reconstitution, and viral control.

Previous studies showed safety of apheresis-derived NK cells, but efficacy remains unclear. We established a method for *ex vivo* expansion of NK cells by stimulation with K562 Clone9.mbIL21 feeder cells to produce mbIL21-NK cells, with dramatically increased cytotoxicity and cytokine production.[25] In this study, we evaluated the effect of multiple high doses of haploidentical NK cells expanded with IL-21 and 4-1BBL (up to $3x10^8$/kg) infused peri-transplant in a relatively small number of patients. We found no infusional reactions and no increased risk of severe acute or chronic GVHD, in contrast to matched allogeneic NK cells expanded with IL-15 and 4-1BBL,[16] confirming the safety of this cellular therapy product. Relapse was remarkably low, with only one of thirteen patients relapsing at last follow-up, despite having poor-risk cytogenetics, high-risk molecular mutations or primary induction failure, which predict high relapse rate post-transplant. Although a phase 1 trial is designed to assess safety and feasibility, these early clinical outcomes are provocative, and support our hypothesis that infusion of mbIL21-NK cells expanded from the same haploidentical donor may enhance the GvL effect. Importantly, this was observed regardless of NK alloractivity or better KIR genotypes, as these features are available only for a minority of patients.

Adoptive immunotherapy with expanded NK cells in the peri-transplant period (Day-2 and Day+7) also improved NK cell immune reconstitution, and correlated with increased NK cell cytotoxicity and cytokine production. This is particularly meaningful because cyclophosphamide was administered after the first infusion, and improved NK cell function was detected before the 3rd infusion of NK cells, two weeks after cryopreserved mbIL21-NK cells were infused (Day+7) and while patients were on full immunosuppression. Additional data will be necessary to probe the persistence of fresh infusions (logistically more difficult) and to show that early discontinuation of immunosuppression (which increases risk of GVHD) is not required for the observed therapeutic effect.[39] The better NK cell numbers and function at Day+28, however, may be independent of phenotype, which is largely similar between those treated or not treated with NK cells. Although we were able to detect an improvement in NK cell

number and function early post-transplant which presumably contributed to an enhanced GVL effect, it was not possible to clearly differentiate between the expanded NK cells that were infused and those that derived from the donor stem cells by phenotype, nor was it possible to determine persistence by HLA typing or STR chimerism analysis, as was done in other studies.[40] We were only able to differentiate phenotypes between the two groups if the analysis was restricted to mature KIR+ subsets. These mature NK cells may represent persistent infused NK cells, or may represent a change caused by the infused cells in maturation of NK cells arising de novo from the new marrow.

Viral reactivation remains a significant complication after haploidentical transplantation with high rates of CMV reactivation and BK virus cystitis.[35] In this study we also observed a marked decrease in viral reactivation in the group treated with mbIL21-NK cells. Remarkably, the incidence of CMV reactivation was halved compared to control group and none developed BKV cystitis. Previous studies demonstrated that NKG2C+ NK cells correlate with both control of CMV reactivation and GvL effect.[33,34] In fact, $NKG2C^+$ NK cells were significantly increased at Day+28 in these patients. Although viral control may be mediated by cross-talk with adaptive immunity, this mechanism is contradicted by the apparent transient absence of $CD8^+$ T cell numbers on Day+28. However, neither $CD4^+$ T cells nor NK cells were lower at this time point.

In conclusion, this phase 1 clinical trial demonstrates that infusions of high doses of *ex vivo* expanded donor-derived haploidentical NK cells in haploidentical bone marrow transplantation is safe and may be potentially effective in controlling leukemia relapse with no major toxicity. These results justify further clinical evaluation with phase 2 and 3 studies to assess the benefit of mbIL21 *ex vivo* expanded NK cells in conjunction with haploidentical transplantation. Future studies will need to explore the scalability of NK cell production required for larger multicenter studies and control production costs, which, so far, represent only a small fraction of transplant costs and should be competitive with new drug therapies which are emerging as post-transplant maintenance strategies.

Authorship contributions

S.O.C contributed with study design, enrollment of patients on study, interpretation of results and wrote the manuscript; J.R.S. and C.J.D. performed the laboratory experiments and contributed with interpretation of laboratory data, R.B. contributed with trial design and statistical analysis, K.C. and D.W. contributed with HLA typing and donor selection, G.R. and J.C. contributed with data collection, D.S., and S.K. contributed with patient enrollment, A.G., S. A. contributed with patients enrollment, I.K., K.R. and E.J.S. contributed with NK cell manufacturing, D.A.L. contributed with study design, NK cell manufacturing procedure, laboratory data and interpretation of results and manuscript writing, R.E.C. contributed to study design, patient enrollment, interpretation of results and manuscript writing. All authors reviewed and approved the manuscript.

Disclosure of conflicts of interest

S.O.C. served as a consultant for MolMed and Spectrum Pharmaceuticals and has equity/leadership in CytoSen Therapeutics. D.A.L served as consultant for Ziopharm Oncology, Courier Therapeutics, and Intellia Therapeutics, and has equity/leadership in CytoSen Therapeutics. The rest of the co-authors have no potential conflict of interest to declare.

Acknowledgment

We thank Robert Igarashi, MD, PhD for critical review of the manuscript.

REFERENCES

1. Copelan EA. Hematopoietic stem-cell transplantation. *The New England journal of medicine.* 2006;354(17):1813-1826.
2. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. *The New England journal of medicine.* 2010;363(22):2091-2101.
3. Michallet M, Thomas X, Vernant JP, et al. Long-term outcome after allogeneic hematopoietic stem cell transplantation for advanced stage acute myeloblastic leukemia: a retrospective study of 379 patients reported to the Societe Francaise de Greffe de Moelle (SFGM). *Bone marrow transplantation.* 2000;26(11):1157-1163.
4. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nature immunology.* 2008;9(5):503-510.
5. Caligiuri MA. Human natural killer cells. *Blood.* 2008;112(3):461-469.
6. Ruggeri L, Mancusi A, Burchielli E, et al. NK cell alloreactivity and allogeneic hematopoietic stem cell transplantation. *Blood cells, molecules & diseases.* 2008;40(1):84-90.
7. Gill S, Olson JA, Negrin RS. Natural killer cells in allogeneic transplantation: effect on engraftment, graft- versus-tumor, and graft-versus-host responses. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation.* 2009;15(7):765-776.
8. Savani BN, Mielke S, Adams S, et al. Rapid natural killer cell recovery determines outcome after T-cell-depleted HLA-identical stem cell transplantation in patients with myeloid leukemias but not with acute lymphoblastic leukemia. *Leukemia.* 2007;21(10):2145-2152.
9. Dunbar EM, Buzzeo MP, Levine JB, Schold JD, Meier-Kriesche HU, Reddy V. The relationship between circulating natural killer cells after reduced intensity conditioning hematopoietic stem cell transplantation and relapse-free survival and graft-versus-host disease. *Haematologica.* 2008;93(12):1852-1858.
10. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science (New York, N.Y.).* 2002;295(5562):2097-2100.
11. Farhan S, Lee DA, Champlin RE, Ciurea SO. NK cell therapy: targeting disease relapse after hematopoietic stem cell transplantation. *Immunotherapy.* 2012;4(3):305-313.
12. Lee DA, Denman CJ, Rondon G, et al. Haploidentical Natural Killer Cells Infused before Allogeneic Stem Cell Transplantation for Myeloid Malignancies: A Phase I Trial. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation.* 2016;22(7):1290-1298.
13. Passweg JR, Tichelli A, Meyer-Monard S, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia.* 2004;18(11):1835-1838.
14. Rizzieri DA, Storms R, Chen DF, et al. Natural killer cell-enriched donor lymphocyte infusions from A 3-6/6 HLA matched family member following nonmyeloablative allogeneic stem cell transplantation. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation.* 2010;16(8):1107-1114.
15. Yang Y, Lim O, Kim TM, et al. Phase I Study of Random Healthy Donor-Derived Allogeneic Natural Killer Cell Therapy in Patients with Malignant Lymphoma or Advanced Solid Tumors. *Cancer immunology research.* 2016;4(3):215-224.
16. Shah NN, Baird K, Delbrook CP, et al. Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. *Blood.* 2015;125(5):784-792.
17. Radvanyi LG, Bernatchez C, Zhang M, et al. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2012;18(24):6758-6770.

18. Miller JS, Soignier Y, Panoskaltsis-Mortari A, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. *Blood.* 2005;105(8):3051-3057.
19. Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer research.* 2009;69(9):4010-4017.
20. Sutlu T, Stellan B, Gilljam M, et al. Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor. *Cytotherapy.* 2010;12(8):1044-1055.
21. Li Q, Ye LJ, Ren HL, et al. Multiple effects of IL-21 on human NK cells in ex vivo expansion. *Immunobiology.* 2015;220(7):876-888.
22. Li X, He C, Liu C, et al. Expansion of NK cells from PBMCs using immobilized 4-1BBL and interleukin-21. *International journal of oncology.* 2015;47(1):335-342.
23. Wang W, Erbe AK, Alderson KA, et al. Human NK cells maintain licensing status and are subject to killer immunoglobulin-like receptor (KIR) and KIR-ligand inhibition following ex vivo expansion. *Cancer immunology, immunotherapy : CII.* 2016;65(9):1047-1059.
24. Kang L, Voskinarian-Berse V, Law E, et al. Characterization and ex vivo Expansion of Human Placenta-Derived Natural Killer Cells for Cancer Immunotherapy. *Frontiers in immunology.* 2013;4:101.
25. Denman CJ, Senyukov VV, Somanchi SS, et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PloS one.* 2012;7(1):e30264.
26. Shilling HG, McQueen KL, Cheng NW, Shizuru JA, Negrin RS, Parham P. Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. *Blood.* 2003;101(9):3730-3740.
27. Chklovskaia E, Nowbakht P, Nissen C, Gratwohl A, Bargetzi M, Wodnar-Filipowicz A. Reconstitution of dendritic and natural killer-cell subsets after allogeneic stem cell transplantation: effects of endogenous flt3 ligand. *Blood.* 2004;103(10):3860-3868.
28. Nguyen S, Dhedin N, Vernant JP, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood.* 2005;105(10):4135-4142.
29. Denman C DA, DA Lee, Ciurea S. GvHD prophylaxis with post-transplant cyclophosphamide for haploidentical stem cell transplantation results in rapid numeric recovery and delayed functional reconstitution of NK cells post-transplant. Abstract presented at NK2013 - 14th Meeting of the Society for Natural Immunity; 2013; Heidelberg, Germany.
30. Gaballa S, Ge I, El Fakih R, et al. Results of a 2-arm, phase 2 clinical trial using post-transplantation cyclophosphamide for the prevention of graft-versus-host disease in haploidentical donor and mismatched unrelated donor hematopoietic stem cell transplantation. *Cancer.* 2016;122(21):3316-3326.
31. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood.* 2010;116(14):2411-2419.
32. Venstrom JM, Pittari G, Gooley TA, et al. HLA-C-dependent prevention of leukemia relapse by donor activating KIR2DS1. *The New England journal of medicine.* 2012;367(9):805-816.
33. Somanchi SS, Senyukov VV, Denman CJ, Lee DA. Expansion, purification, and functional assessment of human peripheral blood NK cells. *Journal of visualized experiments : JoVE.* 2011(48).
34. Lee DA, Verneris MR, Campana D. Acquisition, preparation, and functional assessment of human NK cells for adoptive immunotherapy. *Methods in molecular biology (Clifton, N.J.).* 2010;651:61-77.
35. Ramlal R, Sasaki K, Cerrada SL, et al. Viral Reactivation in Haploidentical Transplants Using Post-Transplantation Cyclophosphamide; a Single Institution Experience. *Biology of Blood and Marrow Transplantation.*22(3):S375-S376.

36. Alyea EP. Modulating graft-versus-host disease to enhance the graft-versus-leukemia effect. *Best practice & research. Clinical haematology.* 2008;21(2):239-250.
37. Satake A, Inoue T, Kubo S, et al. Separation of antileukemic effects from graft-versus-host disease in MHC-haploidentical murine bone marrow transplantation: participation of host immune cells. *International journal of hematology.* 2010;91(3):485-497.
38. van Besien K. Allogeneic transplantation for AML and MDS: GVL versus GVHD and disease recurrence. *Hematology. American Society of Hematology. Education Program.* 2013;2013:56-62.
39. Wang H, Grzywacz B, Sukovich D, et al. The unexpected effect of cyclosporin A on CD56+CD16- and CD56+CD16+ natural killer cell subpopulations. *Blood.* 2007;110(5):1530-1539.
40. Killig M, Friedrichs B, Meisig J, et al. Tracking in vivo dynamics of NK cells transferred in patients undergoing stem cell transplantation. *European journal of immunology.* 2014;44(9):2822-2834.

TABLES

Table 1. Characteristics and clinical outcomes of patients treated on the phase 1 clinical trial.

Pt	Age	Sex	Diagnosis	Disease characteristics	Disease status at SCT	MRD	Relapse risk	Donor	Engraftment	Time to ANC 500	Chimerism at Day 28	Acute gr 2-4 GVHD	Acute gr 3-4 GVHD	Systemic steroids	Chronic GVHD	Outcome of SCT	Last follow-up
1	40	M	CML	Refractory to TKIs	CP1	-	Int	Son	Yes	20	Mixed	No	No	No	No	Died (TRM)	Day +85
2	52	F	AML	FLT3+	CR2	+	High	Son	Yes	17	Donor	Yes	No	Yes	No	Relapsed	Day +582
3	60	M	CML, MDS	Monosomy 7	CP1	-	High	Daughter	Yes	27	Donor	Yes	No	No	No	Remission	Day +755
4	38	F	AML	PIF, cx. cyto, MLL+	CR1	+	High	Sister	Yes	16	Donor	No	No	No	No	Remission	Day +410
5	43	F	AML	PIF, diploid cyto	CR1	-	High	Brother	Yes	18	Donor	Yes	No	Yes	No	Remission	Day +419
6	34	M	CML	BP CNS+	CP2	-	High	Sister	Yes	21	Donor	No	No	No	No	Remission	Day +481
7	23	M	AML	FLT3+	CR1	-	High	Sister	Yes	15	Donor	No	No	No	No	Remission	Day +417
8	18	F	AML	ASXL1+	CR1	+	High	Father	Yes	21	Donor	Yes	No	Yes	No	Remission	Day +316
9	58	F	AML	MLL+	CR1	-	High	Daughter	Yes	19	Donor	Yes	No	No	No	Remission	Day +412
10	45	F	CML	BP CNS+	CP2	-	High	Brother	Yes	19	Donor	Yes	No	Yes	No	Remission	Day +477
11	55	M	AML	MLL+	CR1	+	High	Son	Yes	14	Donor	No	No	No	No	Remission	Day +448
12	25	F	AM	PIF,	CR1	-	High	Sister	Yes	17	Dono	Yes	No	Yes	No	Remi	Day

			L	diploid cyto							r					ssion	+363
13	48	F	CML	Refractory to TKIs	CP1	+	Int	Daughter	Yes	19	Donor	No	No	No	No	Remission	Day +236

Legend: M – male, F – female, CML – chronic myeloid leukemia, AML –acute myeloid leukemia, MDS – myelodysplastic syndrome, TKIs – tyrosine kinase inhibitors, FLT3 – FMS-like tyrosine kinase 3, PIF – primary induction failure, cx. - complex, cyto – complex cytogenetics, MLL – mixed lineage leukemia gene, BP – blast phase, CNS – central nervous system, CP1 – chronic phase 1, CP2 – chronic phase 2, CR1 - first complete remission, CR2 – second complete remission, MRD – minimal residual disease, SCT – stem cell transplant, Int – intermediate, ANC – absolute neutrophil count, GVHD – graft-versus-host disease.

Table 2. Characteristics of the mbIL21-NK cell product.

Pt	Viability (%)	Viable CD32+ cells (APCs) (%)	CD3+ cells (T cells) (%, x 10^5/kg)	CD3- CD(16,56)+ cells (NK cells) (%)	CD19+ cells (B cells) (%)	CD14+ cells (Monocytes) (%)
1	97	0.1	0.02, 0.00002	98.31	Not Detected	Not Detected
2	98	0.35	0.35, 0.0035	98.01	Not Detected	Not Detected
3	96	0.5	0.01, 0.0001	99.38	Not Detected	Not Detected
4	97	0.1	0.01, 0.001	98.77	Not Detected	Not Detected
5	98	0.38	Not Detected	96·73	Not Detected	0.11
6	96	0.07	0.06, 0.006	98.95	Not Detected	Not Detected
7	97	0.15	Not Detected	99.46	Not Detected	0.05
8	98	0.1	0.04, 0.04	98.46	Not Detected	Not Detected
9	98	0.24	0.03, 0.03	99.81	Not Detected	Not Detected
10	96	0.31	0.02, 0.06	99.80	Not Detected	Not Detected
11	97	Not detected	0.01, 0.03	99.72	Not Detected	Not Detected
12	97	0.07	0.07, 0.7	98.98	Not Detected	Not Detected
13	93	0.33	0.03, 0.3	99.33	0.54	Not Detected

Table 3. Characteristics of the NK cell doses, donor/recipient KIR characteristics, and infusional toxicities.

Pt	NK cells per dose	# doses	Patient KIR ligand	Donor KIR ligand	NK alloreactivity	Donor KIR haplotype	# Cen B/B	KIR score	KIR Centromeric	KIR 2DS1	Toxicity
1	1x10^4/kg	2	C2/C2, BW4	C2, Bw4	No	A/A	0	Neutral	Cen-A/A	No	None
2	1x10^5/kg	3	C1/C2, Bw4	C1, Bw4	No	A/B	2	Better	Cen-A/B	No	None
3	1x10^5/kg	3	C1/C2, Bw4	C1/C2, Bw4	No	A/A	0	Neutral	Cen-A/A	No	None
4	1x10^6/kg	3	C1/C1, Bw4	C1/C2, Bw4	Yes	A/B	2	Better	Cen-A/B	No	None
5	1x10^6/kg	3	C1/C1	C1/C2, Bw4	Yes	A/A	0	Neutral	Cen-A/A	No	None
6	1x10^6/kg	2	C1/C2, Bw4	C1/C2, Bw4	No	A/A	0	Neutral	Cen-A/A	No	None
7	1x10^7/kg	3	C1/C2, Bw4	C1, Bw4	No	A/B	2	Best	Cen-B/B	Yes	None
8	1x10^7/kg	3	C1/C1, Bw4	C1, Bw4	No	A/A	0	Neutral	Cen-A/A	No	None
9	1x10^7/kg	3	C1/C1, Bw4	C1/C1, Bw4	No	A/B	0	Neutral	Cen-A/B	Yes	None

10	$3x10^7$/kg	3	C1/C1	C1/C2	Yes	A/B	2	Better	Cen-A, Cen/Tel-B	No	None
11	$3x10^7$/kg	3	C2/C2, Bw4	C1/C2, Bw4	Yes	A/B	2	Better	Cen-A/B	Yes	None
12	$1x10^8$/kg	3	C1/C1, Bw4	C1/C2, Bw4	Yes	B/B	0	Neutral	Cen/Tel-B	Yes	None
13	$1x10^8$/kg	3	C1/C1, Bw6	C1/C1, Bw6	No	A/A	0	Neutral	Cen-A/A	No	None

Legend: KIR – Killer immunoglobulin receptor; Cen – centromere.

FIGURE LEGENDS

Figure 1. NK cell number, phenotype and function in the first year post-transplant for patients treated with haploidentical stem cell transplantation using post-transplant cyclophosphamide on protocol 2009-0266 (without NK cell infusions. A) Absolute lymphocyte count (ALC) was determined from a clinical CBC obtained at the indicated time point. B) Absolute NK cell counts were determined from peripheral blood samples obtained at same time points, from which PBMC were isolated and cryopreserved for batch testing. CD3-CD56+ populations were determined from within lymphocyte gates, and absolute NK count derived according to the percent of CD3-CD56+ cells. C) NK cell maturity was determined according to CD16+ and CD16- fractions of the NK cells in 3B. D) NK cell function at 1 month post-transplant was determined by measuring cytotoxicity against 721.221 targets, wherein PBMC were applied according to NK cell content at a 40:1 NK:Target ratio.

Figure 2. Treatment schema for the clinical trial 2012-0708 of adding infusions of expanded donor NK cells to haplo-identical stem cell transplant with post-transplant cyclophosphamide.

Figure 3. Assessment of phenotype and function of mbIL21-expanded NK cell infusion product (green) or peripheral blood NK cells patients. Mononuclear cells were isolated from peripheral blood (PBMC) of patients on protocol 2009-0266 (without NK cells, red) or 2012-0708 (with NK cells, green) approximately 28 days after stem cell transplant. Samples from patients on protocol 2012-0708 were obtained prior to receiving the 3rd dose of NK cells (approximately three weeks after receiving the 2nd dose). **A.** Cytotoxicity against 721.221 targets. Cells were applied to the cytotoxicity assay at 10:1 NK:Target ratio (according to NK cell content determined by immune phenotyping). **B.** NK cell responses to stimulation with 721.221 targets at 2:1 NK:Target ratio for 3 hours. Degranulation (CD107a), cytokine production, and CD62L cleavage were determined by mass cytometry. **C.** Cytotoxicity and IFNγ production from Figures 3A and 3B stratified according to cell dose received (low $\leq 10^6$/kg/dose, high $\geq 10^7$/kg/dose). P values shown for unpaired t-test. **D.** Differences in phenotype of NK cells at Day+28 post-transplant as determined by mass cytometry. Shown are surface markers on NK cells from Figure 1S that were significantly different between 2009-0266 (no NK cell infusions) and 2012-0708 (with NK cell infusions) using multiple unpaired t-test comparisons followed by the two-stage false discovery approach. Corrected p values are indicated as * (< 0.01), ** (< 0.001), *** (< 0.0001), or **** (< 0.00001). **E.** Heatmap with unsupervised clustering analysis of the NK cell phenotypes from Figure 1S according to relative expression of each receptor in each sample. Sample origin is indicated along the top row: Day +28 PB-NK samples from protocol 2009-0266 (red) or 2012-0708 (blue), or NK cell product (green). **F.** SPADE trees of NK cell subsets present in the NK cell product or peripheral blood at Day+28 post-transplant. Given the small number of samples in each group, KIR expression was excluded from clustering to avoid bias from individual KIR and HLA genotypes. **G.** Heatmap with unsupervised clustering analysis of the NK cell subsets from each sample according to those identified in Figure 3F (using only nodes constituting at least 1% of any one sample). Sample origin color coding are as in Fig 3E. **H.** Principle components analysis based on percent of NK cells in each node of each sample as in Figure 3G. X and Y axis show principal component 1 and principal component 2 that explain 31.9% and 24.1% of the total variance, respectively. Prediction ellipses indicate 95% probability that a new observation from the same group will fall inside the ellipse. N = 22 data points. **I.** ViSNE clustering analysis of NK cells according to sample origin, showing expression levels of surface markers that were visibly different between 2009-0266 and 2012-0708.

Figure 4. Comparison of clinical outcomes between patients treated on protocols 2012-0708 (with NK cells; blue line) and 2009-0266 (no NK cells; red line). Data from 2009-0266 were censored to include patients with only AML/MDS and CML in morphologic CR at the time of transplant. A. C. Kaplan-Meier survival curves were compared using the log-rank method.

Figure 5. Immunologic reconstitution of lymphocyte subsets in the first 6 months post-transplant for patients treated with and without NK cells. Immune subsets were determined by clinical flow cytometry. Absolute cell counts were calculated based on subset percentages and total white blood cell count obtained at the same time. WBC – total white blood cell count; CD3+ - total T cell numbers; CD56+CD3- - NK cells; CD3+CD4+ - total CD4 T cells; CD3+CD8+ - total CD8 T cells; CD25 – T regulatory cells; CD45RA – naïve T cells; CD45RO – memory T cells; CD19+ - B-cells; D30 – day 30 post-transplant; D90 – Day 90 post-transplant; Day 180 – Day 180 post-transplant; 2012-0708 – current phase 1 clinical trial; 2009-0266 – previous clinical trial without NK cells.

Figure 1

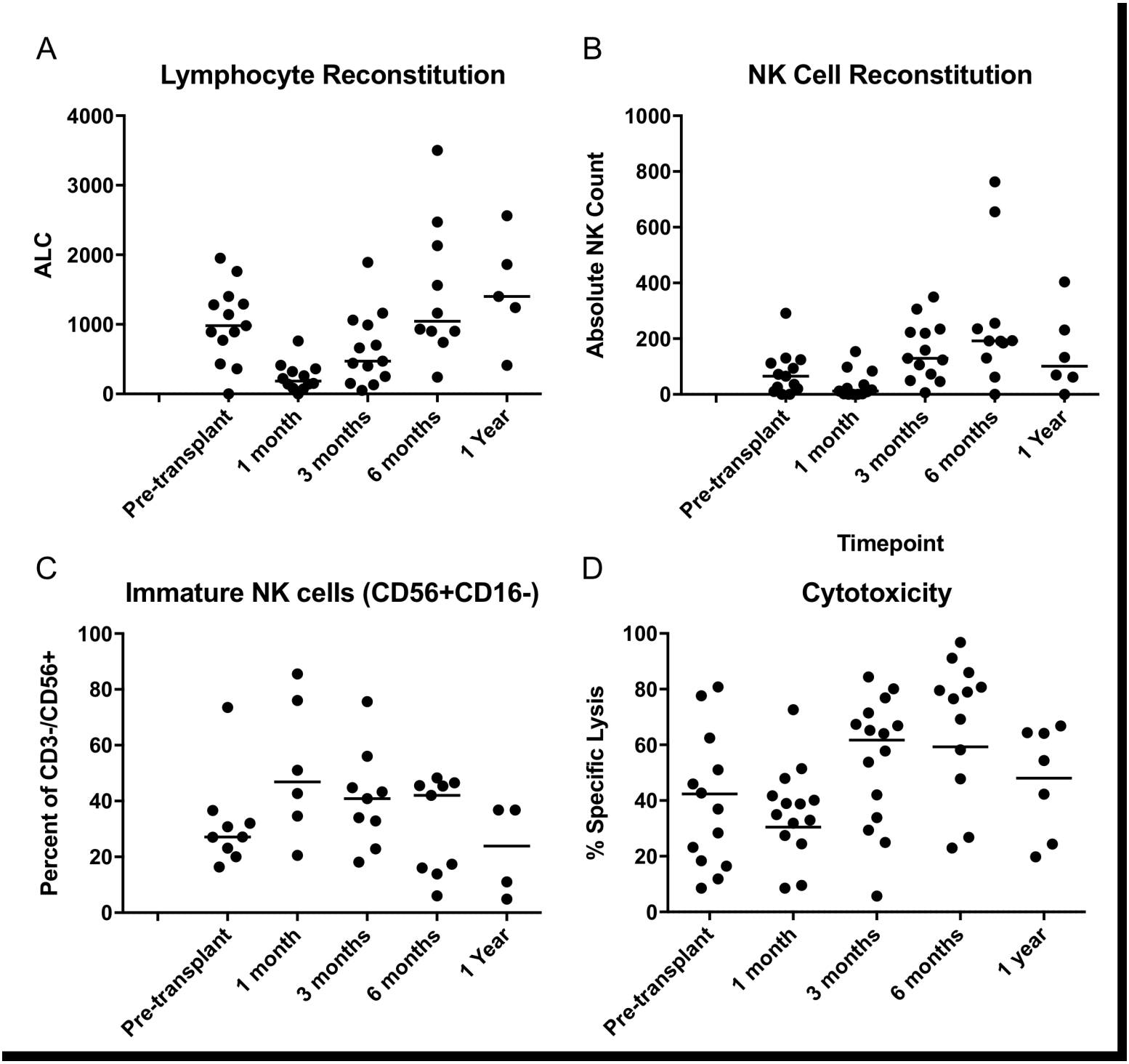
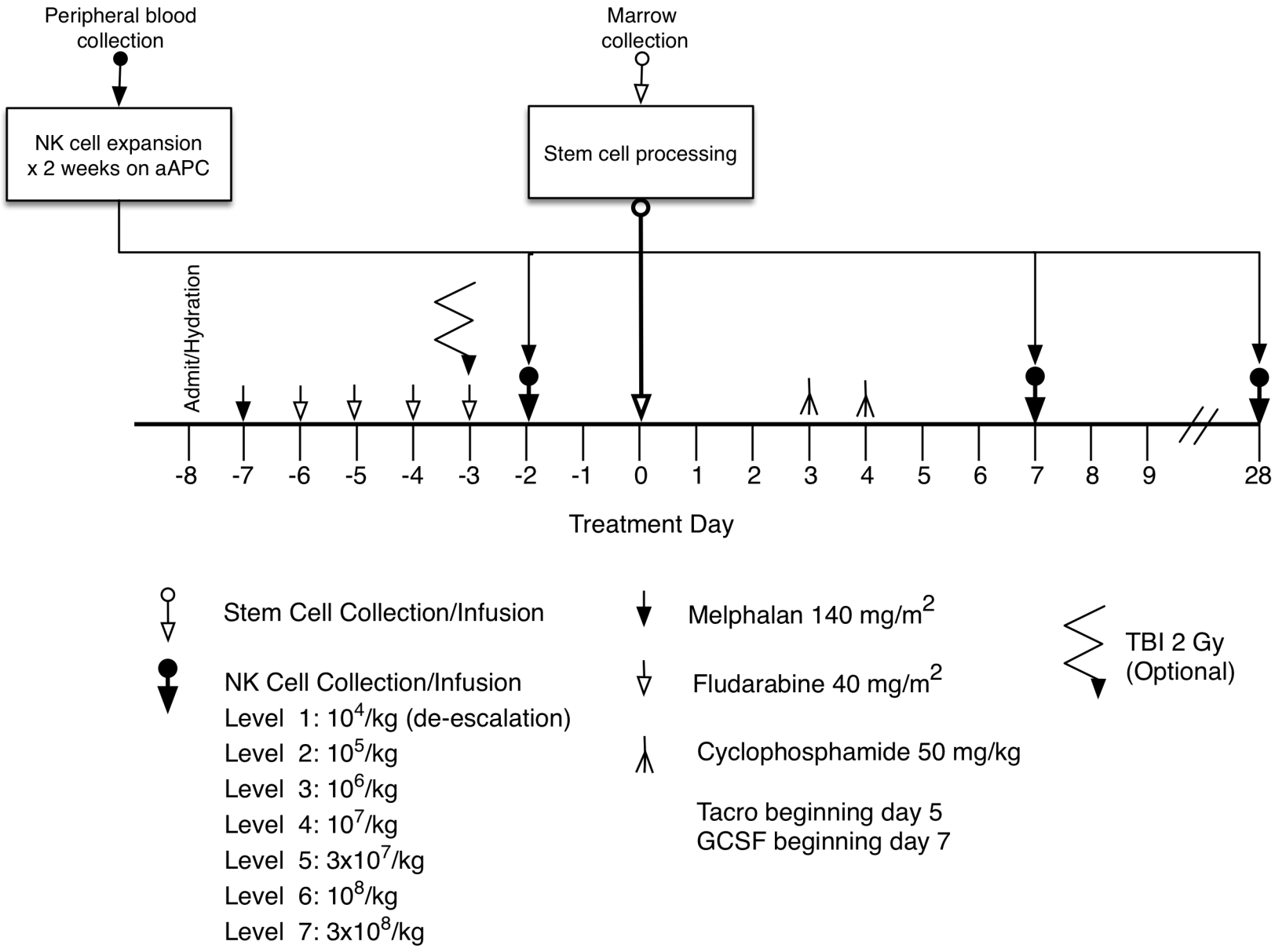


Figure 2



[redacted]

Figure 3

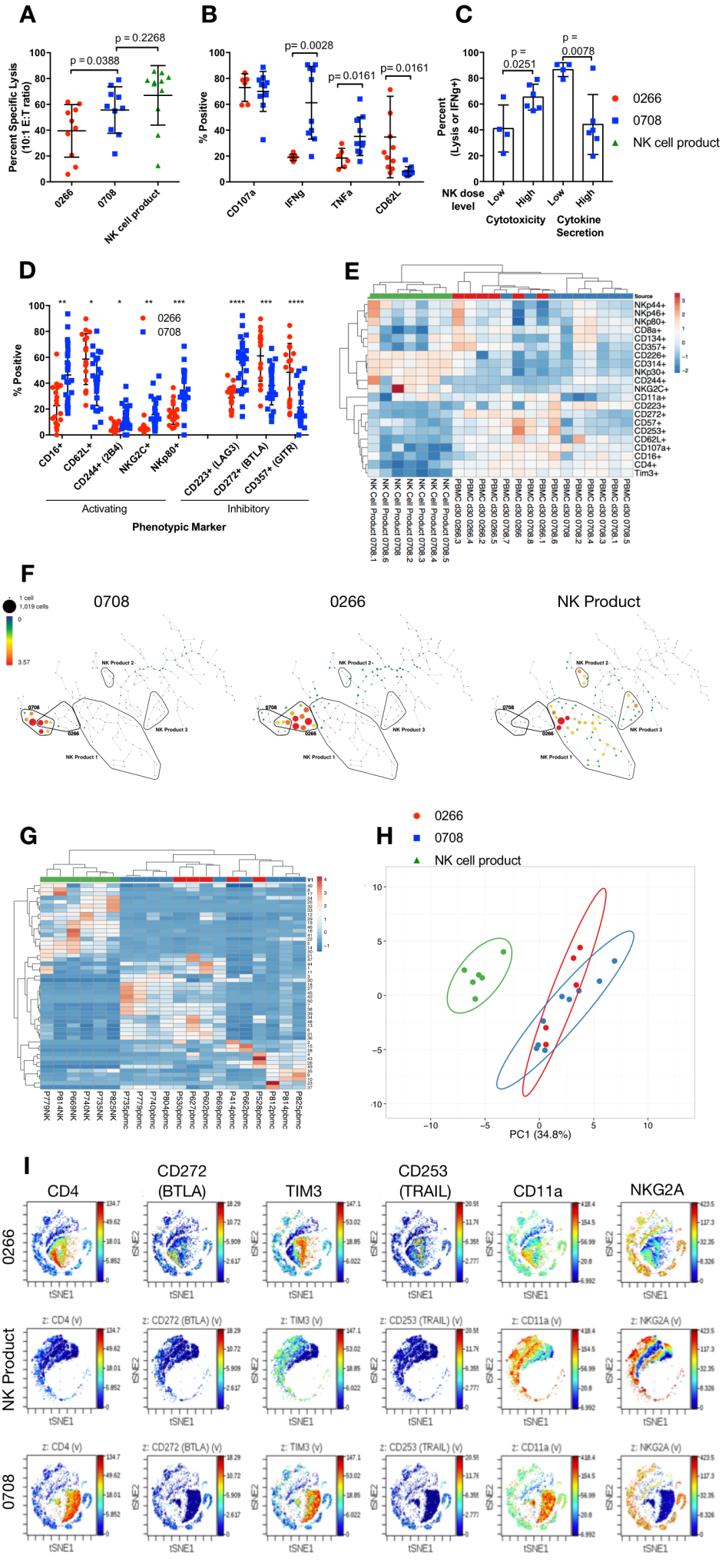


Figure 4

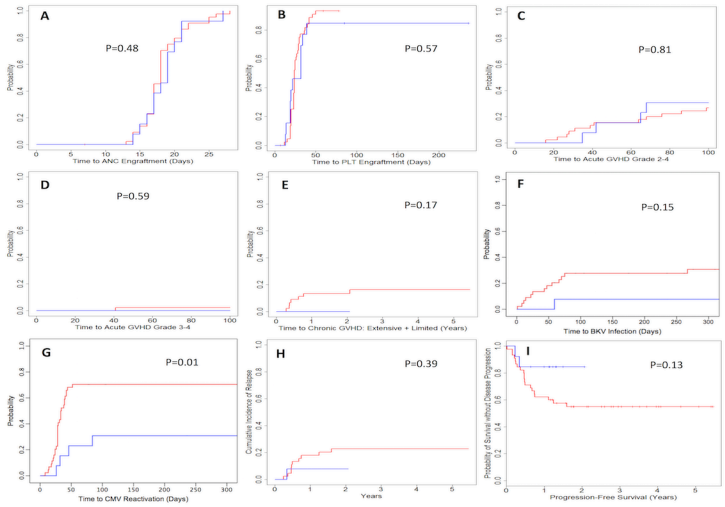
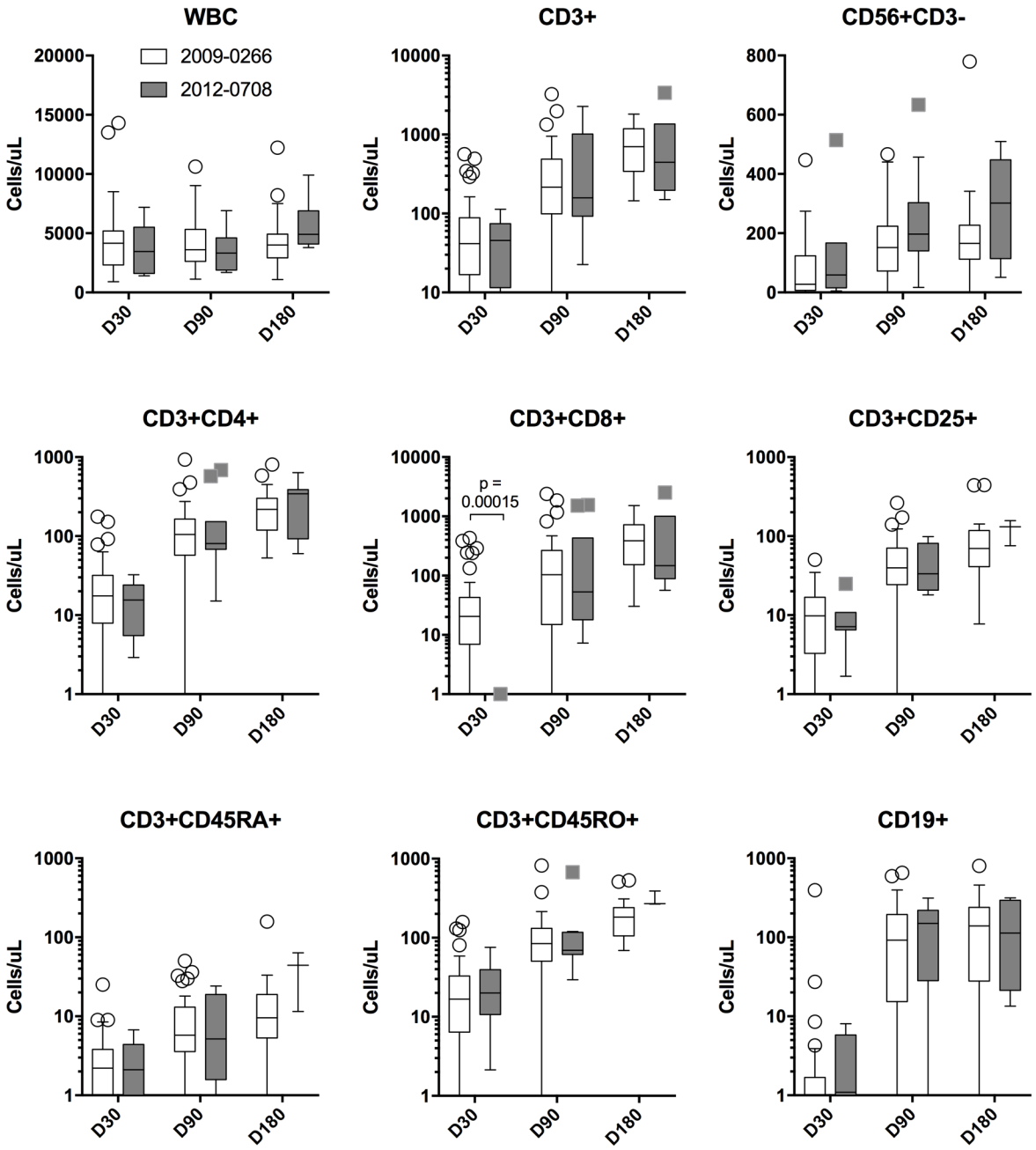


Figure 5



Prepublished online August 23, 2017;
doi:10.1182/blood-2017-05-785659

Phase 1 clinical trial using mbIL21 ex-vivo expanded donor-derived NK cells after haploidentical transplantation

Stefan O. Ciurea, Jolie R. Schafer, Roland Bassett, Cecele J. Denman, Kai Cao, Dana Willis, Gabriela Rondon, Julianne Chen, Doris Soebbing, Indreshpal Kaur, Alison Gulbis, Sairah Ahmed, Katayoun Rezvani, Elizabeth J. Shpall, Dean A. Lee and Richard E. Champlin

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.