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Haploidentical Natural Killer Cells Induce Remissions in Non-Hodgkin Lymphoma Patients With Low Levels of Immune-suppressor Cells

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Abstract

We report a novel phase 2 clinical trial in patients with poor prognosis refractory NHL testing the efficacy of haploidentical donor NK cell therapy (NK dose $0.5\text{--}3.27 \times 10^7$ NK cells/kg) with rituximab and IL-2. ([clinicaltrials.gov](https://clinicaltrials.gov/NCT01181258) NCT01181258) Therapy was tolerated without graft-versus-host-disease, cytokine release syndrome, or neurotoxicity. Of 15 evaluable patients, 4 had objective responses (26.6%) at 2 months: 2 had complete response lasting 3 and 9 months. Circulating donor NK cells persisted for at least 7 days after infusion at the level between 0.6–16 cells/ μ L. Responding patients had lower levels of circulating host derived Tregs (17 ± 4 vs. 307 ± 152 cells/ μ L; $p=0.008$) and myeloid derived suppressor cells (MDSC) at baseline ($6.6\% \pm 1.4\%$ vs. $13.0\% \pm 2.7\%$; $p=0.06$) than non-responding patients. Lower circulating Tregs correlated with low serum levels of IL-10 ($R^2=0.64$; $p<0.003$; $n=11$), suggestive of an immunosuppressive milieu. Low expression of PD-1 on recipients T cells before therapy was associated with response. Endogenous IL-15 levels were higher in responders than non-responding patients at the day of NK cell infusion (mean $\pm$ SEM: 30.0 ± 4.0 ; $n=4$ vs 19.0 ± 4.0 pg/ml; $n=8$; $p=0.02$) and correlated with NK cytotoxicity at day 14 as measured by expression of CD107a ($R^2=0.74$; $p=0.0009$; $n=12$). In summary, our observations support development of donor NK cellular therapies for advanced NHL as a strategy to overcome chemoresistance. Therapeutic efficacy may be further improved through disruption of the immunosuppressive environment and infusion of exogenous IL-15.

Keywords

NK cells; adoptive transfer; cellular therapy; non-Hodgkin Lymphoma; IL-2; refractory lymphoma

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Introduction

Patients with chemotherapy refractory non-Hodgkin lymphoma (NHL) have poor prognosis. [1] Recent advances in cellular therapies and immunotherapies suggest that targeting tumor with an activated immune system overcomes chemo-resistance, and early clinical experience with these approaches has yielded remarkable therapeutic success.[2] Evidence suggests that the immunosuppressive environment surrounding cancer cells contributes to the poor efficacy of therapeutic antibodies and chemotherapy.[3] We hypothesized that natural killer (NK) cells in patients with refractory NHL exhibit poor function with impaired capacity to mediate direct cytotoxic and antibody-mediated killing.[4, 5] NK cells are innate effectors, residing in lymphoid tissues, spleen and peripheral blood at low frequency (5–10% of lymphocytes).

Immunophenotypically defined by expression of CD56 and the lack of CD3, NK cells express a variable combination of activating and inhibitory receptors that bind cognate HLA and HLA-independent ligands and exhibit killing of viral-infected or malignant transformed cells with downregulated self-class I MHC.[6] Several known triggers engage NK cells and transform them into powerful cellular killers including such cytokines as IL-2 or IL-15 and antibodies. The most powerful NK cell activating signal is triggered by antibody Fc binding CD16 receptor, which mediates robust target-cell killing with antigen specificity called antibody-dependent cell-mediated cytotoxicity (ADCC).[7] NK cell killing is either direct (measured by CD107a expression after exposure to target) or mediated by secretion of cytokines such as interferon gamma (IFN- γ) and tumor-necrosis factor alpha (TNF α). We hypothesized that infusion of activated allogeneic donor-derived NK cells combined with CD20-targeting monoclonal antibody rituximab and IL-2 would result in anti-tumor responses against lymphoma expressing CD20 and overcome chemotherapy resistance. We report the results of a phase 2 clinical trial testing the efficacy of haploidentical donor NK cell infusions plus rituximab for refractory NHL.

Patients and Methods

Eligibility criteria and treatment

We conducted a prospective clinical trial approved by the Institutional Review Board of the University of Minnesota (clinicaltrials.gov NCT01181258). Eligible patients had multiply relapsed or refractory NHL or chronic lymphocytic leukemia expressing CD20, Karnofsky performance status (KPS) 80%, and adequate organ function. All patients were evaluated by positron emission tomography (PET) within 4 weeks of treatment and at 2 months post-NK cell infusion. Treatment consisted of lymphodepleting chemotherapy pentostatin 3.75mg $\times$ 2 days, (n=6), cyclophosphamide (60mg/kg IV day -5; n=6) and IL-2 receptor-targeting agent to deplete regulatory T cells (Treg) denileukin diftitox (18 μ g/kg/day IV day -1; n=5). We prospectively planned to increase the dose of denileukin diftitox to 18 μ g/kg/day IV $\times$ 3 days (day -4,-3,-2) for lack of Treg depletion (defined as no increase in Treg at day 14 compared to pre-therapy) in first 5 patients. After we enrolled 1 more patient, denileukin was withdrawn from the market (and our study) by the manufacturer (Eisai). We subsequently amended the protocol and replaced the lymphodepleting therapy with fludarabine 25mg/m² $\times$ 4 days (n=10), cyclophosphamide (60mg/kg IV day -5; n=10) and

methylprednisolone (1 mg/kg day -2 through day +9; n=10) with aim to enhance NK cell function. (Supplemental Figure 1A). All patients received rituximab 375mg/m² IV weekly × 4 doses starting day -8. The NK-enriched cellular product was infused intravenously on day 0 followed by subcutaneous infusion of IL-2 at 9 million IU every other day × 6 doses.

Cellular product and NK cell evaluation

Donor selection criteria included HLA-haploidentical related donor aged 12–75 years who is able and willing to undergo leukapheresis. We preferred younger donors if multiple were available. HLA-haploidentical related donors underwent 5-hour lymphapheresis with COBE Spectra Apheresis System (TerumoBCT, Lakewood, CO). Apheresis product was depleted of T cells (CD3) and B-cells (CD19) using the Miltenyi Biotec CliniMACS® Cell Selection System and CD3 and CD19 MicroBeads and reagent (Miltenyi Biotec, Auburn, CA) and cultured in serum-free media (X-VIVO 15, Cambrex BioScience, Walkersville, MD) supplemented with 10% human AB serum and 1000 U/mL IL-2 (Chiron Corporation, Emeryville, CA).[8] After 8–16 hours of incubation, cells were washed twice with a 5% human serum albumin. An aliquot of the cell product was analyzed by flow cytometry to determine the number of T, B, and NK cells, and NK cell phenotype (fluorochrome-conjugated antibodies detailed in Supplementary Table 1). The planned target NK cells dose to be infused was between 1.5–8×10⁷/kg. Lot release criteria for allogeneic IL-2 activated NK cells product (BB-IND 8847) included viability >70%, NK Cell (CD56+CD3-) 20%, T-cell content 3.0 ×10⁵/kg, B-cell content <3%, Endotoxin <5EU/kg and no organism by Gram stain. If product contained less than 1.5 ×10⁷/kg or <20% NK cells, then all cells were given but NK expansion outcomes was reported separately. In vivo circulating peripheral blood (PB) donor derived NK cells were examined in fresh sampled in all evaluable patients and enumerated as absolute lymphocyte count/μL × % CD56⁺/CD3⁻ NK cells (in lymphocyte gate) × % whole blood donor chimerism using standard very short tandem repeat (VNTR) testing from the whole blood. In recipient-donor pairs differentially expressing HLA-A2 or HLA-A24 antigens, we performed peripheral blood (PB) flow cytometry analyzing donor-specific HLA alleles to quantify the donor NK cells. For each recipient, we determined PRA (Panel Reactive Antibody) by Flow/Luminex assay: flow bead immunoassays utilized purified antigen coated on latex beads as targets for binding of specific antibody in human serum. PRA was correlated with the level of donor NK cell expansion and clinical response.

Sample collection for correlative studies

We collected PB at baseline, after chemotherapy, and on days 3, 7, 14, and 28. We processed mononuclear cells (PBMC) by Ficoll method and stored cells in liquid nitrogen and serum at -80°C. For comparison, we also obtained PB from healthy volunteers and newly diagnosed patients with NHL prior to receiving any therapy. All volunteer subjects and patients signed an IRB-approved consent for study of their PB samples.

Flow cytometry analysis and ELISA

Cryopreserved PBMCs were rested overnight in RPMA medium to recover from freezing and then cultured for 6 hours in the presence of target cells (K562) and IL-12 (5 ng/ml) and IL-18 (50 ng/ml) prior to staining. Cells were stained with fluorochrome-conjugated

antibodies detailed in Supplementary Table 1. Detection of CD107a (degranulation), Ki67 (proliferation), and IFN- γ production was performed after fixation and permeabilization (eBioscience) according to the manufacturer's instructions. Tregs were defined by the expression of Foxp3 and elevated CD25. Myeloid derived suppressor cells were defined as CD33⁺CD14⁺HLA-DR^{-/Low}. All cells were acquired by LSRII and analyzed by FlowJo 10.0. Plasma levels of IL-2, IL-15, IL-10, IL-2R were performed on Luminex-platform (EMD Millipore, Billerica, MA), and IL-6 were determined by commercial enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Correlative studies were performed on cryopreserved samples for 12 patients with stored full-paired sample set (two patients had missing samples).

Study definitions and statistical methods

The primary objective of the prospective trial was overall response rate at 2 months. Secondary objectives were safety and tolerability of this therapy and in vivo expansion of allogeneic NK cells. All patients were evaluated by PET/CT and bone marrow biopsy (if prior marrow involvement) per recent criteria for NHL. [9] Trial size calculation was based on Simon method using a minimax two-stage design with early stopping rule if less than 20% of patients achieve an objective response. Cell frequencies were summarized using descriptive statistics. Statistical analysis was performed using a paired or unpaired Student t test and Pearson correlation (Graphpad Prism, version 6; ElCamino, CA). A P value <0.05 was considered statistically significant. Representative histograms or images were chosen based on average values.

Results

Patient, disease and product characteristics

Sixteen patients were enrolled in the study (Table 1) between 10/19/2010 – 7/9/2015. Patients had a median age of 52 years (range 48–67), and 13 were males. Diffuse large B cell lymphoma was the most common diagnosis (n=11; 5 transformed and 6 de-novo). All patients were rituximab and chemotherapy refractory and failed at least 3 prior therapies. Most patients had B-symptoms and extranodal disease at the time of treatment. The cellular product was infused with a median total nuclear cell (TNC) dose of $4.3 \times 10^7/\text{kg}$ (range 1.9–7.7) and contained 40.2% NK cells (median; range 12–88%) and 48% monocytes (median, range: 12–63%). The final product characteristics are detailed in Table 1. Clinical product NK cell characteristics included high expression of CD16 (median 86%) and high cytotoxicity against K562 targets. Final product NK cells produced higher IFN γ , and had higher proliferation compared to before activation (Supplementary Figure 2A). Expression of NKp44, NKp46, CD69, TIGIT and PD1 on the activated NK product and NK cell inhibitory receptor expression are detailed in Supplementary Figure 2 and Supplementary Table 2.

Clinical response assessment

Clinical outcomes are summarized in Table 3. Fifteen patients were evaluable and 14 had clinical response assessment at day 28. Four responded (26.6%) including two complete responses and two partial responses at 2 months. Three responding patients with extensive

bulky disease at the time of enrollment had robust tumor regressions (80%). One patient developed tumor lysis syndrome and required rasburicase. Three responding patients proceeded to allogeneic donor transplantation in sustained remission at 2, 3, and 9 months after NK cell therapy. There was no correlation between the clinical response and infused cell dose between responders (NK cell dose $1.6 \times 10^7/\text{kg}$; T cell dose 4.5×10^4) and non-responders (NK cell dose $2.1 \times 10^7/\text{kg}$; T cell dose 4.8×10^4). Notably one of the responding patients received low NK cells dose ($1.15 \times 10^7/\text{kg}$, less than the planned target cell dose).

We observed expected hematologic toxicity (NCI CTCAE grade 1–3 thrombocytopenia, anemia and neutropenia) in all subjects. Therapy was associated with NCI CTCAE non-hematologic grade 3 adverse effects (tumor lysis syndrome, infection due *Pneumocystis*; *Clostridium difficile* colitis, neutropenic fever, thrombotic microangiopathy related to pentostatin). Two patients (out of 16) died before disease response assessment; one developed sepsis prior to NK cell infusion, and the second died of overwhelming culture-negative sepsis 10 days after NK cell infusion. IL-6 levels at day 7 in all 15 patients varied from 3.99 to 21.5 pg/ml; these levels did not suggest cytokine release syndrome. We observed no symptoms or signs of graft-versus-host-disease, neurotoxicity, or persistent marrow aplasia.

NK cells in patients with refractory NHL are hypofunctional

As compared to healthy controls, NK cells in patients with newly diagnosed NHL (not enrolled to this trial) and chemorefractory NHL (all enrolled to this trial) exhibited poor degranulation capacity (CD107a) and impaired production of pro-apoptotic cytokines IFN γ (Figure 1A). Moreover, the expression of CD16, which mediates ADCC, was lowest in patients with refractory NHL (Figure 1A). NK cells from patients with NHL expressed higher levels of the suppressive receptor “T-cell immunoreceptor with Ig and ITIM domains” (TIGIT) and lower levels of the T cell immunoglobulin mucin-3 (TIM-3) receptor with activating function on NK cells compared to healthy controls (Figure 1B). [10–12] NK cell expression of program-cell death 1 receptor (PD1) was below 2 % in patients and controls.

Transient donor NK cell persistence and function correlated with endogenous IL-15

We determined the frequency of circulating donor NK cells at defined time-points by two methods designed to differentiate recipient vs. donor by interrogating DNA (VNTR) or HLA expression (flow cytometry). Donor NK cells persisted in the PB for at least a week after infusion (in 7 of 7 tested patients with available antibodies against donor-specific HLA alleles) ranging between 0.6–16 cells/ μl (median 3 cells/ μl), and 0.35–90% of PB NK cells exhibited a donor-specific HLA phenotype (Figure 2A). We separately analyzed 3 patients infused with $<1.5 \times 10^7$ TNC/kg (outside of lot release criteria goal) and determined that all three had evidence of donor cells by HLA-specific immunophenotype (Patients 3,5,7). Donor derived NK cells exhibited higher CD16 expression compared with patients’ NK cell CD16 (mean MFI $\pm$ SEM: 9900 ± 1460 vs. 3590 ± 740 , $p=0.002$, Figure 2B). Whole blood VNTR detected donor DNA in three patients out of 14 tested samples at day 7 at levels 2%, 8%, 18% donor chimerism, suggesting lower sensitivity as compared to HLA allele-directed flow cytometry. In one responding patient, donor NK cells were sustained beyond day 28

(3% on DNA chimerism by VNTR). Although not significant, NK cells proliferated more vigorously in responders as compared to non-responders (day 14: Ki67 mean $\pm$ SEM: 45.1 $\pm$ 7.0, n=4 vs. 28.5 $\pm$ 4.6, n=10; p=0.07; Figure 3A); yet, PB NK cell function as measured by CD107, IFN γ , and TNF α was similar in responders and non-responders at baseline and after treatment (Supplemental Figure 3). Endogenous IL-15 serum levels peaked at the time of NK cell infusion (range 11–33pg/dl). Responders had higher IL-15 levels as compared to non-responders at day 0 (mean $\pm$ SEM: 30.0 $\pm$ 4.0 (n=4) vs 19.0 $\pm$ 4.0 pg/ml (n=8); p=0.02) but not day 14 post chemotherapy (10.5 $\pm$ 6.0 (n=4) vs 4 $\pm$ 2 pg/ml (n=8; p=NS) (Figure 3B). Serum IL-15 levels correlated with functional NK cell killing at day 14 as measured by expression of CD107a ($R^2=0.74$; p=0.0009; n=12, Figure 3C), suggesting that higher endogenous IL-15 improved NK cell cytotoxic capacity. We next examined the effect of pre-existing recipient anti-HLA antibodies on donor NK cells persistence. Fifteen recipients were tested at baseline and 2 patients had high level donor HLA allele-specific antibodies. Both patients with high titer PRA exhibited donor NK cell expansion at day 7 (10 and 6 NK cells/ μ L) and one was a responder, suggesting lack of an adverse effect of PRA on NK cell persistence and clinical outcomes.

High expression of PD-1 and TIGIT correlates with lack of response

We next examined T cells from the NHL patients and random healthy donors for expression of key inhibitory checkpoints: program-cell death receptor 1 (PD-1) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) at baseline and at day 14 post-therapy. Responding patients had similar levels of PD-1 expression to healthy controls at baseline and day 14; however, non-responders exhibited significantly higher levels of PD-1 on T cells before and particularly after therapy. Similarly, T cell TIGIT expression was higher in non-responders at both time points (Figure 4A and 4B). Separating PB T cells into CD3⁺CD4⁺ and CD3⁺CD8⁺, we observed a higher pattern of PD-1 and TIGIT expression on CD3⁺CD4⁺ cells and CD8⁺ T effectors in non-responders as compared to responders (Figure 4B).

Low Treg and myeloid suppressor cell levels correlate with response

We next examined the impact of immune suppressor cells on NK cell expansion and clinical response. Responding patients (n=4) had lower levels of absolute number of Treg at baseline (mean $\pm$ SEM: 17 $\pm$ 4 vs. 307 $\pm$ 152 cells/ μ L; p=0.008) and 14 days after therapy (53 $\pm$ 21 vs. 273 $\pm$ 63 cells/ μ L; p=0.02) as compared to non-responders (n=8) (Figure 5A). Treatment with denileukin diftitox did not result in change in PB Treg frequency (day 14 Treg with denileukin mean $\pm$ SEM: 240 $\pm$ 56 cells/ μ L vs. no denileukin 160 $\pm$ 90) and had no effect on *in vivo* NK cell expansion (data not shown). High PB Treg levels correlated with serum IL-10 ($R^2=0.7$; p<0.001; n=12) and IL-2 receptor- α (IL-2R α $R^2=0.4$; p=0.006; n=12), suggestive of an accentuated immuno-suppressive milieu. Although not statistically significant, frequencies of PB myeloid derived suppressor cells (MDSC) were low in responders and higher in non-responders at baseline (mean $\pm$ SEM: 6.6% $\pm$ 1.4% vs. 13% $\pm$ 2.7%) and after therapy (day 14 mean $\pm$ SEM: 4.8% $\pm$ 0.7%; vs. 10.0% $\pm$ 2.0%; Figure 5B). Notably, low levels of circulating Tregs and MDSCs correlated with NK cell proliferation (n=12, $R^2=0.25$; p=0.035 and $R^2=0.5$; p=0.002; Figure 5C,D).

Discussion

Our clinical experience using haploidentical NK cells with IL-2 and rituximab suggest that this therapy is well tolerated and produces remission in over 1/4th of highly refractory NHL patients. We showed a transient persistence of donor NK cells in most subjects and improved sensitivity of donor NK detection by flow cytometry for donor-specific DNA as compared to PCR techniques. Our data also show that autologous NK cells in refractory NHL patients exhibited poor function, express lower CD16, higher levels of the immunosuppressive receptor TIGIT and lower expression of activating receptor TIM3 as compared to NK cells from healthy controls. These findings suggest several potential mechanisms of immunotherapy resistance in patients with advanced disease. Monoclonal antibodies are often used to focus autologous NK cells to have tumor specificity, however CD16 downregulation can render antibodies less effective. We showed that transient homeostatic expansion of highly functional CD16 expressing donor NK cells may be clinically effective in some refractory NHL patients.

While prior data demonstrated that the tumor microenvironment plays an important role in disease severity and clinical outcomes in B-cell NHL, most studies examined the composition of intratumoral T cells, whereas here, we probed the blood compartment.[13–15] T cell exhaustion is a status of T cell immune response induced by viral infection or tumor which results in reduced function and proliferation.[14] Our findings suggest that refractory NHL patients have a highly suppressive immune environment characterized by increased expression of PD-1 and TIGIT on circulating T-cells. In contrast, low baseline expression of PD-1 and TIGIT on CD8 cells and lower Tregs in the blood compartment were associated with improved clinical responses to adoptive NK cell transfer. Together, these findings highlight the role of the immunosuppressive milieu in modulating an anti-tumor response. TIGIT is a novel key inhibitory checkpoint protein expressed on both NK and T cells with wide variation in its expression among individuals.[16, 17] Non-responding patients had elevated expression of TIGIT on T cells, which was even further upregulated in T cells and NK cells in patients with higher levels of IL-15. These data are consistent with recent *in vitro* reports that the blockade of the TIGIT pathway can increase NK cell IFN γ secretion and abrogate MDSC-mediated inhibition.[18] Our findings suggest that blockade of checkpoint proteins could enhance efficacy of NK cell adoptive therapies and potentially elicit higher clinical response rates in refractory NHL.[4]

In this clinical trial, we showed no benefit of denileukin on Treg depletion, NK cell persistence or clinical response. Notably, three responders received methylprednisone as part of their therapy; suggesting that the effect of steroids in adoptive NK cell therapy may not be detrimental. Our prior experience showed lack of consistent Treg depletion by denileukin [19]; yet, we observed significantly improved response rates in refractory AML, which did not occur in our NHL cohort. Given the relatively small number of treated patients with denileukin in this trial (due to withdrawal of denileukin from the market), our study was not powered to compare the two therapeutic approaches.

In terms of the product characteristics, NK cell enriched product contained variable fraction of other cells, mostly monocytes that likely contributed to *in vivo* cytokine production such

as IL-15, IL-18, IL-6, TNF, and IL-10 [20, 21]. Our prior data suggested that using CD56 positively selected NK cell products produced lower remission rate in AML compared to CD19/CD3 depleted products.[19]. Many pre-clinical and clinical investigations continue to study the impact of NK cell dose, purity and expansion on persistence and clinical outcomes.[22–27] The size of this trial limited the statistical power to examine the effect of KIR genotype or KIR ligand mismatch on clinical outcomes.

Taken together, our observations allowed identification of host factors that may have interfered with response to adoptive NK cell infusion. We showed that high frequencies of MDSCs were associated with lack of clinical response. These data are concordant with pre-clinical results that showed that MDSCs mediate potent inhibition of conventional NK cells. [28, 29] In addition, increased numbers of MDSC has been detected in patients with diffuse large B cell lymphoma at diagnosis and correlated with worse outcomes.[30, 31] Furthermore tumor infiltrating MDSC have been recognized to recruit Treg cells and be permissive of further tumor growth.[32] In our study, circulating Treg appears to limit NK cell proliferation. We suggest that MDSCs and Treg should be studied in future trials as cellular biomarkers of resistance to adoptive NK cell therapy. These findings also highlight the possibility of targeting MDSCs to enhance the efficacy of cellular therapies.[33]

In this study we also confirmed the importance of endogenous cytokines released after lymphodepleting chemotherapy. Levels of IL-15 in PB were almost 2-fold higher in patients who experienced clinical response. This finding supports the development of therapeutic cytokines, such as recombinant human IL-15 (NCI) or IL-15 superagonist ALT803 (Altor), in combination with NK cells to further improve clinical activity. Pilot clinical studies with NK cell therapies are underway (NCT03050216).

Our observation supports development of donor NK cellular therapies for advanced NHL as a treatment strategy. NK cell expansion and therapeutic efficacy might be further improved by checkpoint inhibitors, MDSC-depleting strategies, and IL-15 administration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

Our findings suggest that refractory NHL patients have poorly functioning NK cells and a highly suppressive immune environment that may blunt an anti-tumor response of donor NK cell infusions. In addition, low levels of regulatory T cells and myeloid derived suppressor cells were associated with improved clinical responses to adoptively transferred donor NK cells. Higher levels of PD-1 on T cells before and after therapy were associated with lack of response. We observed short-term persistence of haploidentical NK cells and 28% overall response rate in patients with refractory lymphoma.

Figure 1. NK cell phenotype and function in patients with relapsed/refractory lymphoma as compared to newly diagnosed NHL patients and healthy controls

A) PBMC from NHL patients (n=14) and healthy controls (n=8) were rested overnight in medium and stimulated with target cells (K562, 20:1) and cytokines 6 hours prior to staining. NK cell degranulation (CD107a), IFN γ production, and frequency of CD16-expressing NK cells were evaluated by flow cytometry. **B)** Overnight rested PBMCs from newly diagnosed (n=4) or refractory NHL (8–14) and healthy controls (n=6–7) were stained for TIGIT and TIM3 and evaluated by flow cytometry. Data are shown as mean $\pm$ SEM, and statistical analyses were done on pooled data using the Student's unpaired t test. Each symbol represents an individual donor. New NHL= newly diagnosed NHL.

Figure 2. Donor NK cells persistence

Donor NK cell expansion in peripheral blood at day 7 depicted in histograms showing donor vs recipient HLA gated on CD56⁺CD3⁻ cells. All 7 subjects with differentiating donor/recipient HLA antigens (A2 or A24) are shown. Patients 1,2 and 3 were responders; patients 4,5 and 6 were non-responders and patient 7 died from neutropenic sepsis at day 10 after NK cell infusion. Absolute lymphocyte cell (ALC) in cells/ μ L and whole blood standard very short tandem repeat (WB VNTR) at day 7 after NK cell infusion is shown for each patient.

Figure 3. Correlation of response with endogenous IL-15

A) PBMC from NHL patients (n=14) and healthy controls (n=7) were rested overnight in medium and evaluated for proliferation (Ki67) by flow cytometry. **B)** Endogenous IL-15 serum levels were evaluated at different time points in NHL patients (n=12) comparing responders and non-responders. Data are shown as mean±SEM, and statistical analyses were done on pooled data using the Student's unpaired t test. Each symbol represents an individual donor. **C)** Correlation analyses (n=12) evaluating the relationship between NK cell degranulation and endogenous IL-15 level after therapy (day 14).

Figure 4. Expression of suppressive receptors on circulating T cells

A) PBMC from NHL patients were rested overnight in medium and then staining for PD-1 and TIGIT and evaluated by flow cytometry. Representative histograms showing PD-1 and TIGIT expression in bulk T cells (CD3) in responders vs. non-responders before (upper panel) and 14 days after treatment (lower panel). **B)** Different T cell subsets (CD4, and CD8) were evaluated for PD-1 and TIGIT expression in responders (R) and non-responders (NR). Data are shown as mean $\pm$ SEM, and statistical analyses were done on pooled data using the Student's unpaired t test. Each symbol represents an individual donor. **C)** Correlation analyses evaluating the relationship between TIGIT expression in NK cells and T cells and IL-15 serum level in NHL patients 14 days after treatment. Statistical analyses were done using Pearson correlation.

Figure 5. Circulating MDSC and regulatory T cell correlate with clinical response and NK cell proliferation

Circulating regulatory T cells and MDSC in NHL patients before and after therapy comparing responders ($n=4$) and non-responders ($n=8-10$). **A, B**) PBMCs from NHL patients were rested overnight and stained, and then the frequencies of MDSCs and Tregs were determined by flow cytometry. Each symbol represents an individual donor. **C, D**) Correlation analyses ($n=12$) evaluating the relationship between NK cell proliferation and the numbers and frequency of Tregs and MDSCs in patients with NHL before and 14 days after treatment. Statistical analyses were done using Pearson correlation.

Table 1

Patient, disease, and treatment characteristics (Included are all patients by intention to treat)

	N=16
Age median (range)	52 (48–67 yrs)
Gender : male/female	13/3
Diagnosis	
Diffuse large B cell lymphoma de novo	6
Diffuse large B cell lymphoma transformed	5
Mantle cell lymphoma	2
Follicular lymphoma	1
Lymphoplasmacytic lymphoma	1
Chronic lymphocytic leukemia with 17p del	1
Disease status	
Refractory with prior remission	5
Primary refractory	11
Disease Stage	
I–II	3
III–IV	13
B-symptoms present	10
Extra-nodal disease present	12
Bone Marrow involved	8
Prior therapy	
>3 prior lines of therapy	12
Prior radiation therapy	4
Prior autologous HCT	2
Donor	
Haploidentical child	10
Haploidentical sibling	5
Lymphodepleting chemotherapy	
Rituximab/Cy/Pentostatin/denileukin difitox	6
Rituximab/Cy/Fludarabine/MehtylPND	10
TNC/kg apheresis (median; range)	3.2×10 ⁸ (1.15–4.45)
Infused IL-2 activated product TNC dose/kg (Median; range)	4.3×10 ⁷ (1.9–7.7)
Infused NK cells/kg (Median; range)	1.9×10 ⁷ (0.5–3.27)
Infused NK % (Median; range)	40.2% (12–88%)
Infused monocytes % (Median; range)	
Infused CD3 cell dose median (range)	4.8×10 ⁴ (0.19–18)
Infused CD3% (Median; range)	0.13% (0.02–0.33%)

Abbreviation: Cy Cyclophosphamide, TNC total nucleated cell, NK natural killer, HCT hematopoietic cell transplantation, f/u follow-up

Table 2

Clinical response evaluation (14 subjects evaluable for response)

Overall response at 2 months	4 (28%)
Complete response	2
Partial response	2
Progressive disease	10
Subsequent allogeneic HCT	3 (2,3,8 months post NK therapy)
NK cell detected in PB day 7 post infusion	
Flow cytometry assay for donor HLA	7/7 (positive/tested)
VNTR assay for donor DNA chimerism	3/14 (positive/tested)
Donor PB NK cell present at day 7 in responders	75% (3 out of 4)

Abbreviation: HLA human leukocyte antigen, HCT hematopoietic cell transplantation, NK natural killer cells PB peripheral blood