Phase I Trial of Expanded, Activated Autologous NK-cell Infusions with Trastuzumab in Patients with HER2-positive Cancers

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ABSTRACT

Purpose: Natural killer (NK) cells exert antibody-dependent cell cytotoxicity (ADCC). We infused expanded, activated autologous NK cells to potentiate trastuzumab-mediated ADCC in patients with HER2-positive malignancies.

Patients and Methods: In a phase I trial, patients with treatment-refractory HER2-positive solid tumors received trastuzumab, with or without bevacizumab, and autologous NK cells expanded by 10-day coculture with K562-mb15-41BBL cells. Primary objectives included safety and recommended phase II dose determination; secondary objectives included monitoring NK-cell activity and RECIST antitumor efficacy.

Results: In 60 cultures with cells from 31 subjects, median NK-cell expansion from peripheral blood was 340-fold (range, 91–603). NK cells expressed high levels of CD16, the mediator of ADCC, and exerted powerful killing of trastuzumab-targeted cells. In the 22 subjects enrolled in phase I dose escalation, trastuzumab plus NK cells were well tolerated; MTD was not reached. Phase IB ($n = 9$) included multiple cycles of NK cells (1×10^7/kg) and addition of bevacizumab. Although no objective response was observed, 6 of 19 subjects who received at least 1×10^7/kg NK cells at cycle 1 had stable disease for ≥6 months (median, 8.8 months; range 6.0–12.0). One patient, the only one with the high-affinity F158V CD16 variant, had a partial response. Peripheral blood NK cells progressively downregulated CD16 postinfusion; paired tumor biopsies showed increased NK cells, lymphocytic infiltrates, and apoptosis posttreatment.

Conclusions: NK-cell therapy in combination with trastuzumab was well tolerated, with target engagement and preliminary antitumor activity, supporting continued assessment of this approach in phase II trials.

Introduction

Targeting HER2 in combination with chemotherapy is considered standard-of-care for the treatment of advanced breast and gastric cancers overexpressing HER2 (1, 2). Besides trastuzumab, several anti-HER2 agents have been approved in recent years, with others at various stages of clinical development. While there have been significant advancements in anti-HER2 therapy, treatment options showing consistent survival benefit beyond third-line therapy are lacking (3). Therefore, it is important to explore novel approaches that might potentially bypass mechanisms of tumor resistance and, hence, can augment current therapies.

The main mechanism of action of anti-HER2 agents involves inhibition of the HER2 signaling pathway, but activation of antibody-dependent cell cytotoxicity (ADCC) also plays an important role (4). Natural killer (NK) cells are a major effector of ADCC (4, 5), and polymorphisms of the receptor for the Fc region of IgG (FCGR3A, CD16) have been associated with response to ADCC elicited by therapeutic mAbs (4, 6–8). Methods for *ex vivo* NK-cell expansion and activation based on coculture with stimulatory cells have been developed (5) and, conceivably, such process might reinvigorate NK-cell activity in patients with cancer, which is often impaired (9). We found that coculture of peripheral blood monunucleated cells with leukemia cell line K562 genetically modified to express membrane-bound IL15 and 41BB ligand (K562-mb15-41BBL) allows the generation of large numbers of NK cells for infusion (10–12). NK cells activated with this method had considerably higher cytotoxic capacity than nonactivated NK cells, and showed strong antitumor activity *in vitro* and in xenograft models (10, 11, 1 3–17). These cells also express CD16, and can exert powerful ADCC in the presence of targeting antibodies including trastuzumab (18, 19). NK-cell expansion methods using coculture with K562-mb15-41BBL or other genetically modified K562 cells have been adapted to current Good Manufacturing Practices (cGMP) conditions, and are used to support clinical trials of NK-cell infusion (5, 12).

We postulated that *ex vivo* expansion and activation of NK cells from patients with HER2+ solid tumors might increase their capacity to exert ADCC mediated by trastuzumab. We conducted a first-in-man phase I dose-escalation study to determine the safety and tolerability of expanded NK cells in combination with trastuzumab,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

NK cells enhance antibody-dependent cell cytotoxicity and may potentiate the antitumor effect of the anti-HER2 antibody trastuzumab. In patients with HER2-positive malignancies, we activated and expanded autologous NK cells *ex vivo* and reinfused them one day after trastuzumab administration to investigate safety and efficacy of this treatment combination. Treatment was well tolerated; 6 of 19 patients in the dose-expansion group had stable disease of ≥6 months, suggesting clinical benefit. Findings in serial tumor biopsies and in peripheral blood were consistent with *in vivo* NK-cell activation. The VV genotype of FCGR3A which encodes a CD16 receptor with high affinity for Ig was identified as a potential pharmacogenetic biomarker for treatment response. Our study indicates that NK-cell infusions following trastuzumab are safe and may potentiate its activity. The results warrant exploration of this treatment combination in larger patient cohorts.

and explore the antitumor activity of this treatment combination in patients with HER2-positive advanced solid tumors.

Patients and Methods

Patient population

Patients ages 18–75 years with advanced HER2-positive solid tumors refractory to standard therapy, who had Eastern Cooperative Oncology Group (ECOG) performance status of 0–1, measurable disease according to RECIST version 1.1 (20), and adequate organ function were eligible for enrolment in this trial (ClinicalTrials.gov Identifier NCT02030561). HER2 positivity was defined according to the guidelines of the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP). Patient selection details are in the Supplementary Materials.

The study was conducted in accordance to the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice Guideline and approved by the Singapore Health Sciences Authority and the institution's independent ethics review committee, the National Healthcare Group Domain Specific Review Board (DSRB2013/00566). Written informed consent was obtained from enrolled patients.

NK-cell expansion

Peripheral blood was collected by leukapheresis. Peripheral blood mononucleated cells were cultured with irradiated (120 Gy) K562-mb15-41BBL cells and IL2 for 10 ± 1 days to activate and expand NK cells under cGMP conditions (12). The procedure was performed at the Tissue Engineering and Cell Therapy facility, National University Health System, Singapore. The protocol is described in detail in Supplementary Materials.

Study design

This was a phase I, open-label trial investigating the safety and tolerability of NK-cell therapy administered with trastuzumab in patients with HER2-positive refractory solid tumors, at a single centre at the National University Cancer Institute, Singapore (NCIS).

A 3+3 dose-escalation design was applied in the phase I dose-finding part of the study, with a starting dose of 1×10^6 NK cells/kg (**Fig. 1**; Supplementary Fig. S1). Patients received intravenous trastuzumab on day 1 of 21-day cycles up to 8 cycles or until disease progression. NK cells were administered on day 2 of cycle 1, with subcutaneous IL2 support (1 million IU/m^2) starting on day 1, followed by five additional doses given three times a week to support NK-cell viability and expansion *in vivo*. Patients were eligible for a second NK-cell infusion at cycle 4 if they achieved complete response (CR) or partial response (PR) after cycle 2, or at cycle 6 if they achieved sustained stable disease (SD) or better after cycle 4; patients who achieved sustained SD or better after cycle 6 could receive a third NK-cell infusion at cycle 8, up to a maximum of 3 NK-cell infusions over 8 treatment cycles. Intrapatient dose escalation was permitted.

After determination of recommended phase II dose (RP2D), two additional cohorts of patients were tested in phase IB (Cohorts A and B). Patients in Cohort A received 2 consecutive NK-cell infusions at cycles 1 and 2, while those in Cohort B, 2 consecutive NK-cell infusions at cycles 1 and 2 along with pretreatment with intravenous

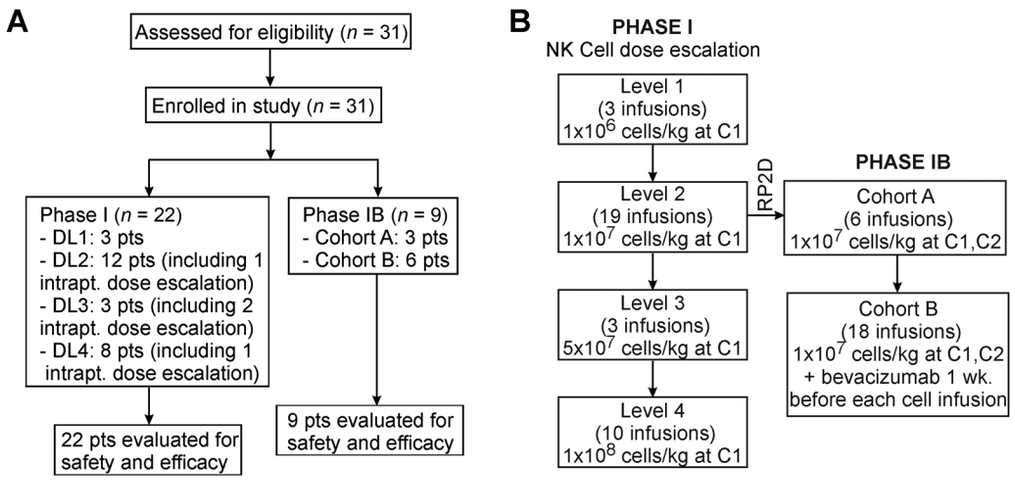

Figure 1.

Patient enrollment and treatment outline CONSORT diagram (**A**). **B**, Patient enrollment and treatment outline. Phase I involved four dose-escalation cohorts, and phase IB was carried out to further optimize treatment strategy based on the RP2D determined in phase I. DL, dose level.

bevacizumab 7.5 mg/kg 1 week prior to each NK-cell infusion. Patients in both cohorts received additional NK-cell infusions if they fulfilled response criteria as per phase I, up to a maximum of 4 NK-cell infusions over 8 cycles of treatment (Supplementary Fig. S1).

Patients who remained stable after 8 cycles of treatment were followed up until disease progression.

Study and efficacy assessments

Patients had vital sign measurements, physical examinations, ECOG performance status determination, full blood count, chemistry panel, ECG, echocardiogram, and CT scan at baseline. Toxicities and laboratory variables were assessed using NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (21). Patients had safety evaluations weekly during cycles with NK-cell infusions and 3-weekly during cycles with trastuzumab alone, and tumor response assessments with CT scans after every 2 treatment cycles. Echocardiography to evaluate left ventricular ejection fraction was performed after every 4 cycles of trastuzumab.

Monitoring NK-cell activity

Serial blood samples were collected to monitor NK-cell numbers and immunophenotype by flow cytometry as described in Supplementary Materials. Optional tumor biopsies were taken from patients with accessible disease within 30 days prior to cycle 1 and within 7 to 14 days after NK-cell infusion and analyzed by IHC for immune cell infiltration and apoptosis, as described in Supplementary Materials. The FCGR3A F158V variant (rs396991) was genotyped with nested PCR followed by Sanger sequencing using germline DNA. Details of the methodology used are in Supplementary Materials.

Results

Patient characteristics

The characteristics of the subjects enrolled in the study are provided in **Table 1**. Thirty-one subjects (29 patients; 2 patients were reenrolled at a different dose level) were enrolled and included in the safety analysis, with 22 subjects in phase I, and 9 in phase IB ($n = 3$ in Cohort A; $n = 6$ in Cohort B; **Fig. 1**). Thirty subjects had breast cancer, while one had gastric cancer. Lines of prior therapy ranged from 2 to 12 (median, 6). All 31 subjects received at least 1 NK-cell infusion and were considered evaluable for toxicity.

Table 1. Demographic and clinical characteristics at baseline of the 31 subjects enrolled.

Characteristic	Phase I	Phase IB
No. of subjects	22[a]	9
Median age, years (range)	59.5 (32–73)	55 (42–67)
ECOG performance status		
0	12	4
1	10	5
Tumor type		
Breast	21	9
Gastric	1	0
Hormone receptor status		
Positive	10	6
Negative	11	3
Unknown	1	0
HER2 positive		
IHC 3+	18	4
FISH+[b]	4	5
Median no. of metastatic visceral sites (range)	2 (1–5)	2 (1–5)
No. of patients with visceral metastasis	17	6
No. of prior metastatic anticancer regimens		
<3	2	0
≥3	20	9
Prior anti-HER2 therapy		
Trastuzumab	22	9
Pertuzumab	8	1
Trastuzumab emtansine	14	2
Lapatinib	16	7

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
[a]Data expressed given as number of subjects in each group unless otherwise specified.
[b]FISH positivity defined as HER2/CEP17 ratio >2.2 in 2013, then updated to HER2/CEP17 ratio of >2.0 in accordance to updated ASCO/CAP guidelines in 2014.

NK-cell preparation

NK cells were expanded from leukapheresis products by coculture with irradiated K562-mb15-41BBL cells and infused immediately after expansion. In 60 expansions with cells from the 31 subjects, the mean percentage (±SD) of NK cells among $CD45^+$ viable cells increased from 8.7% ± 5.4% to 83.6% ± 14.8% after culture (**Fig. 2A**). Total cell viability after expansion, as measured by 7-amino-actinomycin staining and flow cytometry, was 98.7% ± 1.8%. Median NK-cell expansion was 346-fold (range, 91–603), similar to that obtained under the same cGMP conditions from 17 healthy donors enrolled in other trials (median expansion, 386-fold; range, 23–574; **Fig. 2B**). The percentage of $CD3^+$ $CD56^-$ among $CD45^+$ viable cells at the end of the expansion process was 14.0% ± 15.4%. Most remaining cells after culture expressed both CD56 and CD3 (Supplementary Fig. S2). Of the 60 expanded NK-cell products, 59 were infused (in 1 subject, the third infusion was canceled due to ECG changes prior to infusion). The target number of NK cells was achieved for all 59 infusions except one, where 63×10^6 instead of 100×10^6 NK cells/kg were infused (**Fig. 2C**). No significant correlation was observed between rate of expansion and number of previous lines of therapy (**Fig. 2D**).

Expression of CD16 on $CD56^+$ $CD3^-$ NK cells was measured in 54 of 60 cell products before and after expansion (Supplementary Fig. S2). It increased from 84.0% ± 15.0% to 90.8% ± 11.4% ($P = 0.009$ by unpaired t test); mean fluorescence intensity of CD16 in the same samples increased from 7,760 ± 3,972 to 17,818 ± 5,628 after expansion ($P < 0.0001$). Expanded NK cells showed high ADCC activity against the SK-BR-3 HER2+ cell line in the presence of trastuzumab when tested from an aliquot of the infused product within 24 hours of infusion (**Fig. 2E**). Of note, the degree of cytotoxicity was significantly correlated with previous lines of treatment: subjects who had received >6 treatment lines had significantly lower ADCC at all effector:target (E:T) ratios ($P < 0.01$; **Fig. 2F**).

Dose escalation and toxicity

Phase I

Dose escalation of NK cells started at a dose level 1 of 1×10^6 cells/kg (3 infusions) and proceeded to dose level 2 of 1×10^7 cells/kg (3 infusions; **Fig. 1B**), with no dose-limiting toxicities (DLT) observed. Dose level 2 was expanded to an additional 16 infusions. Dose level 3 and 4 consisted of 5×10^7 NK cells/kg ($n = 3$) and 1×10^8 NK cells/kg ($n = 3$), respectively. No DLTs were observed at either level, and dose expansion at 1×10^8 NK cells/kg was applied to another 7 infusions

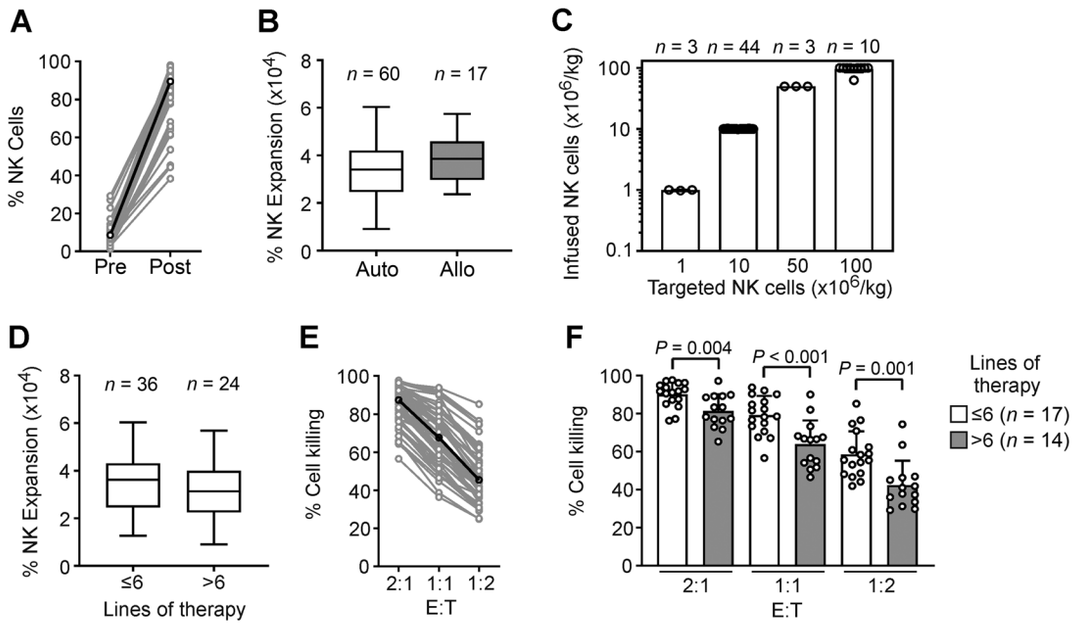

Figure 2.

Ex vivo NK-cell expansion. **A,** Percentage of CD56$^+$ CD3$^-$ NK cells among CD45$^+$ viable cells before and after expansion by coculture with K562-mb15-41BBL cells. The starting product ("pre") consisted of peripheral blood mononucleated cells separated from a leukapheresis product by density gradient centrifugation. The percentage of NK cells was calculated immediately before initiation of the culture. The final product ("post") was obtained after coculture with irradiated (120 Gy) K562-mb15-41BBL in presence of IL2 (40 IU/mL) for 10 ± 1 days. The percentage of NK cells was calculated at the end of the expansion process, immediately before infusion. Results of 60 expansions with cells from the 31 subjects enrolled are shown. **B,** NK-cell expansion in this study (autologous, 60 expansions from 31 subjects) compared with that with cells from healthy donors enrolled in other studies during the same period (allogeneic, 17 expansions from 17 subjects) under the same conditions. Box and whiskers show median, minimum, and maximum values, calculated as in **A**. **C,** Histograms correspond to the number of expanded NK cells planned for each infusion; symbols indicate the number of NK cells actually infused. The target number of NK cells was achieved for all 59 infusions except one, where 63 × 106 cells/kg were infused instead of 100 × 106/kg. **D,** NK-cell expansion according to lines of therapy. Box and whiskers plots show median, maximum, and minimum values. **E,** ADCC activity of expanded NK cells. Data show results of 4-hour cytotoxicity against SK-BR-3 in the presence of trastuzumab at the indicated E:T ratios. The black line illustrates the median value. **F,** Relation between 4-hour ADCC at the indicated E:T ratios and measured as in E with previous lines of therapy; if multiple expansions were performed in a subject, the average value is shown. Bars show mean ± SD, and symbols correspond to individual measurements. *P* values were calculated by two-tailed unpaired *t* test.

(**Fig. 1B**). Overall, there were five grade 3 treatment-emergent adverse events (TEAEs) at 1×10^8 NK cells/kg compared with 1 TEAE at 1×10^7/kg (**Table 2**), with no increase in clinical responses. Balancing between toxicity, efficacy, and feasibility of NK-cell expansion to 1×10^8 cells/kg from a single apheresis in 10 days, 1×10^7 NK cells/kg was declared the RP2D, although the MTD was not reached.

NK-cell infusion was generally well tolerated at the first three dose levels (up to 5×10^7 NK cells/kg) with one grade 3 TEAE deemed unlikely related to study treatment. At 1×10^8 NK cells/kg, five grade 3 TEAEs were observed, including pleural effusion ($n = 2$), pneumonia ($n = 2$), and hypokalemia ($n = 1$), all deemed unlikely to be related to the study procedure. The most common all-grade TEAEs was fever ($n = 6$; **Table 2**). Grade 3 pulmonary toxicities, including pleural effusion and pneumonia, were reviewed and found to be associated with concomitant infection or disease progression, and were regarded not to be related to treatment. All TEAEs were manageable by standard guidelines and resolved without sequelae after treatment discontinuation; no grade 4/5 treatment-related TEAEs were observed.

Phase IB (Cohorts A and B)

In Cohort A, 3 subjects received 2 consecutive cycles of NK-cell infusions at 1×10^7 cells/kg in cycles 1 and 2 (total 6 infusions). No additive toxicities were observed compared with patients in phase I dose level 2 (1×10^7 NK cells/kg), with only 1 patient experiencing grade 1 fever.

In Cohort B, 6 subjects were enrolled and receiving a total of 18 NK-cell infusions. In addition to trastuzumab, patients also received bevacizumab before each NK-cell infusion, in efforts to improve delivery of NK cells to tumor through effects on tumor vasculature (22–24). The most common all-grade TEAE observed was pain ($n = 8$). Three grade 3 TEAEs were observed: hypertension ($n = 1$), hyponatremia ($n = 1$), and ECG change ($n = 1$), with grade 3 hypertension possibly related to bevacizumab; all-grade 3 TEAEs were deemed unlikely to be related to NK-cell infusion. All TEAEs were manageable by standard guidelines and resolved without sequelae after treatment discontinuation, with no grade 4/5 treatment-related TEAEs observed.

Echocardiography was conducted at regular intervals during the study duration to monitor for potential cardiotoxicity associated with anti-HER2 therapy. There were no events of cardiotoxicity observed in either dose escalation or expansion phases of the study.

NK-cell profile and tumor infiltration

CD56$^+$CD3$^-$ NK cells measured in peripheral blood on days 6–13 postinfusion represented 20.1 ± 9.4% of CD45$^+$ lymphoid cells; their absolute numbers were $0.33 \pm 0.26 \times 10^3$/μL in the 59 infusions.

Table 2. Summary of TEAEs reported.

TEAE	Overall		Phase I								Phase IB			
			1×10^6 NK cells/kg ($n = 3$)		1×10^7 NK cells/kg ($n = 19$)		5×10^7 NK cells/kg ($n = 3$)		1×10^8 NK cells/kg ($n = 10$)		Cohort A		Cohort B	
	Total ($n = 59$)										1×10^7 NK cells/kg ($n = 6$)		1×10^7 NK cells/kg ($n = 18$)	
	≥ Grade 3	All	≥ 3	All	≥ 3	All	≥ 3	All	≥ 3	All	≥ 3	All	≥ 3	ALL
Any TEAE	9	60	0	2	1	10	0	0	5	19	0	1	3	28
Pain	0	10							0	2			0	8
Fever[a]	0	6			0	1			0	4	0	1		
Urticaria	0	5			0	1			0	3			0	1
Rash[a]	0	4			0	1			0	1			0	2
Thrombocytopenia	0	2	0	1	0	1								
Constipation	0	3							0	1			0	2
Pleural effusion	2	2							2	2				
Pneumonia	2	2							2	2				
Dyspnea	0	2	0	1					0	1			0	1
Hypokalemia	1	2			0	1			1	1				
Dizziness	0	3			0	2							0	1
Cellulitis	1	1			1	1								
Hypertension	1	2											1	2
Hyponatremia	1	1											1	1
Diarrhea	0	1			0	1								
Dry eyes	0	1											0	1
ECG change	1	1											1	1
Epigastric discomfort	0	1											0	1
Fatigue[a]	0	2			0	1							0	1
Hypercalcemia	0	1											0	1
Hypercholesterolemia	0	1											0	1
Infusion reaction[a]	0	1							0	1				
Insomnia	0	1							0	1				
Nausea/vomiting	0	2											0	2
Myalgia[a]	0	1											0	1
Raised creatinine	0	1											0	1

Abbreviation: TEAE, treatment-emergent adverse event.
[a]TEAE deemed to be related or possibly related to treatment. An AE with relationship missing (unknown) is counted as related.

These values were not significantly different from those measured preinfusion (19.5 ± 14.0%; 0.34 ± 0.31 × 10^3/μL) in the 31 subjects studied. Notably, subjects who had received >6 lines of treatment prior to enrolment had a higher percentage of NK cells postinfusion: 24.1 ± 11.5%, ($n = 24$ total infusions) versus 17.3 ± 6.3% ($n = 35$) for those with fewer lines of treatment ($P = 0.0050$ by unpaired t test); absolute numbers of NK cells were also higher but not statistically different (0.40 ± 0.37 × 10^3/μL vs. 0.29 ± 0.14 × 10^3/μL).

At the first measurement, 1–3 days postinfusion, most NK cells (82.7 ± 11.6%) expressed high levels of CD16. The percentage of NK cells with a $CD56^{dim}CD16^{high}$ phenotype significantly decreased postinfusion with a simultaneous increase of NK cells with high CD56 and heterogeneous expression of CD16 ($CD56^{high}CD16^{het}$; **Fig. 3A** and **B**).

The FCGR3A F158V variant was assessed in all 29 patients. Of these, 14 were homozygous FF, 14 were heterozygous VF, and 1 was homozygous VV genotype. The latter encodes a CD16 receptor with higher affinity for IGs, and has been associated with a better response to antibody immunotherapy (4, 7, 8).

Paired tumor biopsies were obtained in 8 subjects at preinfusion and 1-week post-NK-cell infusion (**Fig. 3C**). In all cases, hematoxylin and eosin (H&E)-stained sections showed tumor cells surrounded by fibrocollagenous stroma containing immune cells, consisting predominantly of lymphocytes, few plasma cells, and occasional macrophages. On H&E, there was a significant decrease in the estimated tumor content postinfusion (54 ± 16% preinfusion and 41 ± 11% postinfusion; $P = 0.038$). There was also an increase in tumor cell apoptotic index postinfusion (mean 0.89 ± 1.04 vs. 1.89 ± 1.64; $P = 0.063$), as determined by cleaved caspase 3 staining. This was paralleled by an increased lymphocyte infiltration in the stromal component postinfusion: the mean cell count in 5 high-power fields (HPF) at 400× was 198.13 ± 56.09 preinfusion versus 245.37 ± 92.82 postinfusion ($P = 0.169$), with a significant increase in $CD56^+$ NK cells (mean cell count in 5 HPF: 4.87 ± 3.09 vs. 19.50 ± 12.30; $P = 0.007$; **Fig. 3C**). Most lymphocytes were $CD3^+$ T cells (pre, 76 ± 12%; post, 82 ± 10%), with a significant increase in $CD8^+/CD4^+$ T-cell ratio noted in postinfusion biopsies (mean 1.22 ± 0.14 vs. 1.88 ± 0.62; $P = 0.026$).

Antitumor responses

All 31 subjects were evaluable for efficacy, and had been taken off study by the time of data analysis. Overall, 1 patient had RECIST PR, 16 had a best response of RECIST SD, and 14 patients progressed. Median progression-free survival was 12.0 weeks (range, 3.0–52.3 weeks; 95% CI, 7.33–16.67).

In phase I, 22 subjects received 35 infusions over four dose levels, with no objective responses observed. Of these, 12 had a best response of RECIST SD; in 7 of these subjects SD was sustained up to cycle 4 and they received a second NK-cell infusion at cycle 6; 6 of these 7 subjects received a third NK-cell infusion at cycle 8. Of the 19 subjects who received at least 1×10^7 NK cells/kg at cycle 1, 6 had SD for ≥6 months (median, 8.8 months; range, 6.0–12.0 months; **Fig. 4A**).

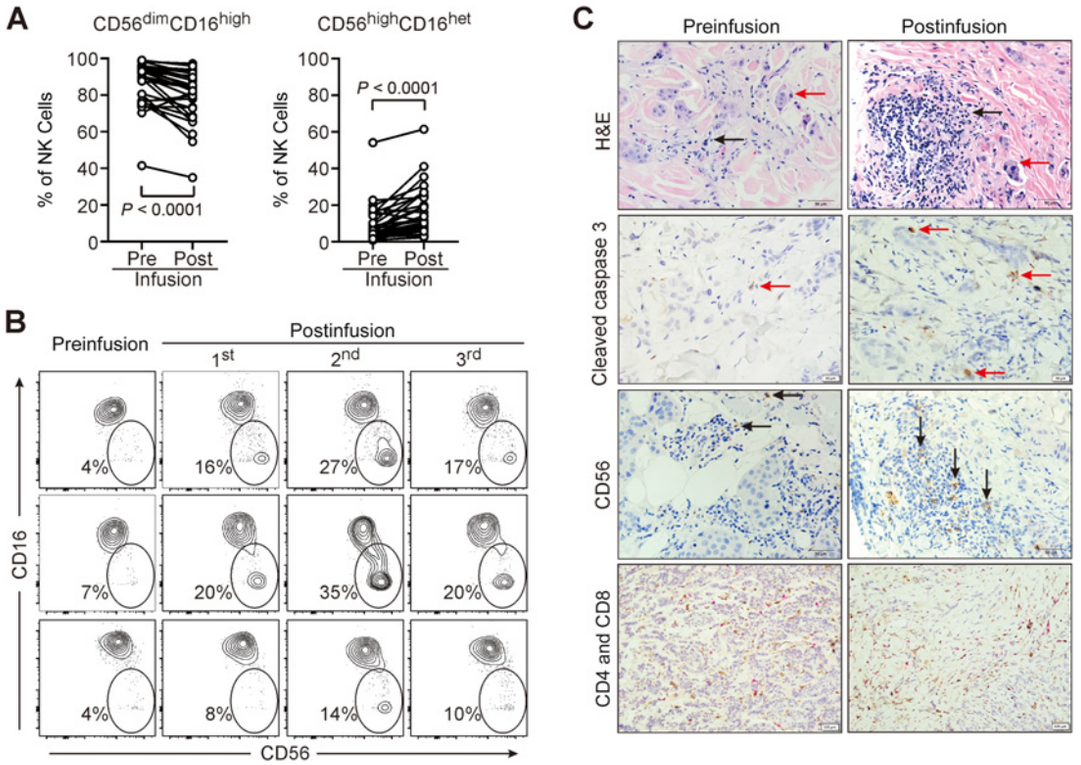

Figure 3.

Monitoring NK-cell activity. **A,** Percentages of peripheral blood CD45$^+$ lymphoid cells with the CD56dimCD16high and CD56highCD16het phenotypes before and after NK-cell infusion. The value postinfusion included in the graphs is the lowest among samples collected 6 to 13 days after NK-cell administration in each subject. $P < 0.0001$ for both comparisons by paired t test. **B,** Flow cytometric contour plots (with outlier events shown) illustrate three representative examples of the data in **A**. Samples were collected at three time points after infusion: 1st, day 1–6; 2nd, day 8–13; 3rd, day 19–22. Ellipses enclose CD56highCD16het cells; percentages among NK cells are shown. **C,** Representative images of paired tumor biopsies obtained before and 1 week after NK-cell infusion, stained with H&E, anticleaved caspase 3, anti-CD56, and anti-CD8 (brown) and anti-CD4 T cells (red). Red arrows point to tumor cells and black arrows point to lymphoid cells. All images at ×400 magnification except CD4 and CD8, which are shown at ×200 magnification.

In phase IB (2 consecutive NK-cell infusions in cycles 1 and 2, with or without bevacizumab), 5 of 9 subjects achieved at least SD as best response, and 1 subject in Cohort B had a PR. This patient was a 55-year-old female with metastatic hormone receptor–positive, HER2-positive metastatic breast cancer who had previously progressed on multiple lines of chemotherapy, anti-HER2 and hormonal therapy. She achieved RECIST PR after 2 consecutive cycles of NK-cell infusions, and remained in PR after cycle 8 NK-cell therapy (**Fig. 4B**). Monitoring of tumor marker CA-153 during study showed downward trend from 146.8 pretreatment to a nadir of 75.4 at cycle 5, remaining stable at 76.6 at the end of study. After completion of cycle 8, she started hormonal therapy with trastuzumab, but progressed with worsening brain and bone metastases 8 weeks later. This was the only patient in the study who had the VV genotype of FCGFR3A F158V variant, associated with greater response to ADCC elicited by therapeutic mAb (4).

Subjects with SD/PR ($n = 17$) and PD ($n = 14$) did not differ in regards to NK-cell expansion *ex vivo* (fold NK-cell recovery at the end of the cultures, 340 ± 90 vs. 327 ± 121), in the capacity of the cells to exert ADCC ($72.7 \pm 13.7\%$ vs. $72.1 \pm 13.7\%$ at 1:1 E:T), percentage or number of NK cells after infusion (19.0 ± 10.3 and $0.32 \pm 0.30 \times 10^3/\mu L$ vs. 22.2 ± 7.4 and $0.35 \pm 0.17 \times 10^3/mL$), or in their proportion of CD56^{high}CD16^{het} among NK cells after infusion ($16.8 \pm 11.6\%$ vs. $15.8 \pm 14.9\%$).

Discussion

In this study, we investigated the use of autologous expanded and activated NK cells to potentiate the effect of trastuzumab in patients with HER2-positive metastatic cancer. To expand NK cells from autologous leukapheresis products, we use a K562 cell line genetically modified to express membrane-bound IL15 and 4-1BBL (K562-mb15-41BBL). We had previously found that coculture of peripheral blood cells with irradiated K562-mb15-41BBL cells in the presence of low-dose IL2 stimulated a robust expansion of NK cells while the number of T lymphocytes in the cultures remained low (10). Indeed, in 60 NK-cell expansions with peripheral blood from the 31 subjects enrolled, the number of NK cells increased dramatically, with a median expansion of 364-fold. Interestingly, the degree of NK-cell proliferation was similar to the one with NK cells from healthy donors, indicating that the proliferative potential of NK cells from patients with advanced breast or gastric cancer is not inherently impaired.

There is increasing evidence that infusion of activated NK cells has antitumor activity (5). For example, cytokine-activated allogeneic NK cells have produced clinical responses in patients with acute myeloid leukemia (25–27). In patients with neuroblastoma, administration of allogeneic NK cells with an antibody targeting the neuroblastoma surface antigen GD2 has yielded encouraging results (28, 29). In preclinical studies, we found that NK cells expanded with K562-

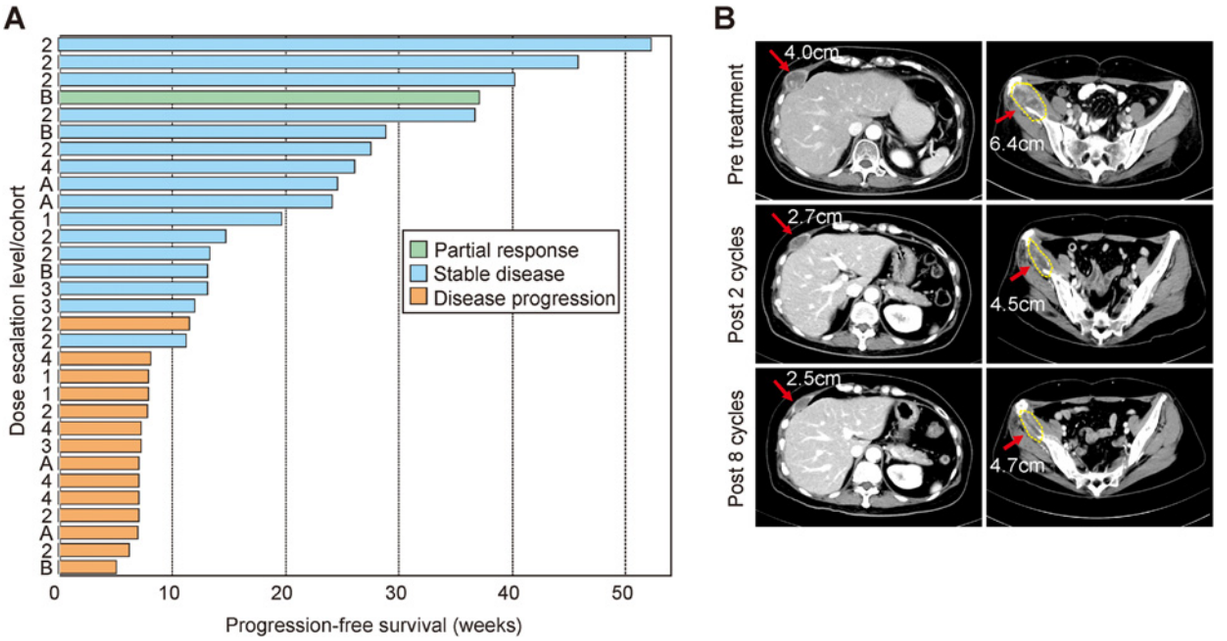

Figure 4.

Treatment response. **A,** Swimmer plot indicating length of treatment effect and best response of patients enrolled in the study. 1, phase I DL1; 2, phase I DL2; 3, phase I DL3; 4, phase I DL4; A, phase IB Cohort A; B, phase IB Cohort B. **B,** Tumor imaging in a patient with metastatic breast cancer who achieved a RECIST PR. CT scans before treatment (top), after 2 cycles of treatment (middle), and postcycle 8 (bottom), demonstrating sustained response in soft-tissue nodules in the right hypochondrial (left) and right iliac regions (right).

mb15-41BBL cells had high cytotoxic capacity *in vitro* and in xenograft models (10, 11, 13, 14), and that this was superior to that of NK cells activated with IL2 (10). This high cytotoxicity together with expression of CD16, and capacity to exert ADCC (18, 19) provided a rationale for infusing these NK cells in effort to enhance trastuzumab-mediated tumor cell killing.

Infusion of up to 1×10^8 NK cells/kg was well tolerated, with no DLT. Notably, no cytokine release syndrome or neurotoxicity was observed, and trastuzumab toxicity was not augmented. We also evaluated the impact of two or more consecutive infusions at 1×10^7 NK cells/kg, with the addition of bevacizumab. There were no significant increased toxicities compared with single infusion, with only 1 patient experiencing grade 3 hypertension that was attributable to bevacizumab and resolved with antihypertensive medications. No toxicities higher than grade 3 were observed in this study. Because we infused autologous, nongenetically modified NK cells, it was not possible to discriminate infused from preexisting circulating NK cells postinfusion. Although there was no increase in NK-cell percentages or absolute numbers in peripheral blood, there was a significant shift in phenotype, with a decrease in cells expressing high levels of CD16 and an increase in cells with high CD56. This would be consistent with NK-cell activation and CD16 engagement (30, 31), followed by CD16 shedding (30, 32).

Of the 19 subjects who received at least 1×10^7 NK cells/kg at cycle 1, 6 had stable disease for ≥6 months. Analysis of paired tumor biopsies showed increased NK cell and lymphocytic infiltration within the tumor tissue, with a corresponding increased tumor cell apoptosis. One patient with advanced metastatic breast cancer who had received multiple lines of prior anti-HER2 therapy achieved RECIST PR postcycle 2 of NK-cell infusion with the addition of bevacizumab. Interestingly, this patient was the only one with the FCGR3A VV genotype, associated with high-affinity CD16 (4). This observation is in keeping with other studies that have shown the VV polymorphism to be associated with high clinical responses to rituximab (33, 34), and suggests that FCGR3A genotyping may be a potential biomarker for response to therapy, although this will need to be validated with larger patient populations.

The addition of bevacizumab was explored in the expansion phase as it has been shown that antiangiogenic agents can aid in normalization of tumor vasculature, breakdown of stromal barriers, and can facilitate immune cell infiltration (22–24, 35). Moreover, a previous study conducted in our institution showed improvement in perfusion to tumor on serial imaging by dynamic contrast enhance-MRI (36). Inclusion of bevacizumab in the trastuzumab–NK-cell treatment showed good tolerability. We think that it is unlikely that the observed PR, which occurred in the bevacizumab group, can be attributed solely or predominantly to it, as single agent bevacizumab yields very low objective response rate in breast cancer and is usually combined with chemotherapy in metastatic breast cancer treatment. In a phase I trial of a nonchemotherapy containing triplet combination of trastuzumab, bevacizumab, and lapatinib in solid tumors, objective response was observed in only 10% of patients (37). Nonetheless, it would be interesting to further investigate the potential benefits of antiangiogenic agents in the context of NK-cell therapy.

The results of our study indicate that NK-cell therapy in combination with trastuzumab in patients with refractory HER2-positive solid tumors is safe and can lead to clinically meaningful disease stabilization in about a quarter of treatment-refractory patients. The autologous NK cells infused in this study were activated and expanded but were not subjected to any further engineering to enhance their cytotoxicity or proliferative capacity. Conceivably, allogeneic NK cells not inhibited by self-human leukocyte antigens might exert more

powerful ADCC (5). Previous studies have also shown that expression of chimeric antigen receptors (10), NK activation molecules (14, 16), and membrane-bound cytokines (17) can considerably enhance NK-cell antitumor activity, and a recent study has indicated activity of chimeric antigen receptor–modified NK cells in patients with B-cell lymphoid malignancies (38). Given the feasibility, safety, and encouraging antitumor activity seen in this study, we propose that a future study of the infusion of genetically modified NK cells with increased potency and *in vivo* expansion potential in HER2-positive tumors is warranted.

Disclosure of Potential Conflicts of Interest

S. Lee reports grants and other from Pfizer (research grant for investigator-initiated studies as well as honorarium for advisory boards and as invited speaker at conferences), other from Novartis (honorarium for advisory board and invited speaker at conferences), Astra Zeneca (honorarium for advisory board and invited speaker at conferences), Roche (honorarium for advisory boards), MSD (honorarium for advisory boards), grants from ACT Genomics (research grant for investigator-initiated studies as well as honorarium for invited speaker at conferences), Eisai (research grant for investigator-initiated studies; honorarium for advisory boards and as invited speaker at conference), Taiho (research grant for investigator-initiated trials), and other from Eli Lilly (honorarium for advisory board), and Amgen (travel support to conferences) outside the submitted work. N. Shimasaki reports a patent 62/477,335 pending on activating NKG2D receptors for NK cells, which is licensed, and with royalties paid from Nkarta. J.S.J. Lim reports personal fees from Astra Zeneca (honoraria and consulting), Pfizer (honoraria and consulting), and Norvatis (honoraria and consulting) outside the submitted work. S.G.W. Ow reports personal fees from AstraZeneca, Pfizer, and Novartis outside the submitted work. Y. Yap reports nonfinancial support from Roche outside the submitted work. E. Coustan-Smith reports that coauthor. D. Campana is her spouse and asks the readers refer to his declaration. R. Sundar reports other from BMS (advisory board), Merck (advisory board), Eisai (advisory board), Bayer (advisory board), Taiho (advisory board), MSD (fees for talk), Eli Lilly (fees for talk), Roche (fees for talk), Astra Zeneca (travel), and nonfinancial support from Paxman Coolers (research collaboration) outside the submitted work. B.C. Goh reports grants from National Medical Research Council, Singapore during the conduct of the study as well as personal fees and nonfinancial support from Bayer Healthcare (clinical trial work, consulting on advisory board), grants, personal fees, and nonfinancial support from MSD (research funds for clinical trials, honorarium for advisory board), other from Seattle Genetics (stock ownership), Mirati Therapeutics (stock ownership), and Gilead Sciences (stock ownership) outside the submitted work; in addition, B.C. Goh has a patent for a method for determining the likelihood of lung cancer pending and a patent for International Patent Application No. PCT/SG2019/050611 pending for a blood based biomarker panel that is developed in collaboration with a company called Sengenics, which is the most likely future licensee. D. Campana reports personal fees and other from Unum Therapeutics (cofounder and stockholder), Nkarta Therapeutics (scientific founder and stockholder), Medisix Therapeutics (scientific founder and stockholder), Juno Therapeutics (royalties) outside the submitted work; in addition, D. Campana has patents 7,435,596, 8,026,097, 9,511,092, 10,428,305, 10,538,739 issued, and patents 62/477,311 and 62/477,335 pending on NK-cell activation, expansion, and genetic modification which are licensed, and with royalties paid from Nkarta, and a patent 10,144,770 on receptors that promote antibody-dependent cell cytotoxicity issued, licensed, and with royalties paid from Unum. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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