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Phase I clinical trial of autologous NK cell therapy using novel expansion method in patients with advanced digestive cancer

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Abstract

Background: NK cells can destroy tumor cells without prior sensitization or immunization. Tumors often lose expression of MHC molecules and/or antigens. However, NK cells can lyse tumor cells in a non-MHC-restricted manner and independent of the expression of tumor-associated antigens. NK cells are therefore considered ideal for adoptive cancer immunotherapy; however the difficulty of obtaining large numbers of fully functional NK cells that are safe to administer deters its clinical use. This phase I clinical trial seeks to address this obstacle by first developing a novel system that expands large numbers of highly activated clinical grade NK cells, and second, determining if these cells are safe in a mono-treatment so they can be combined with other reagents in the next round of clinical trials.

Methods: Patients with unresectable, locally advanced and/or metastatic digestive cancer who did not succeed with standard therapy were enrolled. NK cells were expanded ex vivo by stimulating PBMCs with OK432, IL-2, and modified FN-CH296 induced T cells. Patients were administered autologous natural killer cell three times weekly via intravenous infusions in a dose-escalating manner (dose 0.5×10^9, 1.0×10^9, 2.0×10^9 cells/injection, three patients/one cohort).

Results: Total cell population had a median expansion of 586-fold (range 95–1102), with a significantly pure (90.96 %) NK cell population. Consequently, NK cells were expanded to approximately 4720-fold (range 1372–14,116) with cells being highly lytic in vitro and strongly expressing functional markers such as NKG2D and CD16. This NK cell therapy was very well tolerated with no severe adverse events. Although no clinical responses were observed, cytotoxicity of peripheral blood was elevated approximately twofolds up to 4 weeks post the last transfer.

Conclusion: We successfully generated large numbers of activated NK cells from small quantities of blood without prior purification of the cells. We also determined that the expanded cells were safe to administer in a monotherapy and are suitable for the next round of clinical trials where their efficacy will be tested combined with other reagents.

Trial Registration: UMIN UMIN000007527

Background

Natural killer (NK) cells play critical roles in the early innate response to pathogens and tumor cells [1, 2]. These cells exhibit strong cytotoxic activity against tumor cells without prior sensitization or immunization, and produce numerous cytokines resulting in the subsequent activation of the adoptive immune system.

Tumors often lose expression of tumor-associated antigens and/or MHC molecules as a means of immune escaping detection by T cells [3–5]. NK cells can lyse tumor cells in a non-MHC-restricted manner and are independent of the expression of tumor-associated antigens. Due to this, NK cells are considered ideal for adoptive cancer immunotherapy. In contrast to vaccine therapy or antigen-specific adoptive T cell therapy, it is not necessary to identify

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target tumor antigen for NK cell-based immunotherapy; this makes it more universally applicable and particularly effective for treating solid tumors that frequently lose tumor-associated antigens and/or self-MHC molecules. NK cell-based immunotherapy has been recommended as a means to improving hematologic malignancies [6, 7] and solid tumors [8–12] in clinical settings.

NK cells seem to possess many advantages that would make it ideal for clinical application. However, existing drawbacks are that it is difficult to generate large numbers of fully functional NK cells, and a standard method of ex vivo NK cell expansion has not been established yet. T cells can be expanded more than 1000-fold ex vivo using anti-CD3 monoclonal antibody in combination with cytokines and other stimuli [13, 14]. However in general, NK cells cannot sustain proliferation, therefore, their proliferative responses to cytokines with or without being co-cultured with other cells is modest and temporary [15–17]. To overcome this obstacle, researchers are seeking to develop new methods to obtain larger populations of highly pure NK cells. Examples include the ex vivo expansion of NK cells from (1) leukapheresis products by immunomagnetic beads selection [18–20], (2) from hematopoietic stem and progenitor cells from umbilical cord blood [21, 22], and (3) cytokine-based expansion method co-cultured with transgenic or irradiated tumor cells, and irradiated peripheral blood mononuclear cells [23, 24]. While these methods [18–24] have some merit, they have major drawbacks including: low expansion scale [20], low purity of NK cells [24], high cost [18–20], complicated procedures [18–24], and safety issues for human administration [23]. Developing innovative strategies to generate clinically relevant pure NK cells in large numbers would provide an important breakthrough in NK cell-based immunotherapy.

With this in mind, we recently developed a novel clinical-grade NK cell expansion system using recombinant human fibronectin fragment (FN-CH296, RetroNectin®)-induced T-cells (RN-T cells) as a stimulator. This method delivered a 688 ± 76-fold expansion of total cells in a sample of 31 cancer patients with purity levels of 84.7 ± 3.6 % without prior purification (Additional file 1: Table S1) [25]. Moreover, the majority of expanded cells highly expressed functional markers such as NKG2D (97.3 ± 0.6 %) and CD16 (96.8 ± 0.7 %) and exerted strong cytotoxicity in vitro and in experimental models of human tumors [25]. Having successfully produced these high quality NK cells, our secondary goal was to evaluate their safety when administered to patients with advanced digestive cancers and also assessed their efficacy as a minor objective.

The maximum tolerated dose for NK cells has not yet been established and no general range has been suggested. In fact, there is conflicting data surrounding the maximum tolerated dose. In prior research the administration of a large number of NK cells (almost 5–25 × 10^9 cells for 50 kg patient) in conjunction with lymph depleting chemotherapy and systemic IL-2 administration was found to be effective and safe [6–8]. On the other hand, severe adverse events have been reported when fewer (0.4–2.0 × 10^9 cells for 50 kg patient) NK cells were administered [8]. Taking these conflicting results into consideration, in this phase I clinical study, we evaluated the safety of autologous NK cells generated by our novel system by administered advanced digestive cancer patients with 0.5–2.0 × 10^9 cells (a similar dose to that used in prior studies [6–9, 11, 12]) a total of three times.

Methods

Eligibility

Patients with un-resectable, locally advanced and/or metastatic digestive cancer that was histologically confirmed were enrolled in this study. All patients had failed prior standard therapy and were recruited between September 2012 and June 2013. Eligibility criteria included: age >20 years, Eastern Cooperative Oncology Group (ECOG) performance status ≤2, no plans to receive chemotherapy other than oral fluorouracil prodrugs or radiation therapy, a life expectancy of at least 3 months, absence of serious cardiovascular disease, adequate vital organ function as indicated by leukocyte count ≥3000/mm^3, neutrophil count ≥1500/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥9.0 g/dL, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤100 IU/L, serum total bilirubin ≤2 mg/dL, serum creatinine ≤1.5 mg/dL, and blood urea nitrogen level ≤25 mg/dL. Patients were excluded if they tested positive for hepatitis B or C virus, HIV, HTLV-1, syphilis infection, had active severe infection, serious complications such as severe diabetes mellitus, unstable angina or myocardial infarction within 3 months, were pregnant or lactating, had a medical history of severe hypersensitivity or autoimmune disease.

Study design

This was a non-randomized, open label, phase I clinical trial with dose escalation of NK cells. The primary endpoint of this study was to assess the safety of the NK cells derived from our method, and the secondary end-points were clinical and immunological responses. This study was approved by the ethics committee of Kyoto Prefectural University of Medicine. The trial was designed and conducted in accordance with the Helsinki Declaration and the Ethical Guidelines for Clinical Research (the Ministry of Health, Labor and Welfare, Japan). All

participants provided written informed consent. This trial was registered as the University Hospital Medical Information Network (UMIN) Clinical Trial Registry as ID: UMIN000007527.

Cell processing

Preparation of the FN-CH296 (RetroNectin®)-stimulated T (RN-T) cells.

Peripheral blood (10–20 mL) was taken from each cancer patient. FN-CH296 (RetroNectin®, Takara Bio, Shiga, Japan)-stimulated T (RN-T) cells were prepared by a previously described method [13]. Briefly, peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque PREMIUM (GE Healthcare, Tokyo, Japan). Subsequently, 2×10^6 of cells were re-suspended in GT-T551 culture medium (Takara Bio) containing heat-inactivated autologous plasma (0.5 %) and recombinant IL-2 (Proleukin; NovartisPharma, Nürnberg, Germany), then transferred to a cell-culture immobilized with both anti-CD3 mAb (OKT3: Janssen Pharmaceutical k.k, Tokyo, Japan) and FN-CH296. On day 4, the cells were transferred to CultiLife® 215 bag (Takara Bio), and diluted with GT-T551 medium containing plasma (0.5 %) and IL-2 every 3 or 4 days. Cells were cultured for 1–2 weeks, γ-irradiated and then used as stimulators for NK-cell expansion.

Large-scale expansion of NK cells

PBMCs were obtained from the peripheral blood (20–40 mL) of each cancer patient. Subsequently, 5.6×10^6 of PBMCs were re-suspended in GT-T507α culture medium supplemented with heat-inactivated autologous plasma (1.0 %), IL-2 and OK-432 (Picibanil: Chugai Pharmaceutical Co, Tokyo, Japan). Cells were then transferred to a cell-culture flask and RN-T cells as a stimulator were added. On day 7, the cultured cells were transferred to a CultiLife® 215 bag and stimulated again by RN-T cells in GT-T510 culture medium (Takara Bio) supplemented with heat-inactivated autologous plasma (1.0 %) and IL-2. On day 11, the cells were transferred to a CultiLife® Eva bag (Takara Bio), and GT-T510 containing plasma (1.0 %) and IL-2 were added. Cells were then expanded by adding GT-T510 medium and increasing the number of CultiLife® Eva bag as necessary. On days 21 and 22, the cultured cells were harvested, washed and re-suspended in 100 mL of a saline based-solution supplemented with 1 % of human serum albumin (Albuminar; CSL Behring, PA, USA) then administered to patients immediately.

Quality control testing was conducted by assessing samples taken during the culture period and the final product for sterility by the BacT/ALERT (bioMérieux, Durham, NC, USA) microbiological detection system and for mycoplasma contamination by a MycoAlert Mycoplasma Detection Kit (Lonza Japan, Tokyo, Japan). Sterility tests were contracted to FALCO Biosystems (Kyoto, Japan). The viability of expanded cells was measured by trypan blue exclusion assay, and tested for endotoxin by a kinetic colorimetric LAL assay. After thawing, a small aliquot of the final product was used for in vitro cytotoxicity assay; it was cryo-preserved and examined to identify the proportions of $CD3^-CD56^+$ cells and other cell surface markers by flow cytometry.

Treatment protocol

The eligibility criteria for transferred cells was as follows: ① The cultured cell viability was more than 80 %. ② The mean purity value of the three cultured cells was more than 50 %. ③ The number of transferred cells was 70–130 % of the number that was set in each cohort. The patients were divided into three cohorts of three to four patients each: Cohort 1, 0.5×10^9 cells per dose; Cohort 2, 1.0×10^9 cells per dose; and Cohort 3, 2.0×10^9 cells per dose. Expanded NK cells that passed quality tests were intravenously injected for 60 min on days 0, 7, 14 (Fig. 1). We investigated the dose-limiting toxicity (DLT) occurring over a 28-day period after the third cell infusion. DLT was defined as grade $\geq$3 for any adverse event related to the administration of cultured cells. If no DLT was observed in the previous cohort, another cohort was treated at the next higher dose. There was no intra-patient dose escalation in this study.

Phenotypic analysis

The phenotype of expanded cells and PBMCs at baseline (day 0), before the 3rd administration (day 14), and 4 weeks after 3rd administration (day 42) were analyzed by flow cytometry. Monoclonal antibodies specific for CD3, CD16, CD56 (Beckman Coulter, CA, USA), NKG2D (eBioscience, CA, USA), CXCR3 (R&D system, MN, USA), CXCR4 (Becton–Dickinson, CA, USA), CX3CR1 (Bio Legend, CA, USA) were applied. Each monoclonal antibody was conjugated as follows: CD3 CD16, CD69, CXCR3 with fluorescein isothiocyanate (FITC)-, CD56, NKG2D, CXCR4, CX3CR1 with phycoerythrin (PE)-, CD3 with phycoerythrin-Cyanin 5 (PE-Cy5)-, CD56 with phycoerythrin-Cyanin 7 (PC7-Cy7)-. Cells were analyzed by Cytomics FC500 (Beckman Coulter) and data were acquired by the CXP software, version 2.2 (Beckman Coulter) according to the manufacturer's instruction.

Whole blood cytokine assay

Blood samples were obtained from patients on day 0 and on day 42 after the 3rd administration. All plasma samples were stored at −80 °C until they were analyzed. Following the manufacturer's instructions, we used a

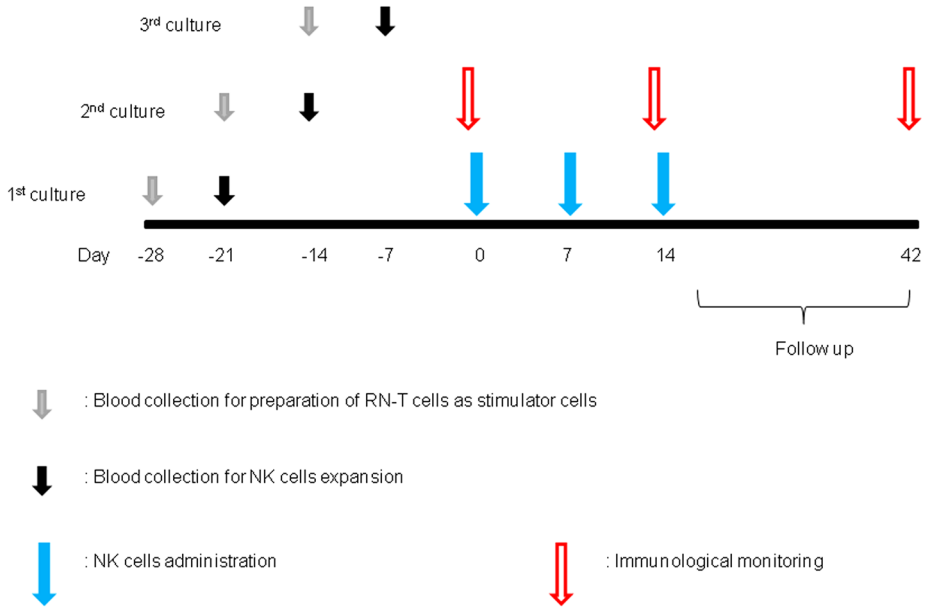

Fig. 1 Treatment protocol. PBMCs were separated to prepare RN-T as stimulator cells. One week later, PBMCs were again separated similarly, and re-suspended in culture medium supplemented with heat-inactivated autologous plasma, IL-2 and OK-432. RN-T cells were added to the same flask or culture bag on day 0 and day 7. On days 21–22, the cultured cells were harvested and administered to the patients immediately. Expanded NK cells were intravenously injected for 60 min on days 0, 7, 14 in a dose-escalating manner (dose 0.5×10^9, 1×10^9, 2×10^9 cells/injection, three patients/one cohort). We investigated the dose-limiting toxicity (DLT) occurring over a 28-day period after the last administration of cultured cells. Blood samplings for immune monitoring were done just before the 1st and 3rd administration and 4 weeks after the 3rd administration. *PBMCs* peripheral blood mononuclear cells, *RN-T cells* RetroNectin®-induced T cells

Bio-Plex multiplex cytokine array system (Bio-Rad Laboratories, CA, USA) with a panel that quantified the following 27 cytokines: IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic FGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF. Data acquisition and analysis were performed with Bio-Plex Manager Software (version 5.0).

In vitro cytotoxicity assay

Blood samplings for in vitro cytotoxicity assay were done on day 0, before the 3rd administration (day 14) and on 42 days after the 3rd administration (4 weeks). To evaluate the cytotoxicity of expanded NK cells (final products) fresh PBMCs against K-562 cell as target cell was assayed using DELFIA® cell cytotoxicity kit (PerkinElmer, USA, CA) according to the manufacturer's instructions. Briefly, PBMCs were added to the well in RPMI 1640 medium with 10 % FCS and incubated for 18 h. Target cells were labeled with Europium-DTPA, and then placed in 96-well tissue culture plates then incubated with PBMCs at various effector-to-target (E:T) ratios. After incubating for 4 h at 37 °C under 5 % CO_2, the release of Europium-DTPA was measured in a time-resolved fluorometer. Cytotoxicity was calculated as follows: % cytotoxicity = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Calcein-AM(Takara Bio) was used to measure cytotoxicity in cryopreserved expanded NK cells instead of Europium-DTPA, and thawed expanded NK cells was mixed with fluorescent-labeled target cells without pre-incubation. Finally, we calculated the values corresponding to the E:T ratio needed to reduce the cytotoxicity of expanded NK cells by 50 % of the maximum lysis value (EC50).

Clinical toxicity and efficacy assessment

Safety and toxicity were determined based on regular patient interviews, physical examination and laboratory tests. Safety was assessed and reported according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0). Objective tumor response was assessed by computed tomography scans in accordance with the Response Evaluation Criteria in Solid Tumor (RECIST VERSION 1.1) criteria. Disease assessment was performed at baseline and every 4 weeks after the final treatment.

Statistical analysis

The Wilcoxon signed-rank test was used to compare paired samples before and after NK cell therapy and

Spearman's rank correlation tested the association between the two. P values of less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism 5 for Windows (GraphPad, San Diego, CA, USA).

Results

Patient characteristics

Between September 2012 and June 2013, fourteen patients (11 males and 3 females) were enrolled in this trial. Table 1 shows patient demographics and clinical characteristics. The median age was 65.3 years (range 48–78). Seven (50.0 %) patients had colorectal cancer, four (28.6 %) had esophageal cancer, and three (21.4 %) had gastric cancer. Two patients in cohort 1 were removed from the study because the purity (no. 1, mean 30 %) or the number (no. 2) of expanded cells did not meet the minimum criteria (0.35×10^9 cells per dose). Two patients whose PS was 2 (no. 4 from cohort 1 and no. 10 from cohort 2), had disease progression during the NK cells preparation period and therefore received only two administration of NK cells.

Characteristics of expanded NK cells

PBMCs from 14 enrolled patients were cultured. The median of NK cell ($CD3^-CD56^+$) in lymphocytes was 13.59 % (range 4.43–34.85). The median of total cell or NK cell expansion rate after 21 and 22 days of culture was 586-fold (range 95–1102) and 4720-fold (range 1372–14,116), respectively (Fig. 2a). The total cell expansion fold did not correlate with the percentage of NK cells in lymphocytes ($\rho = 0.24$, $P = 0.40$), but in the 1st culture it significantly correlated with the cytotoxicity activity of PBMCs on day 0 ($\rho = 0.66$, $P = 0.04$, Fig. 2b). As shown in Fig. 2c, the purity of expanded NK ($CD3^-CD56^+$) cells markedly increased after culture, with the exception of one patient (Pt no. 1). The median purity of NK cell ($CD3^-CD56^+$) was 90.96 % (range 65.94–99.45) in the intention-to treat (ITT) population and 96.14 % (range 65.94–99.45) in the per protocol (PP) population. As shown in Table 2 however, the percentage of $CD3^+CD56^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells was minimal (median 8.60, 3.50 and 0.24 %, respectively). Expanded NK cells highly expressed cell surface markers such as NKG2D and CD16, which are considered viable and functional markers of NK cells. In the ITT population, the median percentage of NKG2D+ and CD16+ cells in NK population was 98.36 % (range 95.20–99.59) and 61.75 % (range 20.84–73.50), respectively. We also found relatively high expression levels of chemokine receptors such as CXCR3, CXCR4 and CX3CR1 on expanded NK cells (median in ITT population, 45.47, 37.71, and 43.74 %, respectively). Additional file 2: Figure S1 shows representative flow

Table 1 Patient characteristics

Cohort	Case	Age/gender	Diagnosis	Disease stage	ECOG/PS	Prior treatment	Combined treatment (chemotherapy)	NK cell administration
1	1	57/M	Rectal cancer	Recurrent	1	Surgery, chemotherapy	None	Excluded from trial[a]
	2	74/F	Rectal cancer	Recurrent	0	Surgery, chemotherapy	S-1	Excluded from trial[a]
	3	61/M	Esophageal cancer	Metastatic	2	Chemotherapy, radiation therapy	None	Complete
	4	62/M	Gastric cancer	Recurrent	2	Surgery, chemotherapy	S-1	Incomplete (2 times)
	5	72/M	Esophageal cancer	Recurrent	0	Surgery, chemotherapy	None	Complete
	6	73/M	Colon cancer	Recurrent	0	Surgery, chemotherapy	None	Complete
	7	66/M	Gastric cancer	Metastatic	0	Chemotherapy	None	Complete
2	8	62/M	Esophageal cancer	Recurrent	0	Surgery, chemotherapy, radiation therapy	None	Complete
	9	65/M	Colon cancer	Metastatic	0	Chemotherapy	S-1	Complete
	10	67/M	Esophageal cancer	Metastatic	2	Chemotherapy, radiation therapy	None	Incomplete (2 times)
	11	69/M	Gastric cancer	Metastatic	0	Chemotherapy	None	Complete
3	12	60/M	Colon cancer	Recurrent	0	Surgery, chemotherapy	None	Complete
	13	78/F	Rectal cancer	Recurrent	0	Surgery, chemotherapy	None	Complete
	14	48/F	Rectal cancer	Recurrent	0	Surgery, chemotherapy, radiation therapy	None	Complete

ECOG Eastern Coorperative Oncology Group, *PS* performance status

[a] Two patients were excluded from trial because the purity (no. 1) or the number (no. 2) of expanded cells didn't exceed eligible value

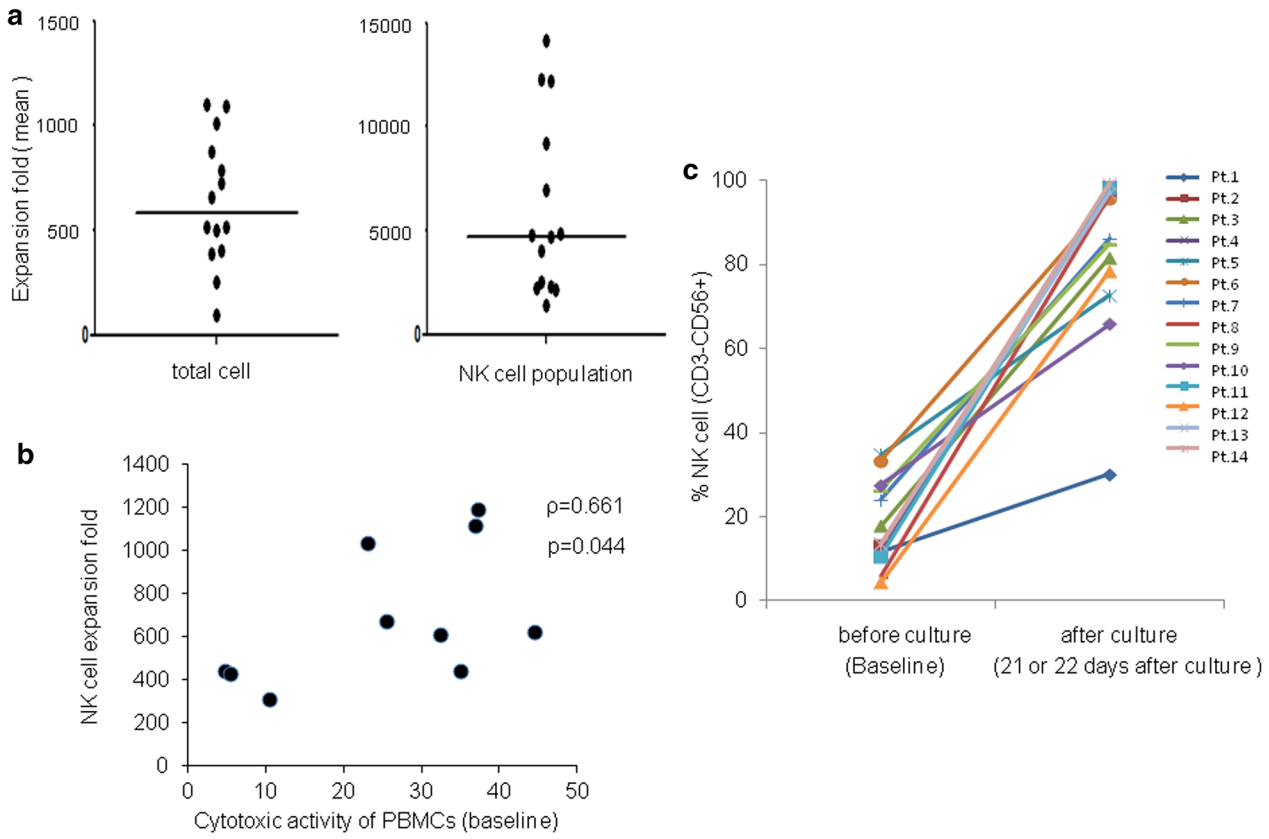

Fig. 2 Expansion and NK purity dynamics of PBMCs obtained from 14 patients. **a** Fold expansion of total cell and NK cell population ($CD3^{-}CD56^{+}$ cell) during the culture period. *Dots* represent mean values of each patient in triplicate cultures. *Horizontal bars* indicate median values. **b** Relation of NK cell expansion fold and cytotoxic activity of PBMCs. Expansion fold of NK cells in the first culture significantly correlated with cytotoxic activity of PBMCs at baseline ($\rho = 0.661$, $p = 0.044$). *PBMCs* peripheral blood mononuclear cells. **c** The purity of NK cell population ($CD3^{-}CD56^{+}$ cell) at baseline and 21 or 22 days after the initiation of the culture

cytometry dot-plots for each population of expanded cells in patient no. 14.

We directly tested the cytotoxicity of each patient's final product using the standard K-562 cells which is a NK-sensitive target. Expanded cells from all patients exerted strong cytotoxic activity against K-562 cells (Fig. 3). At a 6.25:1 effector to target cell ratio, 90.29 % of the K562 targets on average were killed by the expanded cells, and median EC50 value was 1.31 (range 0.39–2.61, Table 2). Representative data from patient no. 5, whose EC50 value was 0.63, appears in Additional file 3: Figure S2.

Safety assessment

The overall toxicity in the 12 ITT patients who were administered NK cells more than twice, is summarized in Table 3. No patient showed toxicity that exceeded grade 3. We observed one patient with grade 3 lymphopenia (no. 3), one patient with grade 3 anorexia (no. 10) and one patient with grade 2 pleuritis (no. 8). These adverse events were caused by cancer progression. Grade 3 anorexia, fatigue, anemia, thrombocytopenia, and total bilirubin elevation were observed in one patient (no. 4). These adverse events were caused by bone marrow carcinomatosis and obstructive jaundice brought on by the progression of gastric cancer. Grade 3 neutropenia and anemia was observed in one patient (no. 11) administered with S-1. These hematological toxicities were transient and were alleviated by drug withdrawal. Grade 1 fever elevation and fatigue associated with NK cell infusion was observed in one patient. The frequency and severity of toxicity did not increase with dose escalation of infused cells, and there were no severe or unexpected toxicity related to the NK cell infusion. Thus, the maximum tolerated dose was not reached.

Clinical efficacy

The evaluation of clinical outcome is shown in Table 4. No patient showed tumor shrinkage during the observation period up to 4 weeks after their last treatment. Of the 12 ITT patients, 5 (41.7 %) presented stable disease

Table 2 Characteristics of expanded cells

Patient no.	$CD3^{-}CD56^{+}$	$CD3^{+}CD56^{+}$	$CD3^{+}CD4^{+}$	$CD3^{+}CD8^{+}$	NKG2D+ in $CD3^{-}CD56^{+}$	CD16+ in $CD3^{-}CD56^{+}$	CXCR3+ in $CD3^{-}CD56^{+}$	CXCR4+ in $CD3^{-}CD56^{+}$	CX3CR1+ in $CD3^{-}CD56^{+}$	NK activity EC50[a]
1	30.18	62.53	NE	NE	NE	NE	NE	NE	NE	2.61
2	98.41	0.93	0.40	0.24	98.91	23.01	28.33	49.79	36.79	1.31
3	81.78	16.28	17.15	0.96	96.70	20.84	48.80	21.98	28.33	1.31
4	98.75	0.91	0.55	0.13	96.28	29.47	36.67	29.78	28.37	1.61
5	72.84	26.50	25.49	1.87	99.59	68.39	44.82	20.32	39.51	0.63
6	95.67	3.93	3.12	0.30	99.36	60.70	56.62	23.31	42.07	0.62
7	86.25	13.27	3.87	0.24	95.20	50.29	29.04	31.34	26.79	1.39
8	96.60	2.27	2.25	0.07	98.54	60.46	42.90	62.89	50.40	1.24
9	84.91	14.91	10.31	2.64	98.17	72.33	41.80	37.88	62.01	0.98
10	65.94	38.83	33.61	0.23	98.67	62.79	44.04	42.40	51.40	1.88
11	98.49	1.39	0.45	0.11	99.45	69.23	49.69	69.29	44.33	1.76
12	78.52	21.40	11.43	0.30	97.88	73.50	61.05	37.54	70.20	1.99
13	97.23	2.57	2.60	0.11	99.00	32.88	57.66	49.55	54.89	0.59
14	99.45	0.44	0.28	0.09	97.82	63.59	46.12	43.73	43.15	0.39
Median (all enrolled patients)	90.96	8.60	3.12	0.24	98.54	60.70	44.82	37.88	43.15	1.31
Median (ITT population)	90.96	8.60	3.50	0.24	98.36	61.75	45.47	37.71	43.74	1.28

[a] The data are presented for EC50, which means the values corresponding to the E:T ratio needed to reduce the cytotoxicity by 50 % from maximum lysis

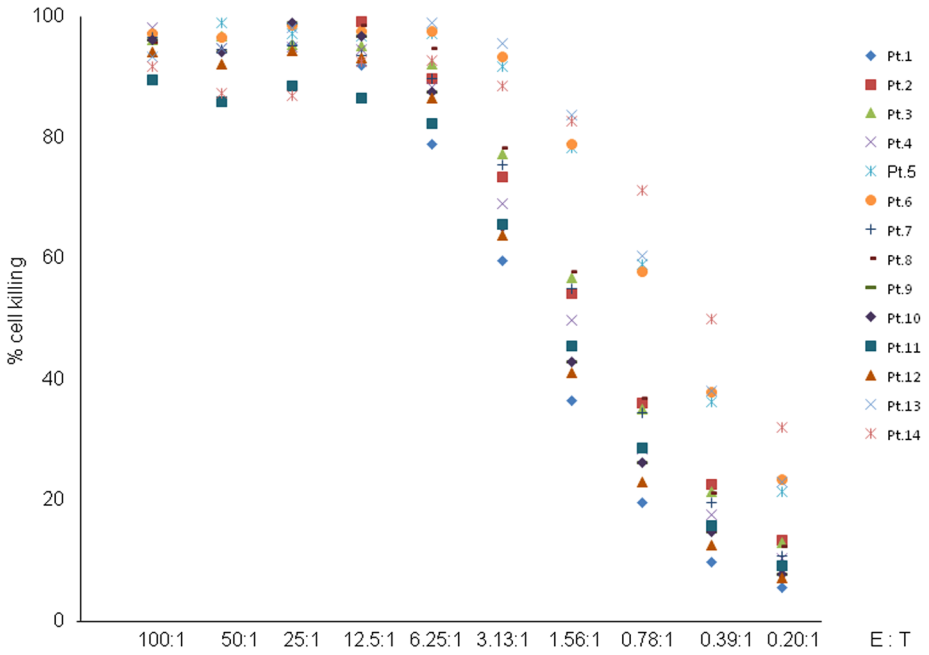

Fig. 3 Cytotoxic activity of expanded NK cells against K-562 cells. 4 h cytotoxicity from 14 patients against K-562 target cells. Mean cell death in each patient at the indicated E:T ratios in triplicate cultures

Table 3 Maximum toxicity per patient

Toxicity	Cohort 1 (n = 5)		Cohort 2 (n = 4)		Cohort 3 (n = 3)		All cohort (n = 12)	
	Grade		Grade		Grade		Grade	
	1–2	3	1–2	3	1–2	3	1–2	3
Hematological								
Neutropenia	0	0	1	0	0	1	1	1
Lymphopenia	3	1	3	0	0	0	6	1
Anemia	3	1	3	1	2	0	8	2
Thrombocytopenia	2	1	1	0	0	0	3	1
Elevated AST	2	0	1	0	0	0	3	0
Elevated ALT	0	0	1	0	0	0	1	0
Elevated total billirubin	0	1	2	0	1	0	3	1
Non-hematological								
Fatigue	2	0	3	0	3	0	8	0
Fever	1	0	0	0	0	0	1	0
Anorexia	0	1	0	1	0	0	0	2
Nausea	0	0	0	0	2	0	2	0
Diarrhea	0	0	2	0	0	0	2	0
Constipation	1	0	2	0	0	0	3	0

(SD) and 7 (58.3 %) presented progressive disease (PD). Of the 10 PP patients, 5 (50 %) presented SD and 5 (50 %) presented PD. Thus, in the PP analysis, the response rate and the disease control rate (DCR, complete response + partial response + SD) in this study were 0 and 50.0 %, respectively.

Table 4 Tumor response

No. of patients	Response				Response rate (%) (95 % CI)	Disease control rate (%) (95 % CI)
	CR	PR	SD	PD		
ITT population n = 12	0	0	5	7	0 (0)	41.7 (15.17–72.33)
PP population n = 10	0	0	5	5	0 (0)	50.0 (18.71–81.29)

ITT intention -to-treat, *PP* per protocol, *CR* complete response, *PR* Partial response, *SD* stable disease, *PD* progressive disease, *95 %CI* 95 % confidence interval

Immunological monitoring

Immunological monitoring was performed for per protocol evaluable patients using their PBMCs before and after the NK cells infusion. As indicated in Fig. 4a and Additional file 4: Table S2, the average NK cell population in peripheral blood lymphocytes (PBLs) slightly increased until day 42 after NK cell infusion. Evaluated by cohort, the population of NK cells in cohort 2 and 3 increased after the infusion of NK cells, whereas there was a gradual decrease in cohort 1 (Fig. 4b). Next, we tested the cytotoxicity of PBMCs against K-562 targets and found that cytotoxicity increased in 80 % of patients after NK cell infusion (Additional file 5: Table S3). On day 14, average PBMC cytotoxic increased more than twice the day 0 baseline value; while it gradually decreased, it remained higher than day 0 until day 42 (Fig. 5a). This change in

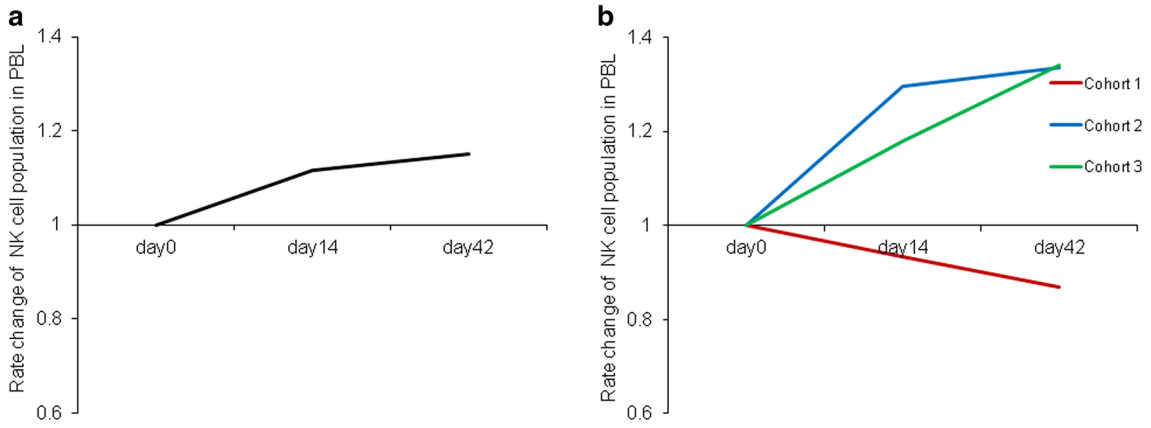

Fig. 4 Longitudinal plots of NK cell population in PBLs plotted according to their deviation from the baseline. Mean levels in all patients (**a**) and levels in each cohort (**b**) are shown. *PBLs* peripheral blood lymphocytes

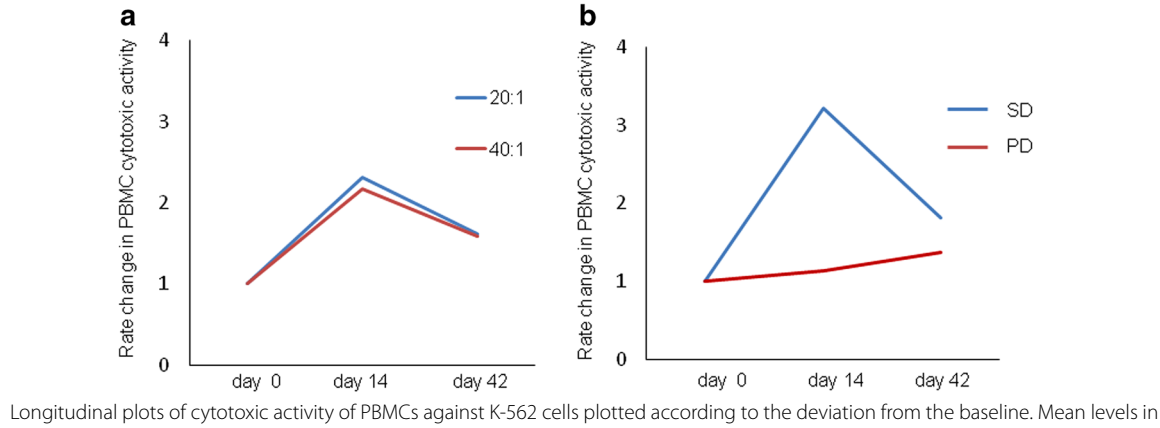

Fig. 5 Longitudinal plots of cytotoxic activity of PBMCs against K-562 cells plotted according to the deviation from the baseline. Mean levels in all patients at indicated E:T ratios (**a** *blue* 20:1, *red* 40:1) and levels for the tumor responses at 40:1 E/T ratio (**b**) are shown. *PBMCs* peripheral blood mononuclear cells, *SD* stable disease, *PD* progressive disease

PBMC cytotoxic activity was similar across all cohorts and seemed to be independent of the number of cells that were infused. However, PBMC cytotoxic activity differed according to objective tumor response, and the average in patients with SD increased more than three times on day 14 and was much higher than in patients with PD (Fig. 5b). Cytokine serum levels did not change significantly after NK cell infusion (Table 5).

Discussion

In this phase I clinical trial consisting of advanced digestive cancer patients, we explored the safety and feasibility of adoptive transfer of NK cells elicited by a novel expansion method using RN-T cells. We generated pure (median 90.96 %) NK cells on a large scale without prior purification. These cells expressed activating markers such as NKG2D and CD16 implicated in cytotoxicity and ADCC activity and exerted strong cytotoxic activity against K-562 cells in vitro. Not only did our proposed method produce fully functional NK cells in large numbers, the process was safe, well tolerated and highly reproducible.

Table 5 Serum cytokines concentration before and after NK cell therapy

	Baseline (day 0) (pg/ml)	Post administration (day 42) (pg/ml)	p value
IL-1β	4.007 ± 7.155	6.438 ± 13.22	0.25
IL-1RA	893.3 ± 2154	983.0 ± 2550	1
IL-2	37.16 ± 22.54	37.45 ± 29.48	0.8203
IL-4	2.456 ± 0.6683	2.600 ± 0.7691	0.4961
IL-5	4.029 ± 2.191	4.171 ± 3.156	1
IL-6	38.62 ± 47.26	45.43 ± 58.69	0.3594
IL-7	3.827 ± 3.984	4.724 ± 6.224	0.8203
IL-8	26.94 ± 9.571	30.73 ± 16.71	0.4258
IL-9	31.33 ± 28.77	32.87 ± 37.69	0.4258
IL-10	26.28 ± 30.72	27.71 ± 39.37	0.3008
IL-12p70	37.28 ± 50.31	47.22 ± 83.46	0.9102
IL-13	14.87 ± 21.99	19.05 ± 36.70	0.8203
IL-15	44.33 ± 20.73	43.67 ± 28.17	0.8203
IL-17	199.2 ± 89.43	185.1 ± 109.2	0.4961
IFN-γ	92.13 ± 119.4	105.4 ± 141.8	0.3594
TNF-α	32.04 ± 45.28	38.69 ± 59.64	0.0977
bFGF	51.92 ± 31.51	53.38 ± 45.84	0.7344
PDGF-BB	172.2 ± 108.3	191.0 ± 219.0	0.9102
G-CSF	55.23 ± 26.89	58.08 ± 40.79	0.9102
GM-CSF	90.58 ± 75.85	94.29 ± 97.23	0.7344
IP-10	287.1 ± 40.88	389.2 ± 204.4	0.3008
MCP-1	133.6 ± 33.18	122.1 ± 52.29	0.2031
MIP-1α	4.972 ± 3.239	4.214 ± 2.073	0.1641
MIP-1β	275.0 ± 86.68	300.6 ± 154.0	1
Eotaxin	116.4 ± 193.8	123.2 ± 233.4	0.7344
RANTES	2157 ± 396.8	2445 ± 580.7	0.2031
VEGF	92.02 ± 30.85	113.2 ± 79.16	0.9102

Values are expressed as the mean ± SD. The paired t test was used to determine statistical differences

NS not significant

NK cells occur in low numbers in PBMCs and obtaining the number necessary for adoptive transfer of NK cells is technically challenging. Unlike T cells, which readily respond to a variety of stimuli, it is generally difficult to expand NK cells from a small amount of blood from patients with advanced cancer who in general tend to have impaired immune function. There are few previous reports in which a NK cell expansion method produced pure cells in sufficient quantities from a small amount of blood without prior purification of the NK cells [26]. IL-2 can induce proliferative responses in human NK cells, but as suggested by existing reports, it can induce up to a 50-fold expansion after being cultured for 2 weeks [27–29]. Recently, Alici et al. reported that anti-CD3 and IL-2 induced 1600-fold NK expansions after 20 days from the blood of patients with myeloma [30]. However, the cytotoxicity of these cells against K562 cells was <10 % at 1:1 E/T ratio [30], which is much lower than the results achieved in this present in vitro study (average cytotoxicity 57.54 or 38.77 % at 1.56:1 or 0.75:1 E/T ratio).

We observed that chemokine receptors CXCR3, CXCR4 and CX3CR1 were relatively highly expressed in expanded NK cells, with a median in the ITT population, of 45.47, 37.71, 43.74 % respectively. These molecules are considered to be involved in the accumulation of intratumoral NK cells [31–34]. Thus, the NK cells elicited by our system are believed to have excellent properties as adoptive transferred cell in cancer immunotherapy. The percentage of CD16+ NK cells in the final product was relatively low compared to that observed in our previous study (average 55.4 vs 96.8 %) [25]. We observed by flow cytometer that CD16 expression on NK cells decreased approximately 30 % after being thawed and this may be attributed to us using thawed samples in the present study, whereas fresh samples were analyzed in our previous study.

Although no clinical responses were observed in 10 PP patients, the in vitro cytotoxicity of PBMCs against K-562 targets increased in 80 % of patients post adoptive NK cell transfer. The average cytotoxic activity on day 14 increased more than two times after NK cells infusion, and the increase continued until day 42 post-transfer. In their clinical trial for patients with metastatic melanoma or renal cell carcinoma, Parkhurst et al. documented the persistence, function and phenotype of autologous NK cells after adoptive transfer [35]. In their study, the peripheral blood lymphocyte 1 week post treatment

consisted of an average of 83 % NK cells, which was much higher than in our clinical study (21 % at 2 weeks post-transfer). This difference in results probably stems from the higher number of transferred cells used in their clinical trial (47×10^9 per dose) versus the current trial (0.5 or 1.0 or 2.0×10^9 per dose). Additionally, their study involved lymph depleting chemotherapy and systemic IL-2 administration combined with NK cell transfer; these procedures were not done in our trial.

As previously mentioned, the focus of this clinical trial was to assess the safety of the NK cells produced with our expansion method. These cells had a relatively high level of functionality which made them promising however, we felt it was also important to know their level of toxicity before focusing on their efficacy. For this reason, although studies like those presented above have received better efficacy with combined treatment such as lymph depletion and any cytokine support, we chose a monotherapy where a relatively small number of NK cells were administered as the best option to assess the safety of the expanded cells.

To overcome the limited clinical efficacy of NK cell-based monotherapy, several strategies have been suggested including the combination of various monoclonal antibodies [26]. Within the setting of NK cell-based therapy, much attention has been given to the KIR-ligand mismatch phenomenon. Lack of KIR-HLA class I interactions has been associated with potent NK-mediated antitumor efficacy in acute myeloid leukemia patients upon haplo-identical stem cell transplantation [36] or NK cells infusion [6, 37] from KIR mismatched donors. Thus, a KIR ligand-mismatched donor is likely to provide the best chance for clinical response. Adoptive transfer of allogeneic NK cells from a KIR ligand-mismatched donor elicited by our novel method is expected to offer advantages over autologous NK cells. Since our method does not stimulate T cell proliferation, this could be an important clinical advantage because it avoids the risk of graft-versus-host disease in allogeneic NK cell therapy. Immune checkpoint blockade with antibodies to CTLA-4 and PD-1 represents a promising cancer therapy that aims to restore an efficient antitumoral response mediated by T cell [38]. As a corollary to targeting negative regulators of T cells, blocking inhibitory signals of NK cells with anti-KIR [39] or anti-Tim-3 [40] antibodies is also an attractive therapy to combine with NK cell therapy. CD16 expression of expanded NK cells produced in our study is relatively high. Therefore, adoptive transfer of these NK cells combined with tumor-specific monoclonal antibodies has the potential to trigger strong ADCC responses. This type of combined therapy with IgG1 monoclonal antibodies is likely to be promising. Having confirmed that our expanded cells are safe to administer, we are currently conducting clinical trials combining them with cetuximab for colorectal cancer and trastuzumab for gastric cancer patients in order to assess their efficacy. (The UMIN Clinical Trials Registry ID: UMIN000013378).

Conclusion

We have shown that our novel NK expansion system using RN-T cells is effective at producing large numbers of fully functional NK cells that are pure. The data here also demonstrates that adoptive transfer of these NK cells is safe and very well tolerated by patients who had failed standard cancer therapy. NK cell transfer as a monotherapy is generally unsatisfactory and although no clinical responses were observed in patients, knowing that these cells can be safely administered will allow us to make an attempt at improving their efficacy by combining them with other reagents. We believe that these expanded cells have the potential to be efficacious in a combination treatment, since we observed that transferred NK cells persisted in the peripheral circulation of patients and exerted cytotoxicity in vitro. The NK cell expansion method suggested here could be applied to various NK cell-based therapies and can be combined with a range of treatment modalities. The advantage of our novel NK cell generation method can be summarized as follows: (1) large number of highly pure and functional cells (2) no need for purification including the use of a magnetic beads sorting system (3) improved safety through the use of autologous RN-T cells rather than transgenic or cancer cells as a stimulator (4) requires only a small amount of blood (5) requires low-serum culture conditions (0.5–1.0 %) and (6) high reproducibility. We believe that this expansion method is an innovative tool for NK cell-based cancer immunotherapy.

Additional files

Additional file 1: Table S1. Summary of NK cell expansion from 31 cancer patients (supplementary information of reference #25).

Additional file 2: Figure S1. Representative flow cytometry dot-plots for each population of expanded cells in patient no.14 (1st culture).

Additional file 3: Figure S2. Cytotoxic activity of the final product from patient 5 against K-562 cells. Mean cell death at the indicated E:T ratios in triplicate cultures. EC 50 is the value corresponding to the E:T ratio needed to reduce the cytotoxicity by 50 % from maximum lysis.

Additional file 4: Table S2. Change in NK population in PBL.

Additional file 5: Table S3. Change in cytotoxic activity of PBMCs against K-562 cells during and after NK cell therapy.

Authors' contributions

Conceived and designed the experiments: NS TI SK TE JM. Performed the experiments: NS TI SK TE MI FS AK TO. Analyzed the data: NA TI TE MI. Contributed reagents/materials/analysis tools: NS TE MI FS AK MT KO. Wrote the paper: NS TI. Intellectual input and data analysis support: YN YI TY. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests
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REVIEW
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Shaping of Natural Killer Cell Antitumor Activity by *Ex Vivo* Cultivation

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Natural killer (NK) cells are a promising tool for the use in adoptive immunotherapy, since they efficiently recognize and kill tumor cells. In this context, *ex vivo* cultivation is an attractive option to increase NK cells in numbers and to improve their antitumor potential prior to clinical applications. Consequently, various strategies to generate NK cells for adoptive immunotherapy have been developed. Here, we give an overview of different NK cell cultivation approaches and their impact on shaping the NK cell antitumor activity. So far, the cytokines interleukin (IL)-2, IL-12, IL-15, IL-18, and IL-21 are used to culture and expand NK cells. The selection of the respective cytokine combination is an important factor that directly affects NK cell maturation, proliferation, survival, distribution of NK cell subpopulations, activation, and function in terms of cytokine production and cytotoxic potential. Importantly, cytokines can upregulate the expression of certain activating receptors on NK cells, thereby increasing their responsiveness against tumor cells that express the corresponding ligands. Apart from using cytokines, cocultivation with autologous accessory non-NK cells or addition of growth-inactivated feeder cells are approaches for NK cell cultivation with pronounced effects on NK cell activation and expansion. Furthermore, *ex vivo* cultivation was reported to prime NK cells for the killing of tumor cells that were previously resistant to NK cell attack. In general, NK cells become frequently dysfunctional in cancer patients, for instance, by downregulation of NK cell activating receptors, disabling them in their antitumor response. In such scenario, *ex vivo* cultivation can be helpful to arm NK cells with enhanced antitumor properties to overcome immunosuppression. In this review, we summarize the current knowledge on NK cell modulation by different *ex vivo* cultivation strategies focused on increasing NK cytotoxicity for clinical application in malignant diseases. Moreover, we critically discuss the technical and regulatory aspects and challenges underlying NK cell based therapeutic approaches in the clinics.

Keywords: natural killer cells, natural killer cell cultivation, natural killer cell expansion, natural killer cell therapy, natural killer cell cytotoxicity, *ex vivo* stimulation

INTRODUCTION

As an important part of the innate immune system, natural killer (NK) cells are deployed as first line of defense against aberrant cells caused by viral infections or malignancies. Human NK cells can be identified *via* their morphology as large granular lymphocytes, and *via* their surface marker profile, as they express by definition CD56, but not CD3. The NK cell compartment can be further divided into subpopulations. There are two main NK cell subsets that can be distinguished, the $CD56^{high}CD16^{neg}$ subpopulation, which has mostly immune modulatory function, mainly accomplished by interferon (IFN)-γ secretion, and the $CD56^{low}CD16^{pos}$ fraction with direct cytotoxic capacity (1–3). NK cell activation is based on a balanced system integrating signals from activating and inhibitory receptors. Inhibitory signals derive mainly from germ-line encoded inhibitory killer cell immunoglobulin-like receptors (KIRs). Ligands for inhibitory KIRs, in humans major histocompatibility complex (MHC) class I molecules, are highly expressed by healthy cells and thereby prevent NK cell activation. Malignant cells often downregulate MHC class I molecules on their surface to evade T cell attack (4). However, these so-called "missing-self" cells are recognized by NK cells through inhibitory receptors, and as signals from activating receptors prevail, NK cells become active and react against the encountered targets. Alternatively, NK cells can be activated by overexpression of stress-induced surface ligands on infected or abnormal cells, which are recognized by activating receptors, such as the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46, and the so-called C-type lectin-like receptors, such as NKG2D (1, 5–9). In this case, activating signals outbalance inhibitory self-signals and lead to NK cell activation. Furthermore, NK cells become activated upon encounter of antibody-coated targets by CD16, which binds to the Fc portion of the antibody and mediates a strong activating signal. By means of activating and inhibitory receptors, NK cells, unlike T and B-lymphocytes, can react immediately without prior priming or antigen presentation.

Activated NK cells execute effector functions through different mechanisms. NK cells mediate direct cytotoxicity *via* the exocytosis pathway with release of cytotoxic granules, which contain granzymes and perforin, resulting in lysis of the target cell (10). In addition, NK cells induce apoptosis of target cells by expression of death receptor ligands, such as Fas ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (11). Production and release of IFN-γ by NK cells after activation also has multiple functional consequences, with particular relevance in tumor surveillance, as IFN-γ inhibits tumor angiogenesis, has antimetastatic activity, and acts pro-apoptotic (12, 13).

The ability of tumor cells to bypass the immune response is a basic prerequisite for cancer formation and progression. Within immune editing, tumors undergo genetic, epigenetic, and phenotypic changes, thereby becoming a heterogeneous cell population that is hardly visible to or assailable by immune cells due to downregulation of tumor antigens and NCR ligands (14). Additionally, malignant cells suppress NK cells by blocking the NKG2D receptor *via* shedding of NKG2D ligands (15–17) or upregulation of inhibitory MHC class I molecules (18, 19). Immunosuppressive cytokines such as transforming growth factor-β, interleukin (IL)-10, or immunosuppressive enzymes, such as indoleamin 2,3-dioxigenase, further impair antitumor NK cell responses of cancer patients (20–22).

Ex vivo modulation of NK cell receptor expression is therefore an important tool to overcome immune response inhibition. A number of studies reported an upregulation of DNAM-1, NKG2D, and other NK cell-activating receptors under certain culture conditions, mostly involving stimulation by IL-2 (23–26). In addition, other ILs such as IL-12, IL15, IL-18, or IL-21 and Type I IFNs shape the NK cell receptor expression profile (27–31).

Natural killer cells can play an important role for cellular immunotherapy and the adoptive transfer of NK cells represents an attractive strategy to treat cancer patients (32, 33). In this context, *ex vivo* expansion of NK cells prior to their clinical application is not only required to increase the applicable cell doses but it is also reasonable to pre-activate and modify their antitumor features. For *ex vivo* cultivation, NK cells from different sources can be stimulated with different cytokines, and, to reach efficient expansion rates, NK cells are cultured among autologous accessory cells or together with different types of growth-inactivated autologous or allogeneic feeder cells (**Figure 1**). Of note, it is possible to genetically engineer NK cells *ex vivo* to further augment their antitumor activity, for example, to integrate chimeric antigen receptors against distinct tumor antigens (34, 35). In this review, we focus on the cultivation of NK cells without genetic modifications. Many different protocols exist for *ex vivo* expansion of NK cells, all with different features and capacities. Here, we give a comprehensive overview of strategies to obtain appropriate amounts of functional NK cells. We will discuss starting material and culture systems as well as the use of cytokines, feeder cells, and other additives.

STARTING MATERIAL FOR NK CELL EXPANSION AND ROLE OF NK CELL PURITY

Until recent, 92% of clinical studies used NK cells from peripheral blood, either donor (79% of recruiting trials) or patient derived (13% of recruiting trials) (36). Alternatives are the use of NK cell lines, or the differentiation of NK cells from umbilical cord blood or pluripotent stem cells (37–39). NK cell lines, such as NK-92, avoid the need for donor selection and enable the production of large cell doses to treat patients on a flexible schedule (40). Nevertheless, NK cell lines require growth inactivation mainly achieved by irradiation, possibly reducing their antitumor potential due to short *in vivo* persistence. Differentiation of NK cells from cord blood $CD34^+$ cells is attractive because of the "off-the-shelf" availability from a cord blood bank. Similarly, NK cells from pluripotent stem cells are a promising concept for the future but still in early development (39, 41). In this overview, we focus on peripheral blood-derived NK cells, currently the main source for NK cells for clinical use.

The NK cell purity, meaning the frequency of NK cells among other cells, is an important factor for the intended therapeutic

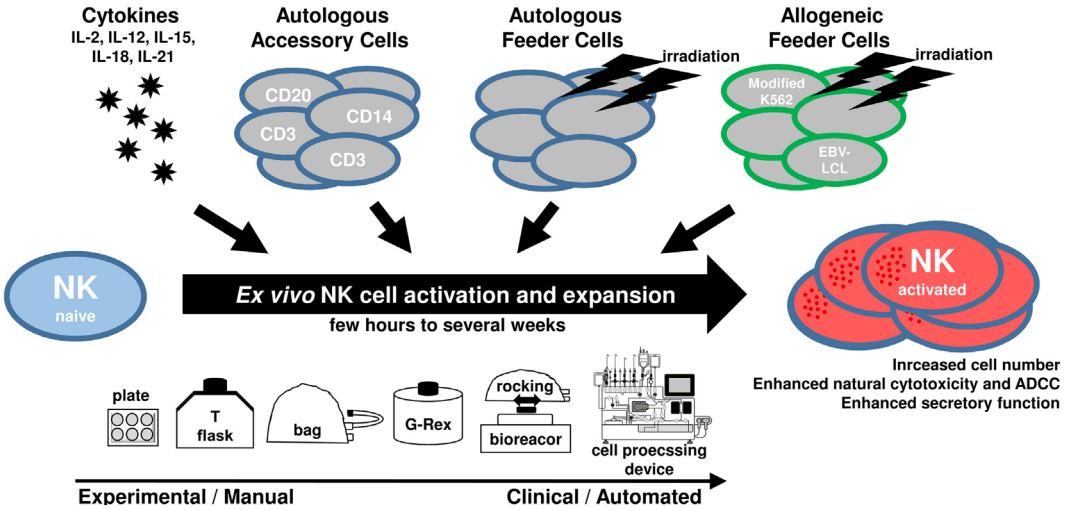

FIGURE 1 | **Scheme showing main components utilized for *ex vivo* natural killer (NK) cell activation and expansion procedures.**

application. For *ex vivo* expansion, NK cells are often cultured within a mixture of cells, such as PBMC, thereby avoiding further purification. Whereas the cultivation of NK cells among other accessory cells is a practical strategy for autologous therapeutic settings, it may be critical for allogeneic applications, since non-NK cells may induce unwanted side effects. Alloreactive T cells are a major risk factor for the patient, as they mediate "graft-versus-host disease" (GvHD), a severe complication following allogeneic hematopoietic stem cell transplantation (HSCT) (42). Furthermore, donor-derived B cells can lead to B cell lymphoproliferative disorder after reactivation of an Epstein–Barr virus (EBV) infection (43, 44), and they can cause the passenger lymphocyte syndrome (45), both critical side effects for the patient. Therefore, purification of NK cells might be required and is realized so far in most clinical settings by magnetic cell separation, for instance, by depletion of CD3-expressing cells and subsequent enrichment for CD56-expressing cells (26, 46–49). In addition, a first proof of concept is shown for good manufacturing practice (GMP)-compliant fluorescence-activated cell sorting to purify for NK cell subsets, such as NK cells expressing a single KIR (50).

CYTOKINE-INDUCED NK CELL EXPANSION

Aims of adoptive transfer of *ex vivo* expanded NK cells are the enhancement of natural cytotoxicity and homing to tumor sites under maintenance of "self" protection. Studies performed with cytokine-stimulated NK cells or PBMC have shown the safety of this approach and indicated some clinical responses upon adoptive NK cell transfer following HSCT. In the next paragraph, we summarize *ex vivo* NK cell expansion protocols starting with purified NK cells (**Table 1**) or PBMC (**Table 2**). Concepts administering cytokines in the presence of growth-inactivated feeder cells will be discussed in later sections of this article.

THE ROLE OF IL-2

Interleukin-2 plays an important role in activation of NK cells *via* binding to the IL-2 receptor (IL-2R), a heterotrimeric protein expressed on NK cells and other immune cells. This led to the interest in both (i) using IL-2 for stimulation of autologous NK cells in cancer patients and (ii) *ex vivo* activation and expansion of allogeneic donor NK cells for adaptive immunotherapy. At the beginning of the 1980s, researchers around Rosenberg and colleagues showed that IL-2 exposed lymphokine-activated killer (LAK) cells were able to attack autologous fresh tumor cells and that this effect could mainly be ascribed to NK cells (71, 72). Nevertheless, in first clinical trials using adoptive transfer of LAK cells and IL-2 therapy, the clinical response did not exceed the efficacy of IL-2 monotherapy (73).

Importantly, during the last 20 years, it has been elaborated that NK cells play a major role in the regulation of the balance between GvL and GvHD after allogeneic HSCT, especially haploidentical HSCT (33, 74–78), demonstrating improved anticancer activity while avoiding GvHD. In order to make haploidentical NK cells available for clinical use, large-scale GMP-conform manufacturing protocols were established. After starting with a leukapheresis product that was depleted for $CD3^+$ cells and enriched for $CD56^+$ cells, cultivation in medium containing IL-2 (1,000 U/mL) for up to 2 weeks yielded 0.1–3 × 10^9 $CD56^+CD3^-$ NK cells (**Table 1**), sometimes sufficient for multiple infusions to patients with hematological malignancies (26). Median NK cell expansion was fivefold and median NK cell purity was >94 with <0.1% T cell contamination (26). *Ex vivo* stimulation with IL-2 induced elevated cytokine secretion by NK cells, enhanced intracellular STAT3/AKT signaling, and upregulation of various NCRs and NKG2D receptors (52). Depletion of CD3 cells from leukapheresis products without subsequent CD56 enrichment and short-term activation with IL-2 overnight led to a final product containing 40% NK cells (**Table 2**). In all cases, IL-2-activated NK cells demonstrated a much higher cytotoxic activity against

TABLE 1 | *Ex vivo* cultivation of pure natural killer (NK) cells with cytokines only.

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
IL-2 + IL-15	PBMC, CD3 depleted and CD56 enriched or PBMC, CD3/CD19 depleted in flasks	>1 (5 days)	75–100% NK ≤0.1% T cells	*Upregulated*: CD69, NKp30, and NKp44	Cytolysis of leukemia cell lines and primary acute leukemic blasts	*In vitro*	(51)
IL-2	PBMC, CD3 depleted, and CD56 enriched in bags and flasks	4–5 (12–14 days)	~92–95% NK <0.1% T cells	increased $CD56^+CD16^-$ frequency; increased p-STAT3 and p-AKT; increased lytic activity, upregulation of CD69, NKG2D, and natural cytotoxicity receptors (NCRs); increasing amount of NK cells without killer cell immunoglobulin-likereceptors	Improved cytotoxic activity against leukemia and tumors	Clinical	(26, 52, 53)
IL-15	Isolated $CD3^-CD56^+$ cells	N/A (2–5 weeks)	94–99% NK	Enhanced killing *via* NCRs, DNAM-1 and NKG2D	Enhanced cytolysis of lymphoma and rhabdomyosarcoma cell lines *via* NCRs	*In vitro*	(54)
IL-2 + IL-21	PBMC, sorted for $CD3^-CD56^+$	None with IL-21 only; strongly with IL-2 + IL-21	N/A	*Upregulated*: CD69, CD25; activation of STAT3	Enhanced cytotoxicity against K562	*In vitro*	(55)
IL-12 + IL-15 + IL-18	PBMC, sorted for $CD3^-CD56^+$ cells PBMC, CD3 depleted and CD56 enriched	N/A (12–16 h)	≥90% NK	*Upregulated*: CD94, NKG2A, NKp30, NKp44, NKG2D, NKp46, CD69, and CD25 *Downregulated*: NKp80	Memory: increased IFN-γ production upon stimulation that is preserved during cell division Responsive to picomolar concentrations of IL-2	*In vitro* Clinical	(56–59)

K562 target cells compared to unstimulated NK cells (26, 43, 52, 53, 60). In addition, after cryopreservation and thawing, NK cells showed a moderate to high viability when activated with IL-2, whereas the viability of unstimulated NK cells was low (26).

Transfer into the clinic in 2004 and 2005 with first patient studies using those IL-2-activated donor NK cells were performed in parallel in Europe and the US, for both, haploidentical HSCT (53), and in the non-transplant setting (43). In the latter one, Miller and coworkers used IL-2 expanded haploidentical NK to treat 43 patients with advanced cancer (43), with 19 of them suffering from acute myeloid leukemia, followed by studies in patient with ovarian and breast cancer and B-cell non-Hodgkin lymphoma (60, 79). Importantly, the authors reported *in vivo* persistence and even expansion of the alloreactive donor NK cells in patients pretreated with high dose preparative regimen, consisting of 5 days of 60 mg/kg intravenous cyclophosphamide and 25 mg/m^2 intravenous fludarabine (43). Of note, successful NK cell engraftment was dependent on the patients' pretreatment regimen, which was also responsible for the patients' elevated IL-15 plasma concentrations (43). In addition, it was demonstrated that *in vivo* persistence of donor NK cells at day 7 after infusion and successful *in vivo* expansion (more than 100 donor-derived NK cells per microliter of patient blood 14 days after transfer) correlated with leukemia clearance (60). Expansion of host regulatory T cells was associated with low numbers of NK cells (60). In parallel, Koehl et al. reported on three pediatric patients with multiply relapsed leukemia (still in blast persistence at HSCT) treated with repeated transfusions of IL-2-activated donor NK cells post-haploidentical HSCT (53), which led to complete remission remaining for several weeks up to some months. In the following clinical study, they also demonstrated a small clinical benefit in patients with various malignancies receiving IL-2-activated compared to patients receiving resting NK cells only (80). Interestingly, IL-2-stimulated NK cells but not unstimulated NK cells promoted NK cell trafficking and changes in the distribution of leukocyte subpopulations in the peripheral blood. In the meanwhile, safety and feasibility using IL-2-activated and -expanded NK cells for adaptive immunotherapy has been demonstrated in various clinical studies as summarized in a recent review by Koehl and others (33).

IMPACT OF IL-15 ON NK CELL EXPANSION

Carson et al. postulated that NK cells might be dependent on other cytokines than IL-2 such as IL-15 (81, 82). The trimeric IL-15 receptor on NK cells shares two subunits with the IL-2R, but not CD25 forming the high affinity IL-2R. Therefore, they also share some functions, e.g., maintenance of NK cell survival (82). Similarities and differences between IL-2 and IL-15 effects on NK cells have been extensively reviewed elsewhere, and IL-15 might be the preferable cytokine for cancer therapy as it inhibits activation-induced cell death and it is considered safe (83–85). In addition, compared to IL-2, IL-15 leads to more sustained antitumor capacity of NK cells *via* signaling through mammalian target of rapamycin and stress-activated gene expression (86). However,

TABLE 2 | *Ex vivo* cultivation of natural killer (NK) cells with accessory cells.

Protocol features	Starting material/ culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
IL-2	PBMC, CD3 depleted in bags and flasks	N/A (overnight)	33% NK 0.1% T cells	N/A	N/A	Clinical	(60)
		N/A (14–16 h)	26.7%		Enhanced cytotoxicity *In vitro*		(61, 62)
		N/A (overnight)	40% NK 0.9% T cells		Enhanced cytotoxicity *In vitro*		(43)
IL-15	PBMC, CD56 enriched	23 (20 days)	98% NK	Expression of NKp30, NKp44, NKp46, NKG2D, and 2B4	Cytotoxic *in vitro*	Clinical	(63)
IL-15 + IL-21	PBMC, CD3 depleted	3.7 $CD56^+/CD122^+$ (2–3 weeks)	>90% $CD56^+/CD122^+$ <0.3% $CD3^+/CD56^-$ <3% $CD3^+/CD56^+$	67% $CD56^+CD16^+$	Cytotoxic against K562 and patient bone marrow blasts	Clinical	(64)
OKT-3 + IL-2	PBMC in plates	193 (21 days)	~55% NK ~22% T cells	N/A	Substantial cytotoxicity against K562	*In vitro*	(65)
	PBMC in flasks	1,625 (20 days)	~65% NK ~22% T cells	*Upregulated*: 2B4, CD8, CD16, CD27, CD226, NKG2C, NKG2D, NKp30, NKp44, NKp46, LIR-1, KIR2DL3, and CXCR3 *Downregulated*: CCR7	Increased cytotoxicity against tumor cell lines and primary MM cells *In vitro*	*In vitro*	(25)
	PBMC	1,036 (total cells) (19 days)	~30% NK ~40% T cells	*Upregulated*: NKG2A, LILR-B1, NKG2D, NKp30, NKp44, and NKp46	*In vitro* cytotoxicity increases during culture	Clinical	(66)
	PBMC in a bioreactor, flasks, and plates	77—bioreactor 530—bags 770—flasks (20 days)	38%—bioreactor 31%—bags 44%—flasks	Bioreactor compared to flasks: higher expression of CD11b, NKG2D, and NKp44	Bioreactor compared to flasks: higher cytotoxicity	*In vitro*	(67)
OKT-3 + IL-2 + Alemtuzumab	PBMC in plates, flasks, and bags	646 (14 days) 1,537 (18 days)	60% NK 37% T cells <0.1% B cells	*Upregulated*: 2B4, NKG2D, NKp30, NKp44, KIR2DL1, LIR-1, and CD16 *Downregulated*: CCR7	Increased cytotoxicity *In vitro* and *in vivo*	Clinical	(68)
OKT-3 + IL-2 + IL-15	PBMC or $CD56^+$ + $CD56^-$ (1:1) in flasks and bioreactor (Cellbag)	PBMC: 112 1:1 Mix: 89 (21 days)	With PBMC: 34% With "1:1 Mix": 92%	*Upregulated*: NKp30, NKp44, DNAM-1, NKG2D, and CD11a	Increased activity against neuroblastoma cell lines *in vitro* and *in vivo*	Preclinical model	(69)
aCD16 mAb + OK432 + IL-2	PBMC in flasks and bags	637–5,712 (day 21)	79% NK 8.4% T cells (day 21)	*Upregulated*: NKG2D, NKp44, and CD69 *Downregulated*: CD16 (transient)	Increased cytotoxicity against tumor cell lines and primary cancer cells *in vitro* ADCC activity	*In vitro*	(70)

recent data revealed that continuous IL-15 signaling causes functional exhaustion of NK cells by decreased fatty acid oxidation, resulting in lower cytotoxicity *in vitro* and decreased tumor control *in vivo* (87). Thus, optimal dosing and timing of IL-15 is critical for *ex vivo* NK cell activation. Purified NK cells expanded using IL-15 exhibit upregulation of NCRs and CD69 and cytolysis of leukemia and primary ALL blasts (51). Enhanced cytotoxicity of IL-15-stimulated NK cells against leukemia and rhabdomyosarcoma cell lines could be attributed to NCRs, DNAM-1 and NKG2D (54). Using IL-15 to expand NK cells from CD56-enriched PBMC for 20 days resulted in a 23-fold expansion of $CD3^-CD56^+$ NK cells with a final purity of about 98% (63). NK cells generated with the latter protocol were transferred to 15 non-small lung cancer patients in a phase I clinical trial in two to four doses of $0.2–29 \times 10^6$ NK cells/kg, showing the safety of the approach (63).

IL-21 ENHANCES NK CELL EFFECTOR FUNCTIONS

The cytokine IL-21, in combination with IL-2 or IL-15, is utilized in some protocols for NK cell stimulation (55, 64). IL-21 belongs to the IL-2 family and signals through a heterodimer consisting of the common γ-chain and the IL-21 receptor α-chain. Activated $CD4^+$ T cells are the main producers of IL-21 and IL-21 affects many different cell types expressing the IL-21 receptor, including NK cells (88). IL-21 plays a role in the development of NK cells from bone marrow progenitors (89), and, in mice, it dampens the expansion of NK cells but is required for functional NK cell maturation (90, 91). Recently, expansion of "memory-like" NK cells has been shown to be IL-21 dependent in the context of tuberculosis infection (92). Wendt et al. observed increased proliferation of $CD56^{bright}$ human NK cells (55), but another group reported no effect of IL-21 on the proliferation of NK cells from healthy human donors and from HIV patients (93). Moreover, IL-21 is known to trigger apoptosis, resulting in a shorter lifespan of NK cells *in vitro* (90, 94). Thus, the time span NK cells are exposed to IL-21 appears critical (95, 96). Besides its effect on NK cell proliferation, IL-21 enhances the effector functions of NK cells, including secretory and cytotoxic functions as well as enhanced ADCC responses (93, 97, 98). Culturing CD3-depleted PBMC for 13–20 days with IL-21 and IL-15 without additional feeder cells yields activated NK cells with a purity of >90%, which were applied in a clinical trial with 41 leukemia patients receiving infusions of donor-derived NK cells 2–3 weeks after HSCT (64). Although the NK cells expanded weakly under this condition (3.7-fold), they possessed potent cytotoxic activity against primary bone marrow blasts prior to transplantation, and infusions with a median dose of 2×10^8 NK cells/kg were well tolerated and correlated with a reduction in leukemia progression compared to historical controls (64).

IL-12/15/18 INDUCED MEMORY NK CELLS

Interleukin-12 was originally discovered as NK cell-stimulating factor, inducing proliferation, enhanced cytotoxicity, and production of IFN-γ by NK cells when added to PBMC (99, 100). IL-12 is produced by DCs, macrophages, and B cells, and its receptor consists of two subunits (α and β), which mediate signaling through members of the JAK–STAT family (101). IL-2 enhances the response of NK cells to IL-12 by increasing the expression of the IL-12 receptor and STAT4, a relevant factor for IL-12 signaling (102). Furthermore, it was revealed that IL-12-mediated IFN-γ production of NK cells requires priming with IL-18, a cytokine also known to enhance IL-15-induced NK cell proliferation (103, 104). Due to the synergistic effects, it seems reasonable to combine the different cytokines for *ex vivo* stimulation of NK cells. In this context, the combination of IL-12, IL-15, and IL-18 raised special interest, as it leads to the so-called "cytokine-induced memory-like" (CIML) NK cells in mice and humans, which exhibit an increased capacity to produce IFN-γ upon re-stimulation at later time points (56, 105). Importantly, this memory response is a cell intrinsic effect that is passed on to offspring cells and is maintained up to several months (56). In mice, the intrinsic ability for mediated IFN-γ production coincided with demethylation of the conserved non-coding sequence 1 in the IFN-γ locus (106). Furthermore, adoptive transfer of CIML NK cells had a clear antitumor activity against established melanoma or lymphoma *in vivo*, which required IL-2 from $CD4^+$ T cells (57, 106). For both, murine and human NK cells, IL-12, IL-15, and IL-18 together induce an increased expression of CD25, making CIML NK cells responsive to low concentrations of IL-2 *in vitro* and *in vivo* (57, 58). Thus, there is a clear rationale to apply adoptive transfer of *ex vivo*-generated CIML NK cells together with IL-2 injections as a combination therapy. Recently, CIML NK cells together with low dose IL-2 therapy were evaluated in a first-in-human phase I clinical trial with promising results, as clinical response was observed in five of nine treated patients (107).

AUTOLOGOUS ACCESSORY CELLS AND AUTOLOGOUS FEEDER CELLS FOR NK CELL EXPANSION

Although cytokines efficiently activate NK cells and result in cell products with advanced effector functions, cytokines alone do not allow pronounced *ex vivo* expansion (**Table 1**). Consequently, in addition to the activation with cytokines, stimuli from autologous accessory cells can be used to further enhance the expansion of NK cells to overcome the hurdle of limited NK cell doses for adoptive NK cell therapy (**Table 2**). Outgrowth of NK cells from the whole PBMC fraction is more effective than cultivation of pure NK cells, because other cell types provide additional factors for NK cell proliferation. $CD14^+$ cells, for instance, enhance the *ex vivo* NK cell proliferation *via* direct cell contact and soluble factors (108, 109). After activation, for instance by concanavalin A, T cells also trigger NK cell proliferation (110).

Stimulation of PBMC with IL-2 and the clinically approved anti-CD3 antibody OKT-3 leads to a profound outgrowth of NK cells (25, 65–67, 111), probably by activation of T cells and this is utilized by several clinical protocols for NK cell cultivation. Nevertheless, starting the culture from PBMC goes along with

extensive coexpansion of unwanted $CD3^+/CD56^-$ T cells and $CD3^+/CD56^+$ NK-like T (NKT) cells, accounting for the majority of cells in the final cellular product. Surprisingly, infusion of this heterogeneous cell product without removal of potentially alloreactive T cells did not cause side effects, such as GvHD, in a safety trial with five cancer patients, evaluating the cultivated cellular product in an allogeneic setting (66). This can be explained by the fact that T cells may lose their alloreactivity during extended *ex vivo* expansion (112). Thus, low NK cell purities may be less critical for long-term cultivated cellular products compared to NK cells directly obtained from a donor, but more clinical data are required to prove this hypothesis. Of note, the approach also allows efficient expansion of functional patient-derived NK cells, as shown for B cell chronic lymphocytic leukemia and multiple myeloma patients, enabling therapy with autologous NK cells and further circumventing possible safety risks of therapy with donor-derived cells (25, 111).

Starting with PBMC enriched for CD56 cells together with the corresponding non-CD56 PBMC in a 1:1 mixture favors a 89-fold NK cell expansion with a final product consisting of 92% NK cells after 21 days (69). Alternatively, adding irradiated autologous PBMC to the culture is a strategy to benefit from these "feeder cells" for NK cell activation and expansion but to avoid their coexpansion (**Table 3**). Of note, to make a clear difference, we use the term "feeder cells" for all inactivated cells that are added to the culture, whereas cocultured non-NK cells that are not inactivated are defined as "accessory cells." Besides its growth inactivating function, irradiation can induce upregulation of stress-regulated surface molecules on PBMC, such as ULBP1–3, that further trigger NK cell activation, e.g., through NKG2D (113). Still, irradiated autologous PBMC induce only weak NK cell proliferation without additional activation of the feeder cells (e.g., only 16-fold expansion within 2 weeks) (24). Whereas irradiated autologous PBMC previously activated with IL-2, OKT-3 and RetroNectin allow a median 4,720-fold NK cell expansion after 3 weeks with a NK cell purity of 91% starting from PBMC (114). To obtain a more pure final product with 98% NK cells, it is possible to start the culture with already CD3-depleted PBMC and add irradiated autologous PBMC as feeder cells together with IL-2 and OKT-3 (23). The highest purity can be achieved by cell sorting, representing also the method of choice to expand defined NK cell subpopulations. As demonstrated by Siegler et al., GMP-sorted and highly pure single KIR^+ NK cells can be expanded 160- to 390-fold in 19 days with IL-2, IL-15, OKT-3, and irradiated autologous PBMC (50).

NK CELL EXPANSION WITH ALLOGENEIC FEEDER CELLS

Using irradiated allogeneic cells as feeder cells is another option to stimulate NK cell expansion *ex vivo* (118) (**Table 4**). Compared to autologous PBMC, allogeneic PBMC may be even more efficient as feeder cells for NK stimulation. Accordingly, in a study testing the expansion of NK cells from patients with advanced lymphomas or terminal solid tumors, 300-fold NK expansion was obtained with irradiated allogeneic PBMC feeder cells from healthy donors, whereas only 169-fold expansion was achieved with irradiated autologous PBMC feeder cells from the patients (115). Furthermore, whereas the availability of autologous feeder cells is limited, as they have to be obtained directly from the patient, for allogeneic feeder cells it is possible to utilize established cell lines. Cell lines can be grown easily to sufficient numbers and different cell lines in fact trigger NK cell proliferation, such as HFWT, K562, RPMI 1866, Daudi, KL-1, MM-170, and different EBV-transformed lymphoblastoid cell lines (EBV-LCL) (99, 119–122).

Culturing PBMC together with the Wilms tumor cell line HFWT and IL-2 leads to significant NK cell expansion (124, 145), and interestingly under this condition NK cells not only arise from mature $CD3^-CD56^+$ NK cells but also from $CD3^-CD14^-CD19^-CD56^-$ NK cell precursors expressing CD122 (146). In 2004, early clinical data showed that adoptive transfer of autologous NK cells generated by coculture with irradiated HFWT is safe and patients with recurrent malignant glioma partially responded to the treatment (125).

Another advantage of cell lines is that it is relatively easy to genetically modify them and to integrate additional factors for NK cell stimulation. In recent years, modified K562 cells have been utilized, such as K562 expressing membrane-bound IL-15 and 41BBL (K562-mb15-41BBL) (126). While unmodified K562 only induce a weak NK cell proliferation (2.5-fold NK cell expansion in 1 week), with K562-mb15-41BBL the NK cell number can be significantly increased by 20- or 1,000-fold in 1 or 3 weeks (126). In addition, stimulation of NK cells with K562-mb15-41BBL demonstrated that NK cells actually have a substantial proliferative potential *ex vivo*, with up to 30 population doublings and 5.9×10^4-fold NK cell expansion (147). NK cells expanded with K562-mb15-41BBL exhibit enhanced natural cytotoxicity against several allogeneic and autologous tumors *in vitro*, efficiently mediate ADCC and showed antitumor efficacy in mouse xenograft models for the treatment of sarcoma and myeloma (128, 148, 149). Of note, in a clinical trial assessing adoptive transfer of K562-mb15-41BBL following HSCT, acute GvHD occurred in five of nine patients, although the donors were completely HLA matched and the doses of injected NK cells and cotransferred T cells were low ($1–10 \times 10^5$ and $\leq 2 \times 10^4$/kg) (131). These observations suggested that the acute GvHD was T cell mediated, but NK cells apparently may promote this severe side effect indirectly (150). Importantly, another group utilized NK cells expanded with a similar K562 variant expressing 41BBL and IL-15 in another treatment setting and did not observe GvHD, although up to 1×10^8 NK cells/kg were administered (129).

Furthermore, Denman and colleagues revealed that K562 expressing 41BBL and membrane-bound IL-21 instead of IL-15 are even more effective for *ex vivo* expansion of NK cells, and weekly restimulation with this cell line supports a sustained NK cell proliferation over several weeks (134). In coculture with K562 expressing membrane-bound IL-21 and 41BBL, NK cells show an increased telomere length and enhanced activation of the STAT-3 signaling pathway, explaining the positive effect for sustained expansion of NK cells over long time (134, 151). Adoptive transfer of NK cells expanded with K562 expressing membrane-bound IL-21 and 41BBL into tumor-bearing mice improved the survival of the animals, indicating a therapeutic effect of these NK cells (135).

TABLE 3 | *Ex vivo* cultivation of natural killer (NK) cells with autologous feeder cells.

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
Irr. autologous PBMC (depleted for $CD3^-/CD56^+$ cells) + IL-2 + IL-15	PBMC, CD3 depleted, and CD56 enriched in flasks	16 (14 days)	97% NK 0.2% T cells	*Upregulated*: NKG2D, DNAM-1, NKp30, NKp44, CD158a, and CD158e	Efficient degranulation and lysis of K562 *In vitro*	*In vitro*	(24)
Irr. autologous PBMC activated with OK432, FN-CH296 and OKT-3 + IL-2	PBMC in flasks and bags	4,720 (21–22 days)	91% NK ~12% NK-like T and T	Strong expression of NKG2D and CD16	Elevated cytotoxicity that is maintained for up to 4 weeks after infusion to patients	Clinical	(114)
Irr. autologous PBMC + OKT-3 + IL-2	PBMC, CD3 depleted, and CD56 enriched in plates	169 (14 days)	84% NK	*Upregulated*: CD16, CD56, NKG2D, NKp30, and NKp44	Increased cytotoxicity against tumor cell lines *in vitro*	*In vitro*	(115)
	PBMC, CD3 depleted in flasks and bags	278–1,097 (21–26 days)	91–98% NK	Most cells express NKG2D, CD16, CD94, NKp46, KIR2DL1, KIR3DL1, and KIR2DL2/3	Efficient lysis of tumor cell lines *in vitro*; persistence in patients up to several months; cytotoxic potential is lost *in vivo*, while ability for ADCC is maintained	Clinical	(116)
	PBMC, CD3 depleted in bags	691 (14 days)	98% NK 0.06% T cells	*Upregulated*: NKG2C, NKp30, NK44, CXCR4, CD25, CD62L, and CD69	Increased cytotoxicity against tumor cell lines *in vitro*; antitumor effect and ADCC activity in a leukemia xenograft mouse model; up to 4 days persistence in patients	Preclinical model	(23)
		758 (14 days)	98% NK 0.4% T cells			Clinical	(117)
Irr. autologous PBMC (depleted for $CD3^-/CD56^+$ cells) + OKT-3 + IL-2	PBMC, CD3 depleted, and CD56 enriched in plates and flasks	546 (14 days)	94.9% NK 2.2% T cells	*Upregulated*: NKG2D, NKp30, NKp44, tumor necrosis factor-related apoptosis-inducing ligand, and DNAM-1 *Downregulated*: NKp80	Increased cytotoxicity against tumor cell lines *in vitro*	*In vitro*	(113)
Irr. autologous PBMC + OKT-3 + IL-2 ± IL-15	PBMC, CD3 depleted, and CD56 enriched in plates and bags	117/63 in bags (±IL-15) 993 in plates (19 days)	Bags: 45% NK 0.6% T cells	*Upregulated*: NKG2D, NKp44	High cytotoxicity against K562 and high productivity of IFN-γ	*In vitro*	(50)
	Good manufacturing practice killer cell immunoglobulin-like receptor (KIR) sorted NK cells in bags	160–390	~100% NK >0.01% T cells	Single KIR + NK cells	Anti-leukemic activity against primary acute myeloid leukemia cells *in vitro* and *in vivo*	Preclinical model	(50)

TABLE 4 | *Ex vivo* cultivation of natural killer (NK) cells with allogeneic feeder cells.

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
Irr. allogeneic PBMC activated with ConA + IL-2	*In vivo* IL-2 primed PBMC depleted for non-NK cells in flasks	1–148 (14 days)	64–98% NK	N/A	Cytotoxic activity against leukemic cell lines	Clinical	(123)
Irr. allogeneic PBMC activated with ConA, PHA and ionomycin + IL-2 + IL-15	PBMC, depleted for CD3, CD4, CD19, and CD33 in bags	80–200 (15 days)	91% CD56 0.3% CD3 (day 12)	*Upregulated*: CD16, CD25	Increased cytotoxicity against tumor cell lines *in vitro*; decreased frequency of INF-g producing cells	*In vitro*	(118)
Irr. allogeneic PBMC + OKT-3 + IL-2	PBMC, CD3 depleted, and CD56 enriched in plates	300 (14 days)	94% NK	*Upregulated*: CD16, CD56, NKG2D, NKp30, and NKp44	Increased cytotoxicity against tumor cell lines *in vitro*	*In vitro*	(115)
Irr. HFWT + IL-2	PBMC in flasks	113 (2 weeks)	86% $CD56^+$/$CD16^+$	N/A	Cytotoxic against tumor cell lines *in vitro*	Clinical	(124, 125)
Irr. Jurkat/KL-1 + IL-2	PBMC in flasks	~130 (2 weeks)	40–90% NK	*Upregulated*: CD54, CD11a, CD48, CD2, CD49d, CD58, NKp30, NKp44, 2B4, DNAM-1, NKG2D, CD25, and CD69 *Downregulated*: CD16	Increased cytotoxicity against tumor cell lines *in vitro* and antitumor activity *in vivo*	Preclinical model	(121)
Irr. K562 expressing membrane-bound IL-15 and 41BBL + IL-2	PBMC in plates	1,089 (3 weeks)	"Virtually pure"	N/A	N/A	*In vitro*	(126)
	PBMC in bags	23, 152, and 277 after 7, 14, and 21 days	96.8% NK 3.1% T cells (day 21)	Marked differences of gene expression profile compared to unstimulated or IL-2-stimulated NK cells	Increased cytotoxicity against tumor cell lines *in vitro* and antitumor activity *in vivo*	Preclinical model	(127)
	PBMC	447 (days 10–14)	88% NK 2.2% T cells (day 14)	Upregulated genes for cytolytic activity, cytokines, chemokines, activating receptors, adhesion molecules, cell cycle regulators, and multiple pathways	Increased cytotoxicity against primary MM cells *in vitro* and *in vivo*; high productivity of IFN-γ	Preclinical model	(128)
	PBMC in G-Rex, bags	442—G-Rex 227—bags (10 days)	70% NK 5–35% T cells	*Upregulated*: NKp30, NKp44, NKG2D, CD26, CD70, and CXCR3 *Downregulated*: CD16, CD62L	Increased cytotoxicity and ADCC against primary tumor cells *in vitro*; robust *in vivo* proliferation post-infusion	Clinical	(129, 130)
Irr. K562 expressing membrane-bound IL-15 and 41BBL + IL-15	PBMC, CD3 depleted, and CD56 enriched	1,000 (21 days)	N/A	*Upregulated*: CD56, NKG2D, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), CD158a, CD158b, and CD158e1	Increased cytotoxicity *in vitro* independent of killer cell immunoglobulin-like receptor mismatch; NK infusion contributed to acute graft-versus-host disease in first clinical trial	Clinical	(131, 132)
Plasma membrane particles of K562 expressing IL-15 and 41BBL + IL-2	PBMC in plates and flasks	1,265 (17 days)	86% NK cells 9% T cells 2% NK-like T	*Upregulated*: NKp30, NKp44, NKp46, NKG2D, 2B4, NKG2A, TRAIL, and Fas ligand (FasL) *Downregulated*: CD16	Increased cytotoxicity against leukemic cell lines and primary acute myeloid leukemia (AML) cells *in vitro*	*In vitro*	(133)
Irr. K562 expressing membrane-bound IL-21, 41BBL, CD64, CD86, and CD19 + IL-2	PBMC in flasks	4.8×10^4 (21 days)	21.7% T cells	High expression of natural cytotoxicity receptors, CD16, and NKG2D	Cytotoxic against tumor cell lines *in vitro*; capable of ADCC; increased telomere length	*In vitro*	(134)
		2,363 (14 days)	83% NK 9.1% T cells	*Upregulated*: DNAM-1, NKG2D, CD16, and CD56	Cytotoxic and capable of ADCC against neuroblastoma cell lines *in vitro* and *in vivo*	Preclinical model	(135)

(*Continued*)

TABLE 4 | **Continued**

Protocol features	Starting material/culture system	NK cell expansion rate	NK cell purity	NK cell phenotype	NK cell function	Setting	Reference
Plasma membrane particles of K562 expressing membrane-bound IL-21 and 41BBL + IL-2	PBMC	825 (14 days) $>10^5$ (28 days)	>90% NK (day 14)	N/A	Increased cytotoxicity against leukemic cell lines and primary AML cells *in vitro*; enhanced proliferation *in vivo*	Preclinical model	(136)
Irr. allogeneic PBMC; irr. EBV transformed lymphoblastoid cell lines (EBV-LCL) (LAZ 388 cells) + PHA + IL-2	PBMC depleted for CD3 and monocytes in bags and plates	~43 (31–21 days)	90% NK <5% T cells	N/A	Increased cytotoxicity against tumor cell lines *in vitro*	Clinical	(137, 138)
Irr. EBV-LCL (TM-LCL) + IL-2	PBMC, CD3 depleted, and CD56 enriched in bags	800–1,000 (2 weeks)	98% NK	*Upregulated*: TRAIL, FasL, NKG2D, NKp30, NKp44, NKp46, CD48, CD25, LTB, MX1, and BAX	Increased cytotoxicity against tumor cell lines *in vitro*	*In vitro*	(139, 140)
Irr. EBV-LCL (SMI-LCL) + IL-2	PBMC, CD3 depleted, and CD56 enriched in bags	3,637 (24–27 days)	99.7% NK			Clinical	(141)
	PBMC, CD3 depleted, and CD56 enriched in CliniMACS Prodigy	850 (14 days)	>99% NK	*Upregulated*: TRAIL, FasL, NKG2D, NKp30, NKp44, and DNAM-1	Increased cytotoxicity and ADCC against tumor cell lines *in vitro*	*In vitro*	(142)
Irr. EBV-LCL (SMI-LCL) + IL-2 + IL-21	PBMC depleted for non-NK cells (research kit) in plates and flasks	2,900 (14 days) 2.7×10^{11} (46 days)	>99% NK	*Upregulated*: TRAIL, NKG2D, and DNAM-1	Cytotoxic against tumor cell lines *in vitro* and *in vivo*; enhanced and sustained production of IFN-γ and TNF-α	Preclinical model	(96)
Lysate of CTV-1	PBMC, CD3 depleted, and CD56 enriched	N/A (overnight)	97–98% NK	*Upregulated*: CD69 *Downregulated*: CD16	Cytotoxic against NK-resistant leukemia cell lines and primary tumors *in vitro*	Clinical	(143, 144)

The stimulatory effect of EBV-LCL on NK cell proliferation was discovered more than 30 years ago (152). In 1994, an early clinical trial already evaluated the adoptive transfer of autologous NK cells expanded with the LAZ 388 cell line to treat 10 patients with metastatic renal cell adenocarcinoma (137). More recently, the cell lines TM-LCL and SMI-LCL were reported for NK cell expansion, allowing around 800-fold expansion of highly pure NK cells within 2 weeks (139–142). NK cells generated with these EBV-LCL feeder cells are currently applied in a study testing them for adoptive transfer in an autologous setting with intended doses up to 1×10^9 NK cells/kg (141). Recently, it was reported that repeated stimulation with SMI-LCL in IL-2-containing medium and adding IL-21 only at start of cultivation enables 10^{11}-fold NK cell expansion after 6 weeks, to our knowledge representing the most efficient protocol to expand NK cells at the moment (96). NK cells generated with the latter method are highly cytotoxic *in vitro*, show a sustained high productivity of IFN-γ and TNF-α, similar to CIML NK cells, and they efficiently controlled melanoma in a xenograft mouse model (96).

Although feeder cells, and allogeneic feeder cell lines in particular, make it possible to generate substantial numbers of NK cells for adoptive therapy, from a regulatory point of view this strategy has drawbacks as feeder cell lines must be qualified as safe for human use. The cell line qualification of modified K562 cells, for instance, includes costly viral testing and assays to prove absence of bacterial and *Mycoplasma* contamination (153). In this context, lysates from cell lines containing the NK cell-stimulating factors could be an alternative to the intact feeder cells to minimize regulatory concerns. It was demonstrated that short cultivation of NK cells with lysate of the leukemia cell line CTV-1 primes NK cells to specifically lyse cell lines that are resistant to resting NK cells (143). Interestingly, the priming effect of CTV-1 on NK cells is KIR independent and does not require supplementation of cytokines, such as IL-2 or IL-15, making this an unique approach for NK cell activation (154). NK cells primed with CTV-1 were evaluated in the first UK clinical trial of a cell therapy regulated as a medicine, with an anti-leukemia effect in four of seven treated patients and no evidence of NK cell infusion-related toxicities (144). Another step forward from a regulatory standpoint could be to add only specific fragments of feeder cells to the culture that are responsible for the desired NK cell activation, instead of using intact feeder cells or their lysates. Of note, NK cells can be expanded *ex vivo* with IL-2 and plasma membrane particles prepared from K562-expressing membrane-bound IL-15 and 41BBL with a rate of expansion that is comparable to stimulation with intact feeder cells and far better than stimulation with soluble IL-15, 41BBL, and IL-2 (133). Plasma membrane particles from K562 expressing membrane-bound IL-21 and 41BBL work for *ex vivo* NK cell expansion as well and may be an option for *in vivo* NK cell expansion, as demonstrated in a first proof of concept using a mouse model (136).

TECHNICAL ASPECTS OF NK CELL EXPANSION

In general, one encounters technical challenges and opportunities when manufacturing NK cells as medicinal products, as reviewed recently (155). In this section, we focus on technical options for NK cell culture, ranging from simple cell culture plates for small scale experiments to highly standardized and automated systems for clinical scale. The selection of the adequate culture system is based on the intended application of the cells. Most preclinical experimental studies grow NK cells in cell culture plates or tissue culture (T) flasks. These are commonly used and very convenient to test and compare different culture additives in parallel, e.g., different cytokine concentrations. However, for clinical applications in large scale, cultivation in plates and flasks is rather inappropriate for different reasons. First, due to the small volume of T flasks, numerous T flasks have to be handled at the same time, with for instance 51 T flasks for the treatment of a single patient (116). In addition, T flasks have to be opened from time to time for medium exchange or harvesting of cells, bearing the risk of contaminating the cellular product. Although the likelihood of contamination for each T flask is reduced to a minimum by sterile workflows in safety cabinets, the remaining risk potentates by the number of flasks.

To overcome the drawbacks of small cell culture vessels, clinical NK cell cultivation is often done in cell culture bags, which make it possible to culture high volumes in a closed system, as all required steps can be done by sterile welding of tubing connections for the transfer of media, harvesting of cells, etc. Unfortunately, different reports describe that the NK cell expansion performance is reduced after transition of a protocol from T flasks to larger scale in cell culture bags (50, 67). In addition, bag systems still require several labor-intensive interventions during the culture, especially when different cultures are set up in parallel.

The G-Rex vessel is another system avoiding frequent processing steps for exchange of medium during the culture. In contrast to normal cell culture flasks, the bottom of the G-Rex is highly gas permeable, ensuring optimal CO_2 exchange and O_2 supply for the cells. Thus, by its design, G-Rex flasks can be filled directly with a high level of cell culture medium and exchange of medium is not necessary for long time. For NK cell culture, G-Rex were used for example for 10 days of culture without any cell manipulation or feeding, and resulted in higher fold expansion of NK cells compared to cell culture bags (130). Unfortunately, although G-Rex are scalable in general, multiple G-Rex flasks are still required to achieve high cell numbers for clinical trials, which can be cumbersome and costly, and G-Rex flasks are still an open system and may require adaption to a closed system (156).

Automated systems combine the need for reduced interventions during the culture with a closed system. Automation of the cell manufacturing ensures constant product quality without the need for highly skilled experts, is finally cost saving, and may be required for cellular therapy to become available beyond specialized academic centers (157). Although early integration of automation is associated with higher capital costs in the development phase, it allows a smooth transition at later stages of clinical development (158). A first feasibility study of automated NK cell cultivation with a stirred bioreactor was already published in 1996, showing advantages of the bioreactor culture over manually handled controls (159). More recently, different investigators report automated NK cell expansion procedures with a rocking motion bioreactor (67, 69, 156, 160), yielding $2–10 \times 10^9$ NK cells

under GMP-compliant conditions. However, the latter system still needs preceding manual cultivation, because relatively high cell numbers are required as inoculum for the automated culture (67, 69, 156, 160). Alternatively, fully automated NK cell expansion with an automated cell processing device can be performed for clinical use, with as little as 10^6 NK cells being sufficient to initiate the automated culture that can yield up to 2.7×10^9 NK cells after stimulation with clinical grade feeder cells (142). Of note, in addition to the culture process, the cell processing device is designed for GMP-compliant cell separation, concentration, and washing applications, so that combined NK cell purification, cultivation, and final formulation of the cellular product is possible fully automated (161). Thus, the whole processing, from the starting material, such as a leukapheresis product, to the finally expanded NK cells, readily prepared for infusion, can be covered by a single instrument.

Centralized processing of NK cell products probably will be carried out mainly in specialized centers for manufacturing of cellular products. Consequently, after *ex vivo* cultivation, storage of the NK cell product and shipment to the location of use will be needed. Compared to naive NK cells, IL-2-activated NK cells are less sensitive to freezing, as they show higher recovery and viability after thawing (26). Still, different groups state that cryopreservation of cultivated NK cells goes along with a drop in cell viability and cytotoxicity, whereas the latter can be restored by a short re-stimulation, e.g., by a short resting in IL-2-containing medium (139, 156). Poor survival of the NK cells can be an issue during further *in vitro* culture post thawing, so that shipping of freshly formulated cells for direct infusion may be advantageous (129). Interestingly, some groups recently claim that freezing and thawing does not influence the cytotoxicity or the proliferative ability of cultivated NK cells in their hands (24, 68). These divergent observations possibly result from different cultivation methods and different protocols for freezing and thawing, which should be investigated further. Without freezing, transport of the readily prepared cells in an appropriate time frame is challenging, and any delay during the shipment affects the quality of the cellular product with critical consequences for the patient. Alternatively, automated and closed systems for cell processing open the way for scale out strategies and de-centralized NK cell manufacturing directly at the location of intended use, avoiding the freezing and shipment process (142). But, although de-centralized manufacturing in the clinics seems promising, cellular therapeutics are very complex and still in early development, so that manufacturing by well-trained specialists in specific facilities is reasonable at that state.

REGULATORY ASPECTS OF NK CELL CULTIVATION FOR CLINICAL USE

Apart from technical difficulties, one has to consider regulatory aspects for the use of *ex vivo*-generated NK cells with regulations varying in time and geographical policies (153). In Europe, for instance, cytokine-activated and -expanded NK cells are currently classified as advanced therapy medicinal products and will be regulated accordingly either centralized or under the hospital exemption by the member states [Regulation (EC) No 1394/2007; Directive 2001/83/EC and Regulation (EC) No 726/2004]. Quality aspects related to somatic cell therapy medicinal product as defined in guidelines (CPMP/BWP/3088/99; EMEA/CHMP/410869/2006; Ph. Eur. 0784: Ph. Eur. 5.14) will apply to the identity, potency, and activity. The establishment of correspondingly adequate in process and quality controls as well as of process target values and product specifications will have to take into account the variability of the primary effector cell as the starting material (162).

CONCLUSION AND OUTLOOK

Comparing different protocols for NK cell cultivation in detail is challenging as these are extremely heterogeneous. The duration of *ex vivo* NK cell cultivation ranges from a few hours for short NK cell activation up to several weeks for long-term expansion, different starting materials are in use with varying NK cell purities, different cytokines are combined at different doses, and NK cells often are cocultured with different feeder cells at different NK-to-feeder ratios. Nevertheless, overall differently *ex vivo* expanded NK cells exhibit some common characteristics.

In general, *ex vivo* cultivated NK cells show an increased cytotoxicity and may become even responsive against tumor targets previously appearing resistant to NK cell lysis. This explains the use of IL-2 or IL-15 in virtually every protocol, as it is known since a long time that both cytokines amplify NK cell activity (81, 163). However, upon NK cell activation with different stimuli, including IL-2 and IL-15, downregulation of CD16 surface levels occurs by metalloproteases-mediated shedding of CD16 (164–166). The Fc receptor CD16 is crucial for NK cells to perform ADCC and would be of particular importance for potential combination therapies using NK cells together with therapeutic antibodies. Of note, although reduced levels of CD16 on NK cells are observed for several NK cell cultivation protocols the NK cells still mediate ADCC (70, 129, 142). Nevertheless, inhibition of the relevant metalloproteases to maintain CD16 on NK cells could be an option to further increase the ADCC function of *ex vivo* activated NK cells (164, 167).

Another clinically highly relevant aspect is the tumor-induced immunosuppression as important challenge for all cell therapeutic strategies. Remarkably, it ruled out from most preclinical and clinical NK cell studies that NK cells may gain the capability to overcome tumor immunosuppression. Different research groups have reported signs of NK cell suppression in cancer patients such as a lower expression of NK cell receptors, e.g., NCRs, NKG2D, DNAM-1, and 2B4 (22, 25, 168–170), the shedding of tumor cell ligands, such as NKp30 and NKG2D (171–174), or the release of blocking NKG2D ligands, such as MICA and ULBP3, *via* tumor-derived exosomes (175, 176). Notably, *ex vivo* cultivation of patient-derived NK cells is often possible with same efficacy as for donor-derived NK cells (25, 111) and can normalize the NK cell phenotype and activation (25). Additionally, elevated levels of NKG2D on *ex vivo*-activated NK cells can scavenge shed NKG2D ligands and counter their inhibitory effect (177). Furthermore, the high cytotoxicity of *ex vivo* expanded NK cells has been shown to be independent of KIR inhibition for some protocols (107, 132).

In comparison to other cell therapeutic approaches using, e.g., T cells, donor-derived allogeneic NK cells mediate GVL without an elevated risk for GVHD or even with a GVHD-reducing effect, as reported in mice and men (74, 75, 77, 78). However, contradictory results regarding GVHD induction have been reported in clinical trials assessing adoptive transfer of NK cells expanded with K562 feeder cell variants expressing 41BBL and IL-15 (129, 131). These reports show that there are still open questions that have to be unraveled to better understand the complex role of NK cells and their specific subsets in the bidirectional regulation of GVL and GVHD.

In conclusion, many different protocols are in use to expand NK cells *in vitro*, each with its specific advantages and disadvantages in regard of cell numbers, function, and handling efforts. The data summarized in this review underline the complexity related to the design of an optimal NK cell therapeutic protocol that should be not only reliable and safe in use but also highly efficient in targeting different forms of malignancies. With this in mind, additional studies need to be envisioned that not only further address *ex vivo* NK cell purification, expansion, and activation strategies but also the final clinical setting including pre-conditioning, dosing, and timing of the NK cell application. Efforts for harmonization of protocols at the European and worldwide level should be undertaken to ensure highest quality and efficacy of the NK cell product for clinical application. Finally, with regard to the possible tumor-mediated immunosuppression, therapeutic concepts have to be developed that either directly strengthen NK cells to deal with the hostile tumor environment and/or specifically counteract tumor-induced immunosuppressive mechanisms.

AUTHOR CONTRIBUTIONS

MG, JW, and EU extensively reviewed the current literature on *ex vivo* NK cell cultivation and expansion and prepared a comprehensive overview that is listed in the tables. MG, JW, UK, AC, VH, and EU wrote and critically reviewed the manuscript.

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Expansion of natural killer (NK) and natural killer-like T (NKT)-cell populations derived from patients with B-chronic lymphocytic leukemia (B-CLL): a potential source for cellular immunotherapy

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B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the Western world. It is currently an incurable disease, making new treatment options such as immunotherapy desirable. Monoclonal antibodies (Mabs) to surface antigens of the tumor cell is one option. Administration of cytotoxic cells such as natural killer (NK) and natural killer-like T (NKT) cells expanded *in vitro* might be a useful treatment modality alone or in combination with MAbs. A limiting step in the development of successful cellular immunotherapy has been the availability of appropriate cytotoxic cells. Here, we report the feasibility of expanding populations of the human killer cells, CD3−CD56+ NK and CD3+CD56+ NKT cells, from peripheral blood mononuclear cells (PBMCs) of B-CLL patients. The influence of tumor B cells on the *in vitro* expansion of killer cells was assessed by depleting B cells from PBMCs by microbead separation before culture. The 21-day cultures from both B-cell- and non-B-cell-depleted PBMC showed a marked expansion of NK cells, and also of T cells, among which almost half had the NKT phenotype. Depletion of B cells before culture did not change the expansion rates of NK and NKT cells significantly. In patients with progressive B-CLL, NK cell expansion capacity was improved after fludarabine treatment when compared to samples obtained before treatment. Repeated samples of PBMCs from individual untreated patients with both indolent and progressive disease cultured under identical conditions gave similar NK cell expansion rates. Expanded killer cell populations had cytotoxic function against the NK-sensitive target K562 cell line and expressed high levels of Granzyme B. From our studies, we conclude that NK cells as well as NKT cells from the peripheral blood of B-CLL patients can be expanded, and that these cells have cytotoxic capacity.
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Introduction

Chronic lymphocytic leukemia of the B-cell type (B-CLL) is the most common subtype of indolent lymphoma characterized by an accumulation of long-lived clonal B cells in the bone marrow, blood, lymph nodes, and spleen.[1] The clinical course of this disease is highly variable. Clinical staging systems as Rai and Binet have been used to predict the prognosis. Some patients with a low clinical stage might live for 20 years or more, while patients with an advanced stage (B or C according to the Binet classification) have considerably reduced survival time.[2,3]

Despite new treatment regimens, CLL remains incurable. Good response to fludarabine has been reported, however, without significant prolongation of survival time.[3,4] Allogeneic bone marrow transplantation may yield complete remissions,[5,6] but its applicability is limited by graft-versus-host disease (GVHD) and the low availability of donors. Autologous stem cell transplantation often leads to good responses but with a high incidence of relapse.[7] Thus, alternative methods of treatment for patients with B-CLL are needed.

Two monoclonal antibodies (Mabs), Rituximab (anti-CD20)[8] and Campath-1H (anti-CD52),[9] have recently been introduced for the treatment of CLL patients. Possible mechanisms for destruction of leukemic cells by these Mabs include antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-mediated lysis. Immunologic effector cells such as cytotoxic natural killer (NK) and T cells are important for ADCC, and cell therapy using peripheral blood-derived, IL-2-activated killer cells might enhance the efficacy of Mab therapy. However, major obstacles in developing this treatment have been the identification and expansion of such effector cells from the patients' peripheral blood in sufficient numbers.

NK cells are a subset of cytolytically active lymphocytes killing virally infected or tumor targets but to spare normal cells.[10,11] In cancer-bearing hosts, NK cells have been considered as an important component of antitumor immunity responsible for rapid elimination of blood-borne metastases.[10,11] The cytolytic activity of NK cells occurs spontaneously (ie in the absence of deliberate, prior immunization) and does not require expression of syngeneic major histocompatibility complex (MHC) antigens by target cells. NK cells do not appear to express one dominant receptor that dictates the specificity of cytolysis. Rather, triggering of NK cell cytotoxicity reflects a balance between activating and inhibitory signals mediated by cell-surface receptors that belong to several different gene families.[12–15]

In B-CLL patients, several phenotypic and functional alterations in NK cells[16,17] have been described such as defective cytotoxic activity.[16–19] The reason as to why NK activity is low in most patients with CLL is unclear. A dilutional effect of leukemic cells and intrinsic functional defects on NK cells have been implicated.[16,20,21] These abnormalities found in NK cells might allow the appearance and/or maintenance of the leukemic B-cell accumulation. However, defective NK activity has been shown to be restored by interferon (IFN)-α and IL-2.[19]

Natural killer-like T (NKT) cells were first described as a distinct subset of T cells less than a decade ago,[22,23] express surface receptors that are also found on conventional T cells, together with receptors that are characteristic of NK cells. One striking property of NKT cells is their capacity to produce a spectrum of cytokines rapidly, including interleukin (IL)-4, IL-10, IL-13, interferon (IFN)-γ, and tumor necrosis factor-α, upon engagement with T-cell receptors.[22,23] The role of NKT cells in antitumor immunity has been investigated both in models of natural immunity and in models where immunity is induced by

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the cytokine IL-12.[24] NK cells and conventional T cells contribute to the therapeutic activities of this cytokine as well.[25] Moreover, NKT cells can prevent GVHD after bone marrow transplantation[25] in an IL-4-dependent manner. Obtaining a large number of NKT cells may be instrumental in controlling certain tumors and GVHD.

Considering the potential usefulness of autologous killer cells such as CD3−CD56+ NK cells and CD3+CD56+ NKT cells in immunotherapy of B-CLL, we explored the feasibility of expanding these populations from the peripheral blood of B-CLL patients.

Materials and methods

Patients

Peripheral blood samples of six patients with phenotypically classical (CD19+CD5+CD23+) B-CLL were included in the study. All patients had a total lymphocyte count of $>5\times10^9$ Lmp/l and fulfilled all diagnostic criteria for B-CLL.[26] The patients were followed from diagnosis until January 2002 at the Clinic of Hematology, Huddinge University Hospital, Karolinska Institutet. At the time of the first blood sampling, all patients were untreated. The clinical characteristics of these patients are shown in Table 1. Four of the patients (AD, KO, LS, and YO) had indolent disease for more than 5 years requiring no treatment. Two previously untreated patients (LB and RL) showed progressive disease requiring therapy,[26] and were retested after therapy. Patient LB had a progressive disease with severe night sweats, increase in the size of the spleen, and rapid increase in the absolute number of circulating lymphocytes. The patient had blood drawn at two time points before treatment and was retested after two cycles of fludarabine/cyclophosphamide, when the disease showed a complete remission in blood. Patient RL showed a rapid progression in lymphocytosis, abdominal lymphadenopathy, and spleen size. Blood samples were drawn just before the first course of fludarabine single therapy and before the next course, when blood lymphocytes were reduced to less than half of the pretreatment value. This study was approved by the local ethics committee at Huddinge University Hospital, Stockholm, Sweden. All the patients studied were untreated unless otherwise indicated. All the samples were encoded with patients' initials. The patients having progressive disease were indicated with '#' symbol after their initials (Patients' initials#). The samples obtained from the same untreated patients were encoded with number (Patients' initials1, patients' initials2, etc); however, the samples obtained from the same patients before fludarabine treatment were encoded with 'Patients' initials and sample number^{Before}', and the samples obtained from the same patients after fludarabine treatment were encoded with 'patients' initials and sample number^{After}'.

Cell cultures and quantification

The *in vitro* culture conditions for the expansion of cytotoxic cells have previously been optimized on peripheral blood mononuclear cell (PBMC) from healthy individuals as described elsewhere.[27] In short, peripheral blood was obtained from B-CLL patients and cultures were initiated on the same day (day 0). PBMCs were isolated by gradient centrifugation, using Lymphoprep (Nyegaard, Oslo, Norway). After washing twice with phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA), cell viability was assessed by trypan blue dye exclusion, and the cells which were plated onto six-well dishes (Falcon by Becton Dickinson, Le Pont de Claix, France) at 100×10^4 cells/ml. CellGro SCGM serum-free medium (CellGenix, Freiburg, Germany) was used in all the cultures with the addition of 5%

Table 1 Patient characteristics of the six B-CLL patients at diagnosis and the times of blood sampling

Patient name	*Samples*	*Time interval between samples (month)*	*Year of birth/ gender*	*Year of diagnosis*	*Binet stage at diagnosis*	*Binet stage at the time of the test and response to fludarabine treatment*	*Tot-lym ($\times10^9$/l)*
AD			1920/M	1988	A		
	AD1					A, nonprog.	28.5
KO			1921/F	1983	B		
	KO1					A, nonprog.	24.6
	KO2	4				A, nonprog.	21.6
LS			1932/M	1990	B		
	LS1					A, nonprog.	5.9
YO			1954/F	1996	A		
	YO1					A, nonprog.	31.0
	YO2	1				B	38.9
LB			1946/F	1993	A		
	LB1^{Before} #					B, prog.	78.5
	LB2^{Before} #	1				B, prog.	82.2
	LB1^{After} #	3				A, CR	2.4
RL			1928/M	1993	B		
	RL1^{Before} #						
	RL1^{After} #	1				B, PR	21.6

#, patients with progressive disease, samples obtained before (LB1 and RL1) and after (LB1 and RL1) fludarabine treatment, respectively.[a]Prog, progressive; nonprog, nonprogressive; CR, complete remission; PR, partial remission.

human serum (Sigma, St Louis, MO, USA) and 500 U/ml IL-2 (Peprotech, London, UK). For the first 5 days, the medium was further supplemented with anti-CD3 antibody (Orthoclone OKT-3, Ortho Biotech Inc., Raritan, NJ, USA) to a final concentration of 10 ng/ml. Total cell numbers were assessed by staining cells with trypan blue dye on days 0, 5–6, 9–10, 14–15, and 19–21. Absolute cell counts were calculated by multiplying the total number of cells in each subset with the percentage of these subsets determined by flow cytometry. On days 5–6, the OKT-3-containing medium was washed out, and fresh medium with IL-2 (500 U/ml) but without OKT-3 was added. The cultures were replenished with fresh medium every 2–3 days throughout the culture period. To prevent contact inhibition of cell growth, the cells were transferred to T25 flasks (TPP, Trasadingen, Switzerland) on days 10–11.

Flow cytometric analysis

The cell phenotype was analyzed by flow cytometry on days 0, 5–6, 9–10, 14–15, and 19–21. Three-color fluorescence was analyzed according to standard procedures. Briefly, cells were mixed with appropriate concentrations of fluorochrome-conjugated Mabs to CD3, CD4, CD5, CD8, CD16, CD19, CD25, CD45, and CD56. All antibodies were obtained from Becton Dickinson (BD, Mountain View, CA, USA). After the addition of primary antibody and incubation for 30 min at room temperature, cells were washed with PBS.

Propidium iodide (PI) staining was used for dead cell exclusion. For data acquisition and analysis, a FACSCalibur (BD) was used with Cell Quest software (BD). In each sample, a minimum of 10 000 cells was acquired in the analysis region of viable cells, using log-amplified fluorescence and linearly amplified side- and forward-scatter signals.

All samples were analyzed by setting appropriate SSC/FSC gates around the lymphocyte population, using PI-negative cells. Consistency of analysis parameters was ascertained by calibrating the flow cytometer with calibrating beads and FacsComp software, both from BD.

B-cell depletion

CD19+ B cells were depleted from PBMC using MACS colloidal super-paramagnetic microbeads conjugated to monoclonal CD19 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, PBMCs were washed with PBS and spun down at 1500 rpm for 5 min. After removal of supernatant, cells were resuspended in 80 μl of the separation buffer per 10^7 total cells. A volume of 20 μl of MACS anti-CD19 microbeads per 10^7 total cells was added, mixed, and incubated for 15 min at 4°C. After incubation, cells were washed with separation buffer in a 10–20 × labeling volume and spun down, after which supernatants were removed completely. Cell suspensions were then applied to an LS+ column, which was prewashed with the separation buffer and placed in the magnetic field of an appropriate MACS separator. The column was rinsed with 4 × 3 ml separation buffer. Negative (B-cell-depleted) and positive (B-cell-enriched) selected cells were collected. After separation, cells were washed with PBS, and viability was assessed with trypan blue dye exclusion.

Transwell cultures

B-cell- and non-B-cell-depleted fractions from one of the patients with progressive disease (RL) were cultured in a transwell (Costar, Cambridge, MA, USA; 24-mm diameter, 3.0 μm pore) with the B-cell-enriched fraction in the lower and B-cell-depleted fraction in the upper chamber. To avoid contact growth inhibition, cells from the upper and lower level fractions, respectively, were transferred on day 10 to separate flasks, and culture was continued until day 21.

Immunocytochemical analysis

On day 21, cultured cells were deposited on microscope slides by cytocentrifugation (Shandon, Cytospin II, Pittsburgh, PA, USA). Slides were frozen and stored at −20°C until staining. Before staining, slides were fixed in 4% formaldehyde in PBS, and antigen was retrieved by incubation in citric acid buffer, pH 7.2, at 60°C overnight.

A polyclonal antiserum to CD3 was obtained from Dakopatts AB (Stockholm, Sweden). The primary antibody to Granzyme B (Mab Grb-7) was obtained from Sanbio (Uden, The Netherlands). For antigen detection, a DAB-based labeled streptavin–biotin immunoperoxidase method (LSAB, Dakoplatts) was used in a Techmate Horizon staining machine (Dakoplatts) according to the manufacturer's instructions.

Cell-mediated cytotoxicity

To assess the cytotoxic capacity of cells obtained after *in vitro* expansion, cells from two patients were analyzed on day 21 of culture. The NK-sensitive K562 cell line was used as a target. The cytotoxic function of cultured cells was measured in a standard 4 h ^{51}Cr-release assay in triplicates at various effector:target ratios. Target cells were labeled with 100 μCi of ^{51}Cr for 2 h at 37°C, washed three times with PBS, and resuspended in 10 ml of RPMI medium. A total of 3×10^4 target cells in 100 μl RPMI medium were placed in triplicates into U-bottomed 96-well plates and incubated for 4 h with 100 μl of effector cells at appropriate concentrations to obtain effector:target ratios from 0.3:1 to 10:1. Cells were then collected by centrifugation, and aliquots of supernatants were counted in a counter. The percentage specific ^{51}Cr release was calculated according to the formula: percent specific release = ((experimental release − spontaneous release)/(maximum release − spontaneous release)) × 100.

Statistics

The results were compared by using nonparametric tests (Mann–Whitney *U*-test). *P*-value <0.05 was considered to be significant.

Results

In vitro *expansion of NK cells and NKT cells from patients with B-CLL*

PBMCs of all patients with B-CLL evaluated here were initially cultured in a concentration of 100×10^4 cell/ml. At the start of the culture, each patient had variable numbers of NK, T, and B cells. However, in all patients, the numbers of NK cells were

Table 2 Absolute cell counts ($\times 10^4$) of NK cells, T cells including subsets, and B cells on days 0 and 21. Cultures were initiated without prior B-cell depletion at a starting concentration of PBMC of 100×10^4 cells/ml

Patients	*AD1*		*KO2*		*LS1*		*YO2*		*LB1^{Before} #*		*LB1^{After} #*		*RL1^{Before} #*		*RL1^{After} #*	
	D0	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*	*D0*	*D21*
NK cells																
Total CD3−CD56+ NK cells	5	2484	3	963	11	2221	3	1704	1	43	17	1226	2	281	3	307
Subsets (of NK cells)																
CD3−CD8+	1	390	0	59	7	111	2	481	2	13	8	298	0	58	0	49
T cells																
Total CD3+ T cells	19	1531	9	1013	42	2303	5	80	3	88	49	2980	8	68	13	71
Subsets (of T cells)																
CD3+CD56−	16	545	7	713	39	1580	4	29	3	22	45	2444	8	18	12	25
CD3+CD56+	3	986	2	300	3	953	1	351	0	66	4	536	0	50	0	46
CD3+CD4+	9	401	4	399	31	1331	3	7	1	13	22	2137	5	9	9	34
CD3+CD8+	5	351	2	331	11	557	2	62	2	46	24	817	3	38	4	26
B cells																
CD19+	52	0	69	1	41	10	88	0	96	62	12	0	82	65	80	0

#, Patients with progressive disease, samples obtained before (LB1^{Before} and RL1^{Before}) and after (LB1^{After} and RL1^{After}) fludarabine treatment, respectively. For LB1, complete remission (CR), and for RL1, partial remission were obtained after fludarabine treatment.

Table 3 Absolute cell counts ($\times 10^4$) of NK cells, T cells, including subsets, and B cells on day 21.[a] Cultures were initiated after B-cell depletion at a starting concentration of PBMC of 100×10^4 cells/ml

Patients	*AD1*	*KO2*	*LS1*	*YO2*
	D21	*D21*	*D21*	*D21*
NK cells				
Total CD3−CD56+ NK cells	4008	773	1812	4333
Subsets (of NK cells)				
CD3-CD8+	629	13	84	1446
T cells				
Total CD3+ T cells	4128	1977	4000	160
Subsets (of T cells)				
CD3+CD56−	1420	1708	2456	59
CD3+CD56+	2708	269	1584	101
CD3+CD4+	533	769	3316	53
CD3+CD8+	743	868	424	88
B cells				
CD19+	0	0	4	3

[a]Owing to very low number of cells at day 0, FACS analysis was not attempted.

low (Table 2). After 21 days of culture, all PBMC samples from the patients with indolent disease showed a marked CD3−CD56+ NK cell expansion both in samples of non-B-cell- (Table 2) and B-cell-depleted PBMCs (Table 3). The expansion approached log-linearity after an initial 'resting' phase. The median NK cell number obtained after 21 days was 1963×10^4 cells/ml (range 963–2484) for non-B-cell-depleted samples and 2883×10^4 cells/ml (range 773–4333) for B-cell-depleted samples (Figure 1). The difference between NK cell expansion rates from these two samples was not significant (*P*-value >0.56).

Under the culture conditions used, not only NK cells (CD3−CD56+) but also T (CD3+CD56−) and NKT cells (CD3+CD56+) were expanded. The median NKT cell number obtained after 21 days was 553×10^4 cells/ml (range 51–986) for non-B-cell-depleted samples and 927×10^4 cells/ml (range 101–2708) for B-cell-depleted samples (Figure 2). The NKT cell expansion rate difference between these two samples was also not significant (*P*-value >0.39). Samples from some patients (AD1 and LS1) had a particularly prominent increase of NKT cells (Table 2). The absolute cell counts for NK, NKT, T, and B cells appear in Table 2 for non-B-cell-depleted cultures and in Table 3 for B-cell-depleted cultures. In cultures in which most of the expanded cells were CD3−CD56+, NK cells had NKT numbers that were relatively low (Table 2, patient YO2). In contrast, under the same conditions, cultured PBMCs from patients with progressive disease (LB1^{Before} and RL1^{Before}) had far less NK cell expansion after 21 days. Moreover, in patient LB1 the NK expansion capacity was restored after fludarabine therapy, when the patient had achieved a CR. B cells remained in the cultures, but were not expanded. B cells were more markedly reduced in cultures from patients with indolent disease (AD1, KO2, LS1, and YO2) than in samples obtained from patients with progressive untreated disease. In contrast, in samples obtained from patients after treatment, the number of B cells was markedly reduced during culture (Table 2).

To study the behavior of NK cells, we subjected PBMCs from a patient with progressive disease, RL, after receiving fludarabine treatment, to a transwell system where we cocultured B-cell-depleted PBMC with tumor B-cell-enriched cells. PBMCs cultured in a transwell system expanded to a slightly better extent than those containing non-B-cell-depleted PBMCs (data not shown).

Expansion rate variability of NK cells in samples obtained at different time points

To assess the reproducibility of our culture conditions, PBMCs obtained from the same patients at different time points were cultured under identical conditions. The time interval between the first and second samples was not longer than 1 month, except in patient KO2, for whom it was 4 months, and for LB with 3 months between the sample before therapy and the sample after therapy. As indicated in Figure 3, the absolute NK cell counts were quite similar in repeated samples, both in those

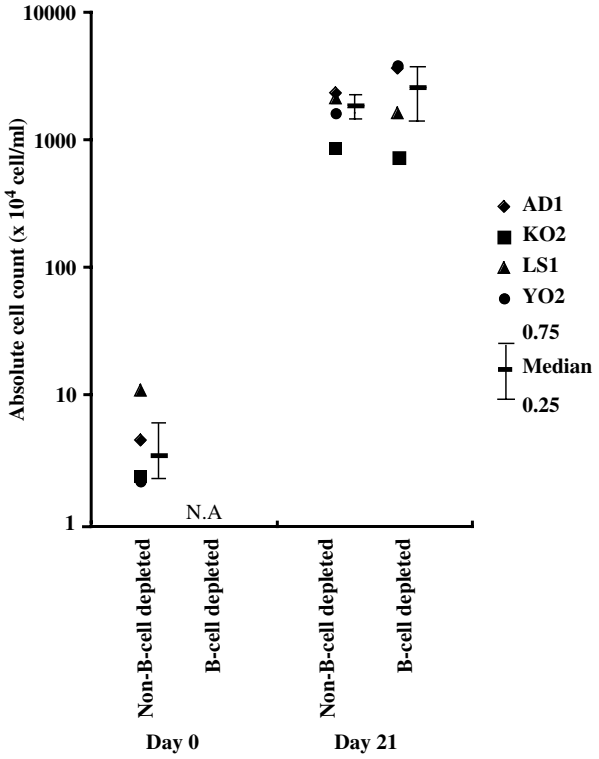

Figure 1 Absolute cell counts of CD3−CD56+ NK cells before and after *in vitro* expansion. Both non-B-cell- and B-cell-depleted PBMC from four CLL patients (AD1, KO2, LS1, and YO2) with indolent disease were cultured at 100×10^4 cells/ml in a medium supplemented with OKT-3 (first initial 5 days, 10 ng/ml) and IL-2 (500 U/ml) for 21 days. Median values and interquartile ranges are indicated. No significant difference in the NK cell count was seen when comparing cultures from non-B-cell- and B-cell-depleted samples (*P*-value >0.56). NA: not analyzed due to low number of cells.

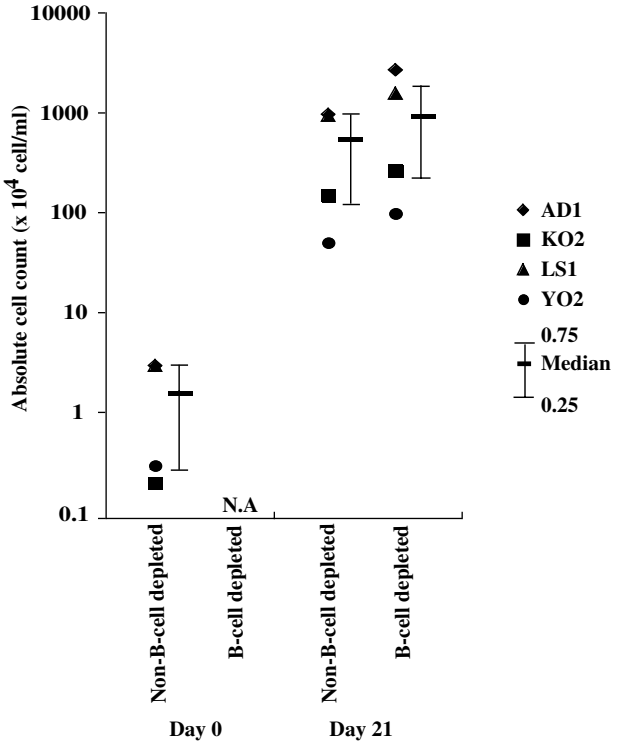

Figure 2 Absolute cell counts of CD3+CD56+ NKT cells before and after *in vitro* expansion. Both non-B-cell-and B-cell-depleted PBMC of four CLL patients (AD1, KO2, LS1, and YO2) with indolent disease were cultured at 100×10^4 cells/ml in a medium supplemented with OKT-3 (first initial 5 days, 10 ng/ml) and IL-2 (500 U/ml) for 21 days. Median values and interquartile ranges are indicated. No significant difference in the NKT cell count was seen when comparing cultures from non-B-cell- and B-cell-depleted samples (*P*-value >0.39). NA: not analyzed due to low number of cells.

obtained from patients with indolent (KO and YO) and progressive disease (LB), indicating a high degree of reproducibility in NK cell expansion capacity from the same patients at different time points.

Effect of fludarabine treatment on killer cell expansion in vitro

Samples from two patients with progressive disease (LB, RL) were obtained for culture before and after fludarabine treatment. Notably, LB received three courses of fludarabine between blood samplings, whereas RL received only one. Accordingly, the response status (response to fludarabine) in LB was better (Table 1). The numbers of NK and T cells obtained after treatment increased markedly more than those cells from samples obtained before treatment, especially for patient LB ($LB1^{After}$, Table 2). Similarly, LB had a lower post-treatment level of B cells compared to RL (Table 2).

NK and T-cell proportions and subset distribution

The percentages of NK cells were rather low on day 0 for all patients (except patient LS1) and even lower for both patients with progressive disease (LB^{Before}, RL^{Before}). However, after 21 days of incubation, samples from patients with indolent disease contained median 74% of NK cells (range 49–94). On day 21, patients who had relatively low proportions of NK cells manifested a high percentage of NKT cells. In general, the percentages of CD3+ T cells expressing the NK marker CD56, in patients with indolent disease (median 53% (range 17–75)), markedly increased by day 21 (data not shown).

Cytotoxicity

Granzyme B staining: In cytospin preparations, both from CD19- and nondepleted cultures obtained at the test period's end (day 21), a high level of Granzyme B was seen in the majority of the cells (data not shown).

Cell-mediated cytotoxicity: Cytotoxicity of the expanded cell populations from the cultured peripheral blood of two patients with indolent disease (AD1 and YO2) was evaluated by Cr-release assay. The NK-sensitive tumor cell line K562 served as a target. On day 21 of expansion, specific lysis of the target cell line could be shown in samples from both patients (Figure 4). At a 1:1 effector:target cell ratio, the median cytotoxic activity was 26 and 44% (percent specific release) in cultures of non-B-cell-depleted cells (Figure 4a) and 17 and 47% in B-cell-depleted cultures (Figure 4b) for patients AD1 and YO2,

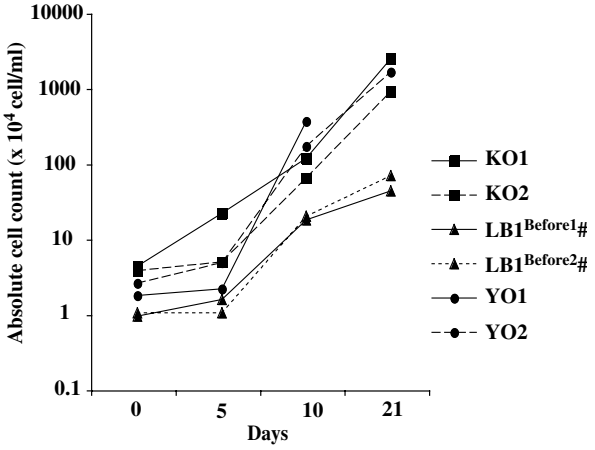

Figure 3 CD3−CD56+ NK cell expansion rates in repeated samples obtained from three individuals and cultured under identical conditions. Two patients had indolent (KO and YO) and one patient had progressive (LB) disease. Samples from the patient with progressive disease were labeled with the symbol '#'. 'Before' after patients' initials refers to samples obtained before fludarabine treatment. Numbers after patients' initials refer to sample numbers. Time interval between first and second samples of the same patients was 1 (LB, YO) to 4 (KO) months.

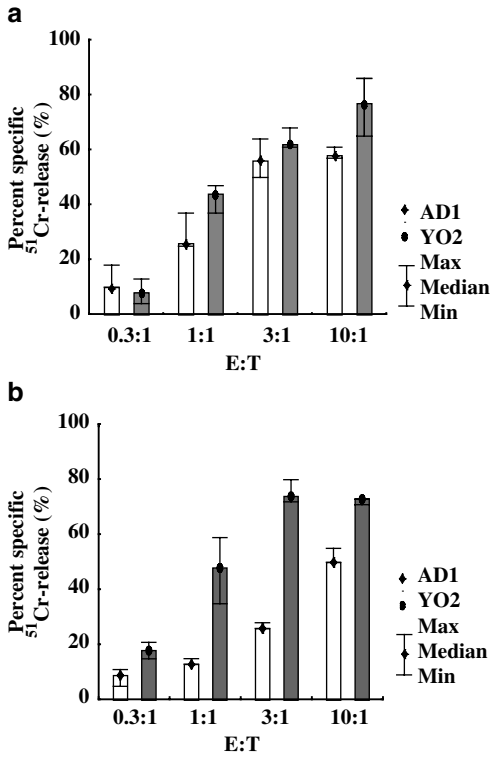

Figure 4 ^{51}Cr-release assay for cytotoxicity of day 21 cultures from (a) non-B-cell- and (b) B-cell-depleted PBMC samples. K562 served as target cells at the effector:target ratios indicated. Values are presented as median with ±min/max.

respectively. The proportion of NK cells in samples AD1 and YO2 differed in proportion (61% for AD1 and 94% for YO2) in unseparated PBMC and 47 and 93%, respectively, in samples from B-cell-depleted PBMC, that is, reflecting slightly higher cytotoxicity for the YO2 sample.

Discussion

We report here the feasibility of expanding killer cell populations of the CD3−CD56+ NK and CD3+CD56+ NKT phenotypes by using anti-CD3 antibody and cytokine (IL-2) induction of PBMC of patients with indolent or progressive B-CLL. To our knowledge, this is the first such study of NK cell as well as NKT cell expansion from peripheral blood of B-CLL patients.

NK cells are known to play a role in the control of solid tumor metastases[28] and also of leukemia.[29] The observation that NK activity is significantly reduced in peripheral blood of CLL patients as compared to healthy donors could argue for an NK cell dysfunction contributing to tumor cell accumulation *in vivo*.[16–19] Reasonably, then, increased numbers and/or activity of killer cells might be therapeutically beneficial for CLL, as it has been shown that NK cells have low cytotoxic capacity in B-CLL patients.[16–19] However, with all leukemic patients, autologous antileukemic effector cells are difficult to collect because of their limited number in the peripheral blood. Previously, others studied expansion of NKT but not of NK cells from the peripheral blood of B-CLL patients.[30] In that report, the T cells that expanded *in vitro* partly coexpressed the NK marker CD56. Moreover, the expansion of CD3+CD56+ T cells from normal donors,[31] patients with CML,[32] and patients undergoing autologous hematopoietic cell transplantation[33] has also been reported.

Recently, we successfully generated cultures of NK cells isolated from the peripheral blood of healthy donors.[27] Therefore, investigating whether PBMC from B-CLL patients could similarly serve as a source of killer cells was of significant interest. In the present study, consistent with those previous results, we successfully expanded NK and NKT cells from patients with indolent phase B-CLL at levels similar to those from healthy donors despite extremely low starting numbers of patients' NK and NKT cells. Our findings that depletion of B cells prior to culture did not significantly influence NK and NKT cell expansion rates indicates that in indolent phase, malignant B cells have no negative effect on the expansion of both NK and NKT cells. Additionally, the average proportion of NK cells achieved after culture was actually higher (74%) in CLL patients than what we have previously seen in healthy donors (55%).[27] In accordance with our previous results, we found a high percentage of T cells coexpressing the NK marker CD56. Our finding that expansion rates were lower for the patients with progressive disease, especially before fludarabine treatment, may indicate that the very low number of NK cells at the start of culture may have a negative effect on the expansion rate. However, since lowering the B-cell count by fludarabine treatment increased the expansion yield, this cytostatic regimen apparently does not negatively influence the post-therapeutic, *in vitro* proliferative rate of NK and T cells.

The analysis of samples obtained from the same patients at different time points revealed that the expansion rates were stable over time and were related to patients' characteristics and tumor burden/therapy. Furthermore, our tumor B-cell depletion and coculture experiments in a transwell system indicated that initiating cultures with large amounts of tumor cells had only a modest influence on the killer cell expansion capacity. Of interest, a small proportion of the cells present when the culture period ended were still B cells. In samples obtained from patients with progressive disease, before therapy, only moderate

reductions in absolute B-cell counts were seen upon culture. Since previous studies have shown that non-neoplastic lymphocytes delay tumor B-cell apoptosis by secreting cytokines and growth factors,[34] it remains to be determined whether the B cells remaining at the end of the culture were neoplastic or active and if they were dependent on NK and T cells for their survival.

Since preceding cytostatic therapy might negatively influence the yield when collecting peripheral blood stem cells for transplantation, a negative effect of fludarabine on the subsequent growth of killer cells *in vitro* might be anticipated. Contrary to that possibility, we found better NK cell expansion in post-therapy in cultures from the patient LB who achieved CR after three courses of fludarabine. Moreover, patient LB had a better killer cell expansion capacity post-therapy than the patient RL, who received only one course of fludarabine, and only achieved PR. It remains to be determined if successful fludarabine therapy consistently enhances the *in vitro* expansion capacity of cytotoxic cells.

Our finding that cells with the NK as well as the NKT phenotype can be expanded in culture indicates that multiple subsets of killer lymphocytes, utilizing partly different killer mechanisms, are generated simultaneously, which may be therapeutically beneficial.

In our cultures, the expression of CD16 was high on NK cells indicating their activated status (data not shown). In as much as CD16 is an Fc receptor involved in ADCC,[16–18] the expanded cell populations generated here could be instrumental as effectors following Mab therapy. The Mab, Campath-1H, had only a marginal effect on CD16 + NK cells compared to that on T and B cells,[35] but deficiency in the number of NK and NKT cells remains several months after Campath-1H therapy (personal communication). Thus, following Mab therapy, the infusion of IL-2-activated killer cells may be of clinical value. Still another phenotypic indicator of the cytotoxicity of the expanded killer cells was the presence of high levels of Granzyme B within cytoplasmic granules. Additonally, the NK-sensitive CML tumor cell line K562 was lysed at a 1:1 effector:target ratio resulting from a non-B-cell-depleted population of killer cells. Compared to NK cells taken from healthy donors and expanded, killer cells derived from CLL patients appeared to be equally cytotoxic after expansion *ex vivo*.[33]

In conclusion, our results clearly demonstrate that several potentially cytolytic subtypes of lymphocytes from B-CLL patients can be expanded *in vitro*. These cells express CD16 and Granzyme B and have cytolytic capacity, indicating that their use alone or in combination with Mab may be a feasible clinical strategy for controlling B-CLL.

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Review Article

NK Cells in Healthy Aging and Age-Associated Diseases

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NK cells exhibit the highest cytotoxic capacity within the immune system. Alteration of their number or functionality may have a deep impact on overall immunity. This is of particular relevance in aging where the elderly population becomes more susceptible to infection, cancer, autoimmune diseases, and neurodegenerative diseases amongst others. As the fraction of elderly increases worldwide, it becomes urgent to better understand the aging of the immune system to prevent and cure the elderly population. For this, a better understanding of the function and phenotype of the different immune cells and their subsets is necessary. We review here NK cell functions and phenotype in healthy aging as well as in various age-associated diseases.

1. Cellular Senescence

Aging, at the whole organism scale, is a very complex process, involving many different mechanisms. Studying the normal senescence process is also complex as we are permanently surrounded by pathogens, have different lifestyle habits, and exposed to different levels of stress which all influence the senescence process. Senescence biology really started in 1961 with Drs. Hayflick and Moorhead [1]. At this time, cells were supposed to be immortal *in vitro* and death occurring because of nonoptimal conditions. He shattered this dogma by stating that cells were programmed to divide a certain number of times before entering a replicative senescence state, where cells stop to divide. In 1971, Olovnikov discovered that this phenomenon was due to a DNA shortening, occurring at each division [2, 3]. Then, telomeres were studied first in 1978 in *Tetrahymena thermophila* [4] and few years later in human [5]. The first hints that telomere length could be a cause of senescence came from the observation that its length was not the same in every tissue [6]. One year before, Drs. Greider and Blackburn discovered the telomerase [7], and Hayflick phenomenon could be explained by the fact that telomerase activity and telomere length could be the main actors of normal senescence [8]. Here, we will focus on the senescence of a very important part of the human body, the immune system, and especially the natural killer (NK) cells subsets.

2. NK Cell Biology

NK cells are a very important population of cytotoxic cells linking innate and cellular immunities. They originate from common lymphoid progenitors, like B and T cells, and mature in lymphoid tissues (spleen, bone marrow, tonsil) before entering the blood circulation [9]. A major difference with lymphocytes is their lack of CD3, BCR, or TCR expression. They can be defined as $CD3^{-}CD56^{+}CD16^{+}$ cells. These cells can react very quickly upon stimulation, faster than T cells, as they can kill directly "missing-self" cells that lack MHC class-I molecules without any need of previous sensitization, antibody binding, or peptide presentation [10]. These cells are very important in antiviral and antitumoral responses. This very fast and efficient ability to kill is still very strictly regulated. The NK cell takes the decision to kill by measuring the balance between signal received by its inhibitory and activating receptors expressed at its surface, inhibition being dominant [11]. These signals are transmitted by 2 families of receptors, the Ig-like and the C-type lectins. Among Ig-like inhibitory receptors there are KIRs

(killer-cell immunoglobulin-like receptors) recognizing HLA molecules and sending a strong inhibitory signal [9] and LIR (leucocyte inhibitory receptor) also binding to class-I HLA. Concerning inhibitory C-type lectins, Ly49 and the heterodimer CD94/NKG2A-B, recognizing HLA-E molecules [9] are able to prevent NK cells to kill. The activating receptors are also part of these 2 families. The NCR (natural cytotoxicity receptors) such as CD16 (FcγIIIA), allowing antibody-mediated toxicity, or NKp46, NKp30, and NKp44 belong to the Ig-like family. CD94/NKG2C-E (recognizing HLA-E) as well as NKG2D (recognizing nonclassical HLA) are activating C-type lectins receptors. A part of their role at the interface of innate and adaptive immunities is influenced by the level of CD56 and CD16 they express [12–14]. $CD56^{dim}CD16^{+}$ are terminally differentiated cytotoxic cells that act more like innate immunity, although they are also cytokine producers. On the other hand $CD56^{bright}CD16^{-}$ cells are less differentiated, cytokine secreting cells able to sustain innate and adaptative immunity [15]. There is a third NK cell subpopulation, $CD56^{-}CD16^{+}$, originally described in HIV-1^{+} patients [16], also described in hepatitis B and C [17], with poor proliferative and cytotoxic capacity low cytokine production, and high chemokine production [18]. $CD56^{bright}CD16^{-}$ cells are considered immature cell precursors of $CD56^{dim}CD16^{+}$ cells [19, 20]. However, it is not clear the relationship between $CD56^{-}CD16^{+}$ cells and the other NK cell populations.

3. Aging and Overall Immunity

Immunosenescence is defined as the progressive loss of immune functions through aging, and all types of immune cells are concerned. Hematopoietic stem cells (HSCs) become less and less able to renew the blood cells populations due to the shortening of telomeres and the accumulation of DNA lesions due to free radical created during their metabolism [21]. Macrophages lose their bactericidal capacities and their number decreases [22]. Antibody-producing B cell number decreases and leads to a smaller immunoglobulin diversity and affinity [23]. Dendritic cells antigen presenting function decreases with age causing profound changes in cellular immunity [24]. The lymphocytes homeostasis is modified as less and less naïve immune cells are created, and memory populations start to lose their functions, leading to a greater susceptibility to pathogens and cancers [25]. To estimate the immunosenescence, T cells activity is used as a biomarker as nearly all of their functions are modified by aging. They produce less cytokines [26], the repertoire diversity decreases [27], the homeostasis is modified [26], their proliferation is impaired [26], their intracellular signal transduction capability is deregulated [28], and they are less cytotoxic [29].

4. NK Cells in Healthy Aging

During aging, like for lymphocytes, NK cell number functions and phenotype are modulated and modified. Several studies indicate that in the elderly there is an increase in number and a redistribution of NK cell subpopulations, with a decrease of $CD56^{bright}$ population, more immature, and an increase of $CD56^{dim}$ mature cells, particularly those highly differentiated who express CD57, as well as the $CD56^{-}CD16^{+}$ cells [30–34]. While the $CD56^{bright}$ cells phenotype does not change during healthy aging, the terminally differentiated $CD56^{dim}$ population displays higher levels of HLA-DR and CD95 (Fas) surface expression and a decrease in CD69 (a C-type lectin and early activation antigen) compared to young individuals [30]. When NK cells cytotoxicity was tested in healthy elderly individuals, it was noted that age does not affect it [35], but the increase of $CD56^{dim}$ population in blood does not correlate with an increase of overall cytotoxicity (Figure 1). This supposes an impairment in NK cells cytotoxic activity at the single-cell level although no default was identified in binding to target or in perforin content [36]. The explanation for this intrinsic reduced cytotoxic activity is still under investigation.

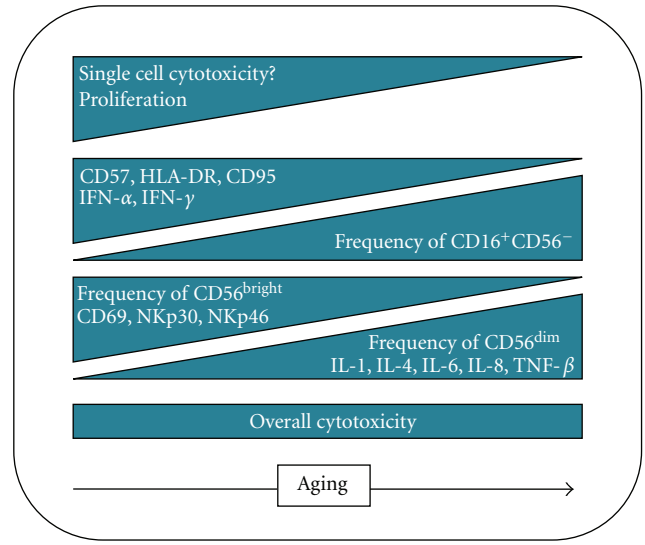

Figure 1: NK cell phenotype and functions in healthy aging. Increase and decrease in the NK-related parameters during aging are depicted. For example, NK cell proliferation is reduced.

One of the most important cytokines for NK cells is IL-2 as it binds adaptive immune response and NK cells. Treating NK cells with cytokines such as IL-2, IL-12, IFN-γ, and IFN-α increases their killing aptitudes and allows them to kill cells usually "NK resistant." In healthy elderly people, if cytokine stimulation is not impaired, the ability to kill "NK resistant" cells still decreases [35]. IL-2 can also induce NK cells proliferation, but, in elderly people, the intensity of the response varies from a very slight decrease to nearly no proliferation [30]. IL-2 also modifies the NK profile for cytokine secretion. In elderly, compared to young people, IL-2 induction of IFN-γ and IFN-α is decreased whereas IL-1, IL-4, IL-6, IL-8, IL-10, and TNF-α increase [37]. NK cells from elderly also produce less IFN-γ upon IL-2 stimulation whereas perforin and TNF-α were not modified [36]. Almeida-Oliveira et al. recently did a very interesting study about the modulation of NK markers throughout life,

from childhood to death [38]. They noticed an expansion of the $CD56^{dim}$ population and shrinkage (in frequency and number) of $CD56^{bright}$ in elderly people, increasing cytotoxic cells while diminishing the NK $CD56^{bright}$ amount of cytokine like IFN-γ, TNF-α, GM-SCF, or IL-10 and IL-13 (Figure 1). Moreover, activated NK cells secrete less IFN-γ. $CD56^{bright}$ cells from children and elderly subjects express more KIR receptors, and in their cohort, most of them express only inhibitory KIR or both inhibitory and activating at the same time. Concerning NCR, they find a decline in the expression of NKp30 and NKp46 in elderly people. NKp30 is known to participate in the crosstalk with dendritic cells leading to the link between innate and adaptive response [39]. They also found a decline in CD94 expression in elderly people in both NK subsets and only in $CD56^{dim}$ cells in children. Interestingly, they found no decline in NKG2D expression neither in children nor in elderly. This could be a form of adaptation to a deficient adaptive immune system as during aging it becomes less effective while during childhood it is mostly naïve regarding antigen encounter.

5. Age-Associated Diseases

Countless diseases are correlated with aging, so in this present paper, we will talk about the most common and immune-related diseases to link up with NK cells. Emerging at alarming speed is certainly Alzheimer's disease (AD) that is associated with aging, with the exception of the early onset congenital form that can occur at any time. Usually this disease is diagnosed after 65 years and is becoming a real problem worldwide, as in 2006, there were 26.6 million patients, and it is predicted to affect 1 in 85 people globally by 2050 [40]. The causes of AD are still unknown, except for the congenital form. Several hypotheses have been proposed to explain the disease. Among them, the amyloid hypothesis postulates that amyloid β (Aβ) accumulation, forming plaques in the brain, is the causative agent of AD [41]. This is supported by several facts: Down syndrome patients got an extra chromosome 21, bearing the Aβ-related gene APP, and develop AD before 40 [42]. Finally, apolipoprotein E 4 gene (APOE4) is a known AD-associated marker as different genotypes in the APOE gene lead to differential accumulation of Aβ in the brain [43]. Nonplaque Aβ oligomer may also be very important as they can bind neuron surface receptor and disrupt synapses [44]. Moreover, one of these receptors may be the prion, responsible for Creutzfeldt-Jakob disease [45]. In 2009, this theory was modified, suggesting that a neighbor of Aβ, and not necessarily the protein itself, could be a major causative agent of the disease. N-APP, a fragment of APP, is adjacent to Aβ and is cleaved from APP by one of the same enzymes. N-APP triggers the self-destruct pathway by binding to a neuronal receptor called DR6 (Death Receptor 6) [46]. DR6 is highly expressed in the part of the brain that is the most affected by AD. It is possible that the N-APP/DR6 pathway might be hijacked in the ageing brain to cause damage. In this model, Aβ only plays a side role. One of the other hypotheses is the Tau hypothesis [47]. Tau protein is a factor that stabilizes microtubules abundant in central nervous system and neurons. It has been shown that hyperphosphorylated tau proteins can cluster together and form neurofibrillary tangles shattering neuron transport system by disintegrating microtubules and leading to the death of the cell [48–50].

Several cancers have been linked to aging so, far and we will discuss here those occurring frequently and in the majority of elderly people. According to the US National Cancer Institute (NIC), after 65 years, there are 10 times more cancer cases than before, and the probability to develop a cancer rises after 40 years of age [51]. The most common cancers in elderly people are prostate, breast, colon, pancreas, bladder, stomach, lung, and rectum. The reasons why it happens mainly in elderly are not clear, but some explanations can be provided. First, some of the cancers develop slowly due to their intrinsic aggressive potential or by its control by the immune system that for some reasons becomes deficient after years or decades of constant immune surveillance. Secondly, with advancing age the immune system starts to weaken, lowering our natural shield against cancer [52]. Thirdly, a longer lifespan is logically associated to higher risks of exposition to carcinogens like those due to pollution, smoking, chemicals, or UV. Finally, the mechanisms involved in cellular detoxification are impaired in aged cells rendering their capacity to prevent (antioxidant levels) and repair DNA damage or protein modifications (e.g., chaperones such as heat shock proteins) that ultimately lead to faulty functions, cell death, or cell transformation [53, 54].

Bone and joint-associated diseases reduce the quality of life of the elderly population rendering them more dependent. One of the most painful age-related diseases is arthritis, which is a generic term covering more than 100 pathologies. The most common form of arthritis is osteoarthritis (or degenerative arthritis) and is predominant in 65+ people (70% of cases in USA). It consists in a loss of cartilage in joints leading to an increased friction between bones inducing pain and a progressing disability [55]. Inflammation of the area is often noted, and the epiphyses damages force a compensatory bone growth that can prevent natural movements. It is related but not caused by aging. The most concerned body parts are hands and knees. Osteoporosis is an age-related condition where bones become porous, leading to an increased risk for fractures [56]. During the disease, bone mineral density is reduced, and the inner architecture is impaired making the bone much more fragile. It occurs in women, after menopause for type 1 osteoporosis while type 2 osteoporosis is prevalent for both genders after 75 (2 : 1 ratio for female). The main problem of this disease is the increased risk of fall plus the fact that fracture can severely disable elderly people [57]. The most common fracture related to osteoporosis is the hip fracture often leading to hip replacement.

Cardiovascular issues like atherosclerosis or high blood pressure are also correlated with age, and the risk of stroke doubles every decade after 55 years of age [58]. It could be partly explained by the fact that the vascular wall loses part of its elasticity, which plays a great role in the control of blood pressure. Moreover, cholesterol levels increase with age, and its accumulation hinders blood flow

in small arteries like coronaries. Atherosclerosis is a much more complicated disease as it involves an immunological component [59]. It consists in an accumulation of lipids in the vascular wall generally close to area where there are turbulences in blood flow. It induces an inflammation due to monocyte accumulation and transformation in "foam cells" that make the plaque, or atheroma, growing thicker and thicker. Disruption of this complex may lead to release of thrombogenic molecules in the blood causing clotting. It is usually asymptomatic but often associated with heart attack or sudden death.

To conclude our presentation of age-related diseases, we will discuss about destructive eye diseases like glaucoma, cataract, and age-related macular degeneration (AMD) which are causing severe disruption in daily life. Glaucoma is induced by an increase in pressure leading to the optic nerve compression and destructing of retinal cells and, if left untreated, to blindness [60]. It affects 1 in 200 people aged 50 and younger and 1 in 10 over the age of 80. It is the second cause of blindness worldwide after cataract. Cataract consists in a clouding that develops in the crystalline lens or the lens capsule of the eye and induces a loss of vision that can be complete. The senile cataract, occurring in the elderly, begins with a clouding of the lens followed by its swelling, and finally it shrinks with a complete loss of transparency. Moreover, with time, the cataract cortex liquefies to form a milky white fluid which can cause severe inflammation if the lens capsule leaks [61]. Age is an important risk factor for senile cataract and is often present with AMD which manifests by damages to the retina, in the macula area, leading to a progressive loss of (central) vision. Around 10% of 66–74 people will develop AMD. The prevalence increases to 30% in 75–85 elderly [62].

6. NK Cells in Age-Related Diseases

Among the samples of age-related diseases described in this present paper, NK cells play a role but with differences in their implication in the corresponding diseases (Figure 2). In Alzheimer's disease, NK cells have an increased IL-2-mediated cytotoxic activity that is negatively correlated with cognitive status [63]. Another study from the same team showed that it may be due to a deregulation of protein kinase C (PKC), a regulatory enzyme playing a role in NK exocytosis and cytotoxic response after induction by IL-2 and IFN-β [64]. Cytotoxicity increased by 102% after IL-2 stimulation and 132% after IFN-γ in AD patients compared to controls (healthy elderly and younger people). After IL-2 and IFN-γ stimulation, a physiological decrease in cytosolic PKC concentration was observed in controls but not in AD patients, and cortisol (as immunosuppressor) did not decrease PKC activation in the AD cohort. Finally, IL-2 was shown to induce a greater release of IFN-γ and TNF-α in AD patients compared to controls (healthy elderly), and here also these releases were negatively correlated with cognitive status [65]. Altogether, these data suggest that NK cell cytotoxic activity and overall functionality participate actively in neuroinflammation related to the neurodegeneration observed during AD. It was even suggested to use NK activity as a biomarker of AD progression [66].

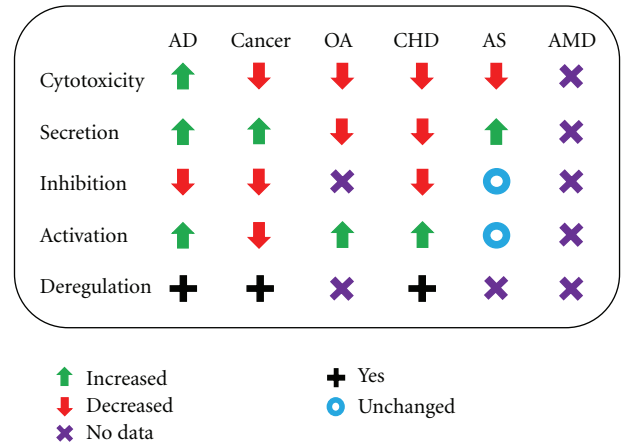

Figure 2: NK cell phenotype and functions in age-associated diseases. The functionality of NK cells in the different diseases is depicted. AD: Alzheimer's disease; OA: osteoarthritis; CHD: cardiovascular/heart diseases; AS: atherosclerosis; AMD: age-related macular degeneration.

Concerning age-related cancers and NK cell function, some evolved an NK escaping process. The perfect example is prostate cancer. Tumor cells can secrete soluble NKG2D that will induce a fake NK response by competing with the true NKG2D for binding on the receptor site and at the same time evade CD8 recognition, as it is expressed on 80% of NK cells, by inhibiting MHC-I expression [10]. This is a fact that shows how cancers evolve to escape from an important population, the NK cells. Concerning pancreatic cancer the major problem is that tumor is surrounded by a fibrotic shield that allows very few cells to reach the core. Among these tumor-infiltrating cells, only a very small number of NK cells were observed [67]. But treating this disease locally with autologous NK cells may be relevant to support for the cure as it was showed that apoptotic pancreatic tumor cells are a very good activator of NK and T cells [68]. Moreover, NK cells stimulated by dendritic cells pulsed with tumor-derived RNA can reverse the resistance of pancreatic carcinoma cells [69]. For colon cancer, NK cells reduced activity was showed to be related to colon cancer metastasis as patient with normal NK response remained free of metastasis whereas low NK response patient showed relapse [70]. NK activity could be used as a marker of colorectal progression and help to identify patients with higher risks of metastasis. In colorectal carcinoma tissue, despite high levels of chemokines and cytokines, tumor infiltrating NK cells are very rare [71]. The tumor escaping mechanism is not yet enlightened but it also promotes the important role of NK cells and seems to be present even in the early stages of the disease. Moreover, NK cells presence in colorectal carcinoma tissue has been negatively correlated with the age of the patients maybe due to an age-related decrease in adherence molecule expression [72]. The decreased expression of activating receptors NCR and DNAM-1 is not only seen in healthy individuals >65 but

also in young acute myeloid leukemia patients [31, 73]. This is attributed to increased expression of CD122 and CD155 (DNAM-1 ligands) in leukaemic blasts [34]. Considering the relevance of DNAM-1 in NK recognition/killing of cancer cells, its reduced expression on NK cells from AML patients may represent another mechanism of tumor escape. For stomach cancer, NK cells activity has been correlated to clinic-pathological parameters including tumor size, lymphatic and vascular involvement, and lymph node metastases. The 5-year survival was higher in responding NK group (~95%) compared to nonresponding NK group (72%) [74, 75]. Here also, NK activity could be a good marker for tumor volume and dissemination and prognosis. In lung cancer, a study showed that tumor-infiltrating NK cells are mainly $CD56^{bright}$ and able to secrete cytokines but are unable to kill tumor cells [76]. Cells were $CD56^{bright}$ and $CD16^{-}$, highly enriched in the tumor, but their cytotoxicity was lower than those from NK cells in peripheral blood. They were also found in the tumor stroma, not in direct contact with tumor cells. Intratumoral NK cells display great phenotypic alterations such as reduced NK cell receptor expression. These defects lead to an impaired degranulation and secretion of cytokines, like IFN-γ. As tumor expresses activating and inhibiting NK cells ligands, it seems that it is a tumoral NK escaping mechanism, and because of this, NK cells are not correlated to the clinical outcome of patients [77].

In osteoarthritis and periprosthetic inflammation, synovial tissue was removed and studied to analyze its immune cell composition [78]. It has been showed that the main infiltrating population was NK cells and that synovial fluid was very rich in NK attractants like CCL-4, CCL-5, CXCL-9, CXCL-10, and chemerin. These NK cells express receptor consistent with an exclusive $CD56^{bright}$ phenotype (Figure 2). They also produce less IFN-γ than peripheral NK cells, which does not prevent further development of the disease as IFN-γ can induce osteoclast differentiation and thus bone repair. This can also have significance in osteoporosis, as elderly possess less IFN-γ secreting NK cells, but this has not been investigated so far.

NK cells have also been linked to coronary heart disease (CHD) [79]. CHD patients had lower NK cytotoxic activity, less $CD56^{dim}$ cells, less $CD56^{bright}$ regulatory cells, and less IFN-γ secreting NK cells than age-matched controls. In idiopathic pulmonary hypertension (PAH), NK cells impairments have also been identified [80]. They revealed that PAH patients' NK cell phenotype was modified. They displayed decreased levels of the activating receptor NKp46 and KIRs, reduced secretion of the cytokine MIP-1β, and a significant impairment in cytolytic function associated with decreased KIR3DL1 expression. These NK cells were more responsive to TGF-β, known to decrease KIR expression. Recent hypotheses suggest the links between innate immunity, TLR, and cardiovascular diseases [81]. During cardiac injuries, some TLR ligands may activate innate immune cells, like NK cells through TLR-2, and thus creating a potentially critical inflammation of the heart.

As discussed, NK cell distribution in tumors is fairly low suggesting mechanisms preventing their recruitment or the possibility that these cells are not the best chemoattractable cells compared to others, at least for certain tissue. In a site like the eye there have been very few reports but the existing ones also suggest a poor presence of NK cells [82]. This rare study also identified large amounts of IgG, IgA, and IgE as well as C1q, C3c, and C3d complement components in the connective stroma and within the new blood vessel walls in AMD patients. A common treatment for cancer and age-related macular degeneration is photodynamic therapy. Other cancers may be treated similarly as suggested by a study showing synergy between photodynamic therapy and other proapoptotic treatments such as FasL and TRAIL [83]. AMD can be subdivided in wet or dry AMD. The wet AMD refers to consequences of choroidal neovascularization. Together with increased levels of cytokines related to innate immunity in the vitreous fluid experiments using KO mice (NK T cell deficient and Jα18 deficient) show significant reduction of the effect of experimentally induced choroidal neovascularization-related diseases [84]. *In vitro* experiments confirmed the fact NK-like cells could produce VEGF in cocultures with retinal pigment epithelial cells [85]. This suggests that NK-like family may be involved in such diseases (Figure 2). This was partly confirmed by the fact HLA-Cw*0701 allele in combination with the inhibitory KIR AA haplotype was significantly associated with AMD ($P = 0.006$, OR = 4.35). This genotype combination suggests that NK cells are indeed involved in the pathogenesis of AMD [86].

7. Conclusion

NK cells are important immune cells as they provide a rapid and intense response to challengers. The exact link between NK cell phenotype and their function is still poorly understood and should be pursued to enable a better understanding of diseases, especially in the elderly as this population is showing slow but continuous immune erosion. Immunosenescence of NK cells is recognized more and more as a major player in age-related pathologies and hyporesponsiveness. While the role of NK cells is clearly established in certain pathologies (cancer), their role in other such as autoimmune diseases or immunosurveillance of chronic infectious diseases is less established. As innate cells, NK also participates in the interplay with adaptive immunity by leaving the host with reasonable immune surveillance and cytotoxic activity performed by $CD8^{+}$ T cells. Thus, altering NK cell functionality naturally, that is, with aging or during diseases, will irreversibly impact on immunity. When both factors are present (aging and diseases), the patients are probably even more at risk. Before NK cells to be used as biomarkers for certain pathologies, as suggested by others, one should first identify NK cell aging as many diseases where NK is involved are seen in the elderly population.

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ORIGINAL ARTICLE

Relationship between natural killer cell activity and glucose control in patients with type 2 diabetes and prediabetes

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ABSTRACT

Aims/Introduction: Natural killer (NK) cells are cytotoxic lymphocytes critical to human immunity. Previous studies showed correlations between NK cell function and blood glucose concentrations. The purpose of the present study was to assess the NK cell activity and various metabolic parameters in people with type 2 diabetes, prediabetes and normal glucose tolerance.

Materials and Methods: A total of 49 participants were enrolled in the study. Anthropometric and biochemical parameters including age, sex, body mass index, smoking status, blood pressure, fasting plasma glucose, C-peptide, insulin, glycated hemoglobin, total cholesterol, triglyceride, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were assessed. The 75 g oral glucose tolerance test was carried out for 2-h postload glucose level. Homeostatic model assessment was calculated for insulin resistance and β-cell function. NK cell activity was measured by detecting the circulating interferon-gamma level secreted from NK cells.

Results: NK cell activity was lower in patients with type 2 diabetes (768.01 ± 650.35) compared with those with prediabetes (2,396.08 ± 653.76, $P < 0.001$) and normal glucose tolerance (2,435.31 ± 633.22, $P < 0.001$). In patients with type 2 diabetes, there was a significant inverse linear relationship between NK cell activity and fasting plasma glucose, glycated hemoglobin, and 2-h postload glucose level (all $P < 0.001$). Multiple regression analysis showed glycated hemoglobin to be an independent predictor of NK cell activity in patients with type 2 diabetes.

Conclusions: Compared with individuals with normal glucose tolerance or prediabetes, type 2 diabetes patients have a reduced NK cell activity, and it is significantly related to glucose control.

INTRODUCTION

People with type 2 diabetes mellitus have increased morbidity and mortality from diabetic vascular complications[1]. Recent studies have shown that patients with diabetes are also prone to various cancers, and efforts are being made to understand the underlying mechanisms for this increased cancer incidence[2–4]. The chronic inflammatory state in diabetes patients might be involved in the impaired immune function and, consequently, the higher susceptibility to infection[5,6]. Natural killer (NK) cell activity, in particular, has been shown to be involved in the immune dysfunction and increased risk of cancer in diabetes patients[7].

NK cells engage in innate immunity to remove pathogens and cancer cells[8]. They also release cytokines to transmit adaptive immunity[9]. Previous research has shown that different levels of cytokines produced by NK cells result in various levels of cell toxicity. NK cells, which strongly express cytokines, have strong cytotoxicity to target cells, whereas cells that have degraded cytokine expression have little or none of this function[10]. A method was recently developed to easily assess the activity of NK cells by measuring the level of secreted interferon-gamma (IFN-γ) derived from NK cells[7]. NK cell activity,

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expressed as a concentration of serum IFN-γ, is directly related to changes in the function and level of NK cells, and can indicate the degree of innate and adaptive immunity[11]. In light of this, several recent studies showed that the decline in NK cell activity is related to various types of cancer. As a result, NK cells are now known to play a role in cancer cell immunological surveillance[12–14].

Several studies were carried out to evaluate the NK cell function in diabetes patients. NK cells lost their function when exposed to hyperglycemia, as in patients with uncontrolled diabetes[15], and physical inactivity and poor metabolic status were associated with decreased NK cell activity[16]. Type 1 diabetes, as well as type 2 diabetes, patients were presented with a lower number of NK cells and a decline in NK cell function[17–19]. However, little is known about the NK cell activity in patients with prediabetes – who are in a hyperglycemic state, but not high enough to be diagnosed with diabetes – nor its relationship with metabolic parameters in these patients.

In the present study, we assessed and compared the NK cell activity in participants with type 2 diabetes, prediabetes and normal glucose tolerance (NGT), and analyzed the relationship between NK cell activity and various metabolic parameters in these participants.

METHODS

This was a cross-sectional study carried out on individuals with type 2 diabetes mellitus, prediabetes and NGT who visited the Endocrine & Diabetes Center of Gangnam Severance Hospital, Seoul, Korea, from April to June 2017. Type 2 diabetes was defined as both previously diagnosed and undiagnosed type 2 diabetes mellitus. Diagnosed diabetes mellitus was based on self-reported responses. The diagnosis of diabetes was based on the following American Diabetes Association criteria: fasting plasma glucose (FPG) ≥126 mg/dL, or symptoms of diabetes plus casual plasma glucose ≥200 mg/dL, or 2-h postload glucose level (2hPG) ≥200 mg/dL during a 75-mg oral glucose tolerance test, or glycated hemoglobin (HbA1c) ≥6.5%. Prediabetic patients were defined as those with FPG 100–125 mg/dL, 2hPG 140–199 mg/dL or HbA1c 5.7–6.4%. The NGT group was defined as individuals with FPG <100 mg/dL, 2hPG <140 mg/dL and HbA1c ≤5.6%. Individuals with human immunodeficiency virus, splenectomy, immunosuppressive therapy after organ transplantation, rheumatic diseases, Crohn's disease, use of other drugs that might affect immune status, severe liver or kidney disease, current or previous malignancy, blood aminotransferase level of ≥100 IU/L, or blood creatinine level of ≥1.4 mg/dL were excluded from the study.All procedures carried out in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the institutional review board of Gangnam Severance Hospital, and was carried out with prior informed consent from all participants.

The height, weight, and systolic and diastolic blood pressure were measured in the morning with light clothing without shoes. Body mass index was calculated as bodyweight in kilograms divided by height in meters squared (kg/m^2). Smoking and past medical and surgical history were assessed at the time of screening by a single interviewer. Fasting blood samples were drawn and analyzed for HbA1c, serum glucose, cholesterol, triglyceride, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, insulin and C-peptide. The 75 g glucose tolerance test was carried out in all participants for post-loading glucose, insulin and C-peptide. To indirectly evaluate the insulin secretion function of pancreas β-cells and insulin resistance in the human body, the following formula was calculated using homeostatic model assessment (HOMA)[20].

$$\text{HOMA-IR} = \text{Fasting blood insulin (uU/mL)} \times \text{fasting plasma glucose (mmol/mL)}/22.5$$

$$\text{HOMA-\%B} = (20 \times \text{fasting blood insulin (uU/mL)})/(\text{fasting plasma glucose (mmol/mL)} - 3.5)$$

NK cell activity was measured using the NK-Vue Kit® (ATgen, Sungnam, Korea)[7]. A total of 1 mL of whole blood was collected from all participants and placed in an NK-Vue Tube®, which is a vacuum filter containing Promoca®. To ensure sufficient blood coating on the entire inner surface of the tube, it was gently mixed 10 times and then placed in an incubator at 37°C within 30 min. To ensure successful secretion of IFN-γ from activated NK cells, NK-Vue® Tubes were set up for 24 h in an incubator. The supernatant was then collected with a pipette and transferred to a 1.5 mL tube. After centrifugation at 16,000 r.c.f. for >1 min, the supernatant was collected in another 1.5 mL tube, and sent to enzyme-linked immunospecific assay plates to measure and quantify IFN-γ levels released from NK cells[7,21].

Statistical analysis

Statistical analysis was carried out with the SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). The baseline characteristics were expressed as the mean ± standard deviation for continuous variables. The comparison of baseline characteristics and NK cell activity among patients with type 2 diabetes, prediabetes and NGT was carried out by Kruskal–Wallis tests with Bonferroni's method and the Mann–Whitney method. Correlation analysis and univariate, multivariate regression analysis were used to assess relationships between biochemical parameters and NK cell activity, and to identify factors that predict the NK cell activity. *P*-values <0.05 were considered statistically significant.

RESULTS

Of 49 study participants, 21 had type 2 diabetes, 15 had prediabetes and 13 had NGT. Patients with diabetes had lower NK cell activity compared with participants with NGT and prediabetes (768.01 ± 650.35 vs 2435.31 ± 633.22, 768.01 ± 650.35 vs 2396.08 ± 653.76, respectively, all $P < 0.001$). There was no significant difference in NK cell activity between NGT and prediabetes patients ($P = 0.821$; Table 1).

Correlation analysis was carried out to determine the association between NK cell activity and biochemical parameters. When all the study participants were included for analysis, NK cell activity was in negative correlations with FPG ($r = –0.745$, $P < 0.001$), 2hPG ($r = –0.778$, $P < 0.001$) and HbA1c ($r = –0.827$, $P < 0.001$). Insulin ($r = 0.357$, $P = 0.023$) and HOMA of β-cell function (HOMA-B; $r = 0.787$, $P < 0.001$) showed significant positive correlations with NK cell activity. These correlations remained significant after adjusting for age, sex, blood pressure and smoking status (Table 2).

When diabetes patients were divided into different levels of glucose control and separate analysis was carried out, NK cell activity showed significant negative correlations with FPG ($r = –0.705$, $P = 0.002$), 2h-PG ($r = –0.795$, $P = <0.001$) and HbA1c ($r = –0.790$, $P < 0.001$), and a significant positive correlation with HOMA-B ($r = 0.649$, $P = 0.005$) after adjusting for age and sex, blood pressure, and smoking status. In the prediabetes and NGT groups, no significant relationships were observed between measured parameters and NK cell activity (Table 2).

Multiple regression analysis with all study participants showed HbA1c and HOMA-B to be independently associated with decreased NK cell activity ($B = –317.849$, 95% confidence interval –384.927 to –250.771, $P < 0.001$; and $B = 5.596$, 95% confidence interval 3.199–7.993, $P = 0.024$, respectively). Among patients with type 2 diabetes, HbA1c was independently associated with decreased NK cell activity ($B = –280.787$, 95% confidence interval –347.053 to –214.521, $P < 0.001$; Table 3).

DISCUSSION

Impaired immune function has been proposed as a possible underlying mechanism linking the increased risk of infection and cancer incidence among diabetes patients[3,4]. In the present study, NK cell activity was measured in normal individuals, prediabetes patients and type 2 diabetes patients, and diabetes patients had lower NK cell activity compared with the other two groups, whereas prediabetes and NGT participants had

Table 1 | Baseline characteristics of study participants

	Type 2 DM	Non-DM		*P*-value
		Prediabetes (IGT or IFG)	NGT	
n	21	15	13	
Sex				
Men (*n*)	8	5	4	
Women (*n*)	13	10	9	
Age (years)	60.71 ± 6.99	58.80 ± 10.27	53.69 ± 13.76	0.153
Current smoker (%)	38.1	40	15.4	0.307
SBP (mmHg)	128.00 ± 14.84	130.53 ± 17.79	118.62 ± 14.00	0.104
DBP (mmHg)	76.33 ± 9.79	84.27 ± 14.42	79.00 ± 13.47	0.307
BMI (kg/m^2)	23.55 ± 2.75	24.71 ± 2.35	23.10 ± 3.54	0.304
FPG (mmol/L)	10.55 ± 3.47†‡§	5.82 ± 0.53¶	5.13 ± 0.31	<0.001
HbA1c (%)	8.86 ± 1.61†‡§	5.85 ± 0.36¶	5.35 ± 0.35	<0.001
2hPG (mmol/L)	15.54 ± 4.75†‡§	6.94 ± 1.48	5.85 ± 0.69	<0.001
Total cholesterol (mmol/L)	5.13 ± 0.96	4.90 ± 0.93	4.86 ± 1.15	0.699
Triglyceride (mmol/L)	2.08 ± 1.21	1.72 ± 0.80	1.43 ± 0.79	0.182
HDL cholesterol (mmol/L)	1.31 ± 0.33	1.36 ± 0.26	1.40 ± 0.35	0.708
LDL cholesterol (mmol/L)	3.39 ± 0.75	3.25 ± 0.92	3.26 ± 1.03	0.865
Fasting C-peptide (nmol/L)	0.59 ± 0.25†§	0.75 ± 0.23	1.05 ± 0.43	0.007
Fasting insulin (pmol/L)	46.53 ± 37.78†§	62.30 ± 31.67¶	118.76 ± 129.59	<0.001
HOMA-B	34.40 ± 17.09†‡§	100.33 ± 22.56¶	152.15 ± 35.38	<0.001
HOMA-IR	1.89 ± 1.45	1.73 ± 0.52	2.21 ± 0.94	0.224
NK cell activity	768.01 ± 650.35†‡§	2396.08 ± 653.76	2435.31 ± 633.22	<0.001

Data are presented as the mean (standard deviation). The *P*-value represents the analysis of variance *P* for the baseline measures among the groups. †$P < 0.05$ between normal glucose tolerance (NGT) and type 2 diabetes mellitus (DM), ‡$P < 0.05$ between prediabetes (impaired glucose tolerance [IGT] and impaired fasting glucose [IFG]) and type 2 DM, §$P < 0.05$ between non-DM (NGT and prediabetes) and type 2 DM, ¶$P < 0.05$ between NGT and prediabetes (IGT and IFG). 2hPG, 2-h postload glucose; BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-B, homeostatic model assessment of β-cell function; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; NK, natural killer; SBP, systolic blood pressure.

Table 2 | Correlation analysis between biochemical parameters including natural killer cell activity after adjusting for age, sex, blood pressure and smoking status

	NK cell activity							
	Type 2 DM		Prediabetes		NGT		All participants	
	r	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
BMI	0.193	0.459	0.409	0.240	−0.040	0.925	0.162	0.294
FPG	−0.705	<0.001	−0.208	0.565	−0.395	0.333	−0.745	<0.001
HbA1c	−0.790	<0.001	−0.470	0.170	−0.751	0.032	−0.827	<0.001
2hPG	−0.795	<0.001	0.268	0.453	−0.608	0.109	−0.778	<0.001
Total cholesterol	−0.264	0.306	0.032	0.929	−0.407	0.317	−0.245	0.109
Triglyceride	−0.003	0.990	0.624	0.054	−0.419	0.302	−0.183	0.235
HDL cholesterol	−0.131	0.616	−0.427	0.219	0.332	0.422	0.036	0.818
LDL cholesterol	−0.267	0.300	0.188	0.604	−0.448	0.265	−0.186	0.228
Fasting C-peptide	−0.350	0.169	0.179	0.621	0.196	0.642	0.219	0.153
Fasting insulin	−0.297	0.247	−0.009	0.980	0.749	0.032	0.302	0.046
HOMA-B	0.649	0.005	0.387	0.269	0.244	0.560	0.738	<0.001
HOMA-IR	−0.489	0.046	0.187	0.606	0.090	0.833	−0.212	0.166

Data are presented as the mean (standard deviation). 2hPG, 2-h postload glucose; BMI, body mass index; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-B, homeostatic model assessment of β-cell function; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; LDL, low-density lipoprotein; NK, natural killer; NGT, normal glucose tolerance.

Table 3 | Multiple stepwise regression analysis between natural killer cell activity and biochemical parameters in type 2 diabetes mellitus and all participants

	B (95% CI)	β	R^2	*P*-value
NK cell activity in type 2 DM				
FPG	4.499 [0.894, 8.104]	0.432	0.459	0.233
HbA1c	−280.787 [−347.053, −214.521]	−0.697	0.459	<0.001
2hPG	−2.381 [−4.724, −0.038]	−0.313	0.459	0.327
HOMA-B	14.096 [4.745, 23.447]	0.370	0.459	0.154
NK cell activity in all participants				
FPG	4.702 [0.208, 9.196]	0.277	0.713	0.302
HbA1c	−317.849 [−384.927, −250.771]	−0.599	0.713	<0.001
2hPG	−2.345 [−4.602, −0.088]	−0.228	0.713	0.305
Fasting C-peptide	−96.849 [−249.246, 55.548]	−0.098	0.713	0.529
Fasting insulin	5.527 [−3.117, 14.171]	0.059	0.713	0.526
HOMA-B	5.596 [3.199, 7.993]	0.295	0.713	0.024

Data are presented as the mean. 2hPG, 2-h postload glucose; CI, confidence interval; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HOMA-B, homeostatic model assessment of β-cell function; NK, natural killer.

comparable NK cell activity. Also, NK cell activity was associated with various parameters related to glucose metabolism only in diabetes patients.

The present study results are in line with previous studies that demonstrated a reduced NK cell activity in diabetes patients compared with controls. The role of NK cells is to engage in innate immunity and transmit adaptive immunity[8,9]. NK cells have characteristic activating receptors, such as NKp45 and NKG2D, that are activated by contact with ligands on the surface of infected cells or tumor cells[22,23]. Several cytokines, such as interleukin-12 and interleukin-15, promote NK cell activation through differentiation and maturation[24]. Activated NK cells release proteases called perforins and granzymes[25] that have cytotoxic effects on target cells. They also regulate the immune response by releasing cytokines, such as IFN-γ and tissue necrosis factor-alpha[26]. Therefore, the impairment in immune system maintenance, as well as restriction of cancer growth resulting from the reduced NK cell activity in diabetes patients, might lead to the increased susceptibility to infectious diseases and cancer.

Meanwhile, there was no difference in NK cell activity between prediabetes patients and individuals with NGT, whereas there was a difference between participants with diabetes and prediabetes. Also, a significant correlation was noted only in the diabetes group, and not in the NGT and prediabetes groups between HbA1c, FPG, 2hPG and NK cell activity.

In multiple regression analysis, HbA1c independently showed a significant relationship with NK cell activity only in the diabetes group. This is in agreement with a previous study that showed that the cytolytic function of NK cells significantly deteriorates with the exposure to elevated glucose concentrations in the range seen in poorly controlled diabetes[15]. In other words, it can be inferred that while NK cell activity deteriorates linearly with the degree of hyperglycemia, hyperglycemia starts to interfere with NK cell activity once it reaches a certain degree, at least higher than a prediabetes range.

HOMA-B, which indirectly represents the β-cell function in pancreas, clearly had a significant positive correlation with NK cell activity in type 2 diabetes. Also, fasting plasma C-peptide and insulin showed significant correlations with NK cell activity. A study by Zhang *et al.*[17] found that type 1 diabetes patients with severely reduced insulin levels also had decreased NK cells. In addition, expression of NKG2D, an NK cell receptor, was reduced. NK cell production of IFN-γ, which is essential for immune system function, was also impaired. A study by Rodacki *et al.*[18] also found that NK cell activity in patients with long-standing type 1 diabetes was lower than that of normal controls, in addition to NKG2D expression. These data, along with the present results, suggest that insulin deficiency might be associated with impaired NK cell function. In the prediabetes state, there is a compensatory increase in insulin secretion to overcome hyperglycemia, and it might explain why there was no reduction in NK cell activity in prediabetes patients. However, when prediabetes patients progress to overt diabetes, their islet function is reported to be approximately 50% of individuals with NGT, and consequently might be related to a reduced NK cell activity.

In addition, previous reports show that low physical inactivity or unhealthy metabolic status contributes to impaired NK cell function[16]. Based on these reports, we investigated correlations between NK cell activity and biochemical parameters related to diabetes, obesity and metabolic syndrome. However, other than FPG, 2hPG and HbA1c, no significant correlation was noted between NK cell activity and body mass index, total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. Furthermore, although some studies showed differences in NK cell function at different levels of hyperglycemia, when we carried out subgroup analysis on the relationship between NK cell activity and these metabolic parameters, we did not find any significant differences. The lack of significance might be due to a small number of study participants in our study.

The present study had several limitations. First, this was a small cross-sectional study of 49 people at a single center. Accordingly, there is a risk of bias in this study. Due to the characteristics of a cross-sectional study, changes in NK cell activity related to differences in fasting plasma glucose or HbA1c over time cannot be identified, and it is difficult to explain the actual relationship between blood glucose management and NK cell activity in patients with type 2 diabetes. A future large-scale study with more participants is required to investigate the relationship between NK cell activity and diabetes, blood glucose control, and dyslipidemia. It is also necessary to determine the relevance of changes in NK cell activity through classification and analysis of blood glucose or HbA1c results.

In conclusion, NK cell activity is significantly lower in patients with type 2 diabetes compared with NGT and prediabetes patients. NK cell activity significantly decreases linearly with the increase in blood glucose level. The study supports the hypothesis that the increasing prevalence of infectious diseases and malignancy in type 2 diabetes patients is associated with decreased immune function. Future efforts are required to redefine the relationship between type 2 diabetes mellitus and NK cell activity, and to provide a way to reduce the incidence of infectious diseases and cancer by carrying out large-scale studies of patients with type 2 diabetes.

DISCLOSURE

The authors declare no conflict of interest.

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LETTER TO THE EDITOR

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Natural killer cells associated with SARS-CoV-2 viral RNA shedding, antibody response and mortality in COVID-19 patients

Changqian Bao[1†], Xiandong Tao[2,3†], Wei Cui[4†], Yuanyuan Hao[1], Shuaike Zheng[5], Bin Yi[2,3], Tiewen Pan[2], Ken H. Young[6*] and Wenbin Qian[1,7*]

Abstract

Coronavirus disease 2019 (COVID-19) is a novel infectious viral disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Two consecutively negative SARS-CoV-2 viral RNA test (interval ≥ 24 hours), improved respiratory symptoms and obvious absorption of inflammation in pulmonary imaging are the discharge criteria for COVID-19 patients. The clearance profile of viral RNA in the upper respiratory tract specimens, including nasopharyngeal swab and/or oropharyngeal swabs, is related to innate immune cells such as Natural Killer cells. A total of 168 patients were included for the study. In this cohort, non-severe and severe groups showed significant differences in white blood cells, neutrophils, lymphocytes, basophils and platelets counts, as well as in infection related parameters such as CRP and serum cytokine IL-6. For lymphocyte subsets tests at admission, the severe group displayed significantly lower cell counts than the non-severe group. Higher counts of total T cells, CD4 + T cells, CD8 + T cells, and NK cells in peripheral blood showed a significant correlation with the shorter time taken to obtain the first negative viral RNA test and first positive IgM/ IgG antibody test. The number of B cells was only correlated with time to achieve the first positive IgM/IgG test. The count of NK cells was also correlated with a higher level of IgG antibody ($p = 0.025$). The lymphocytopenia group had a significantly worse survival rate ($p = 0.022$) and a longer duration ($p = 0.023$) of viral shedding than the normal lymphocyte count group. A lower NK cell count correlates the most with the worse survival rate ($p<0.001$) and a longer duration ($p<0.001$) of viral shedding. This study suggests the potential value of allo-Natural Killer cell therapy as an universal COVID-19 treatment strategy.

Keywords: COVID-19, SARS-CoV-2, Natural killer cell, RNA viral shedding, Antibody response

To the editor,

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread globally since December 2019. Factors that affect the duration of viral shedding of SARS-CoV-2 [1] have not been fully declared yet. The association between lymphocyte subsets, including Natural Killer cell count, viral RNA shedding, and antibody response remain unclear. A total of 168 COVID-19 patients were retrospectively enrolled, followed and analyzed as methods indicated (Additional file 1: Methods) for this study (Additional file 2: Table S1). Non-severe and severe groups showed

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significant differences in white blood cells, neutrophils, lymphocytes, basophils and platelets counts, as well as in infection related parameters such as CRP and serum cytokine IL-6. The severe group had higher level of CRP and IL-6 than non-severe group. For lymphocyte subsets tests at admission, the severe group displayed significantly lower cell counts than the non-severe group (Additional file 3: Table S2). SARS-CoV-2 viral RNA detection and specific IgM and IgG tests were taken at least twice per week after admission. Higher counts of total T cells, CD4 + T cells, CD8 + T cells, and NK cells in peripheral blood showed a significant correlation with the shorter time taken to obtain the first negative viral RNA test and first positive IgM/ IgG antibody test.The severe group required a longer time to achieve the first positive IgM/IgG test (either IgM or IgG more than 10 IU/mL) (Additional file 4: Table S3). Inflammatory monocytes were reported to secret IL-6 and GM-CSF in COVID-19 patients,resulting in elevated IL-6 and other cytokines such as IFN-γ and TNF-α [2]. IL-6 mediated JAK/STAT signalling which could elicit cytokines release storm (CRS) and correlated with worse prognosis. According to our data, higher CRP and IL-6 had a significant correlation with a longer time taken to achieve the first viral RNA negative and IgM/IgG positive tests. The number of B cells was only correlated with time to achieve the first positive IgM/IgG test. The count of NK cells was also correlated with a higher level of IgG antibody ($p = 0.025$) (Additional file 5: Table S4 and Additional file 1: Additional material). The lymphocytopenia ($<1 \times 10^9$/L) [3] group had a significantly worse survival rate (log-rank $p = 0.022$) and a longer duration (log-rank $p = 0.023$) of

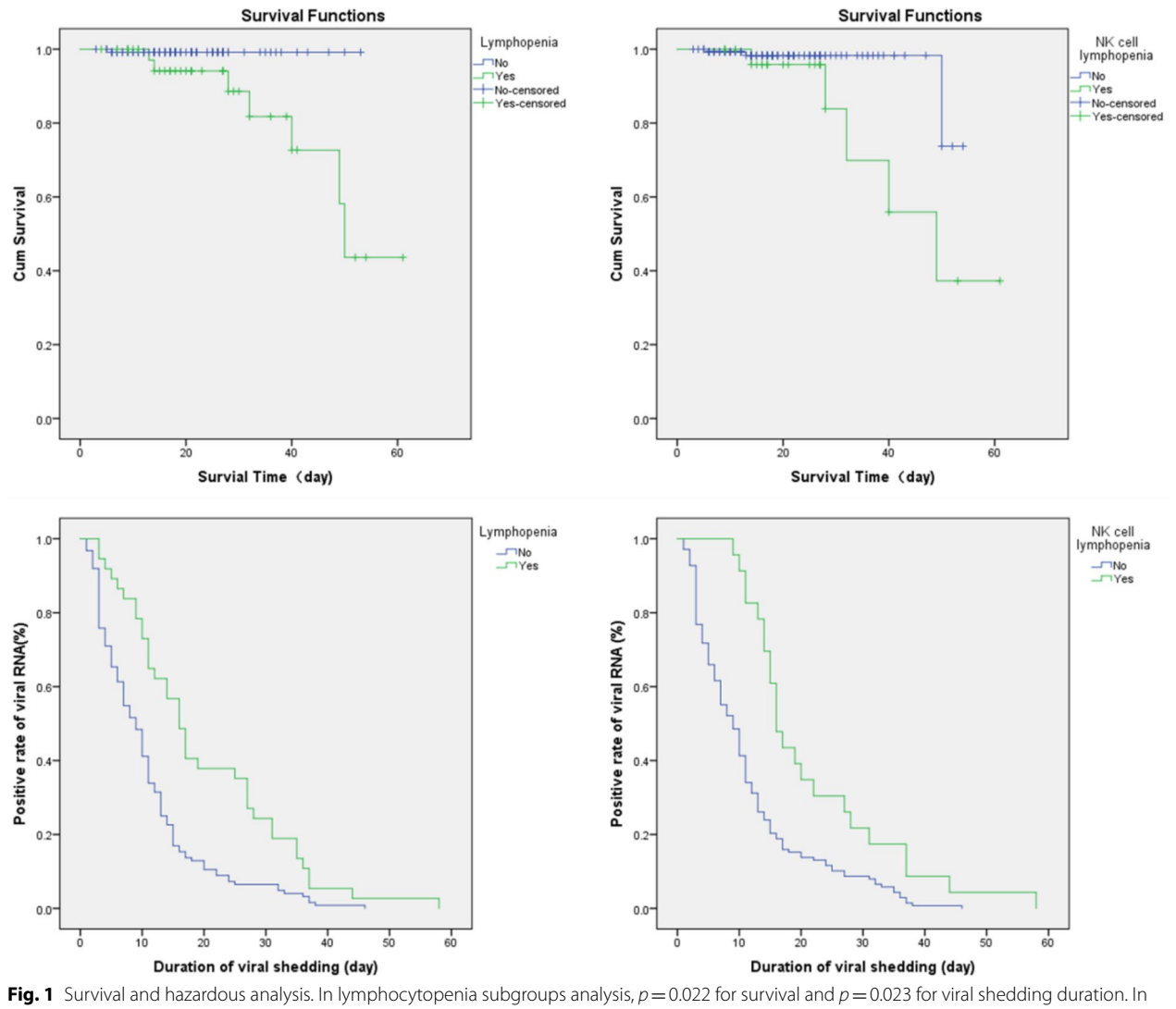

Fig. 1 Survival and hazardous analysis. In lymphocytopenia subgroups analysis, $p = 0.022$ for survival and $p = 0.023$ for viral shedding duration. In NK cell lymphocytopenia $p < 0.001$ for survival and $p < 0.001$ for viral shedding duration

viral shedding than the normal lymphocytes count group. The NK cell lymphocytopenia ($<0.1 \times 10^9$/L) [4] group had a significantly worse survival rate (log-rank $p < 0.001$) and a longer duration (log-rank $p < 0.001$) of viral shedding (Fig. 1).

Both innate and adaptive immune responses are essential to control viral infections. NK cells, as a part of innate immune system, play an important role during acute viral infection [5]. The strength and duration of IgG after infection is the key point of immunity from SARS-CoV-2 [6]. NK cells have a direct killing effect on virus-infected cells through the killer-cell immunoglobulin-like receptors (KIR) [7], lysis-infected cells and releasing antigen. NK cells interact with dendritic cells [8] and may play a part in processes of antigen-presentation and adaptive immunity against SARS-CoV-2. NK cell counts could be critical to the IgG immunity for COVID-19. Engagement of CD16 on NK cells by antibody-coated virus-infected cells results in antibody-dependent cellular cytotoxicity [9]. The innate immune system serves as a frontline of clearance for coronavirus and regulates immune response. We hypotheses that NK cells can directly kill virus-infected cells through degranulation, receptor mediated apoptosis, and antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig. 2) [10, 11]. Also, it has been proven that NK cells conduct active crosstalk with autologous dendritic cells (DC) through a process that requires NK cell-DC cell interaction and the secretion

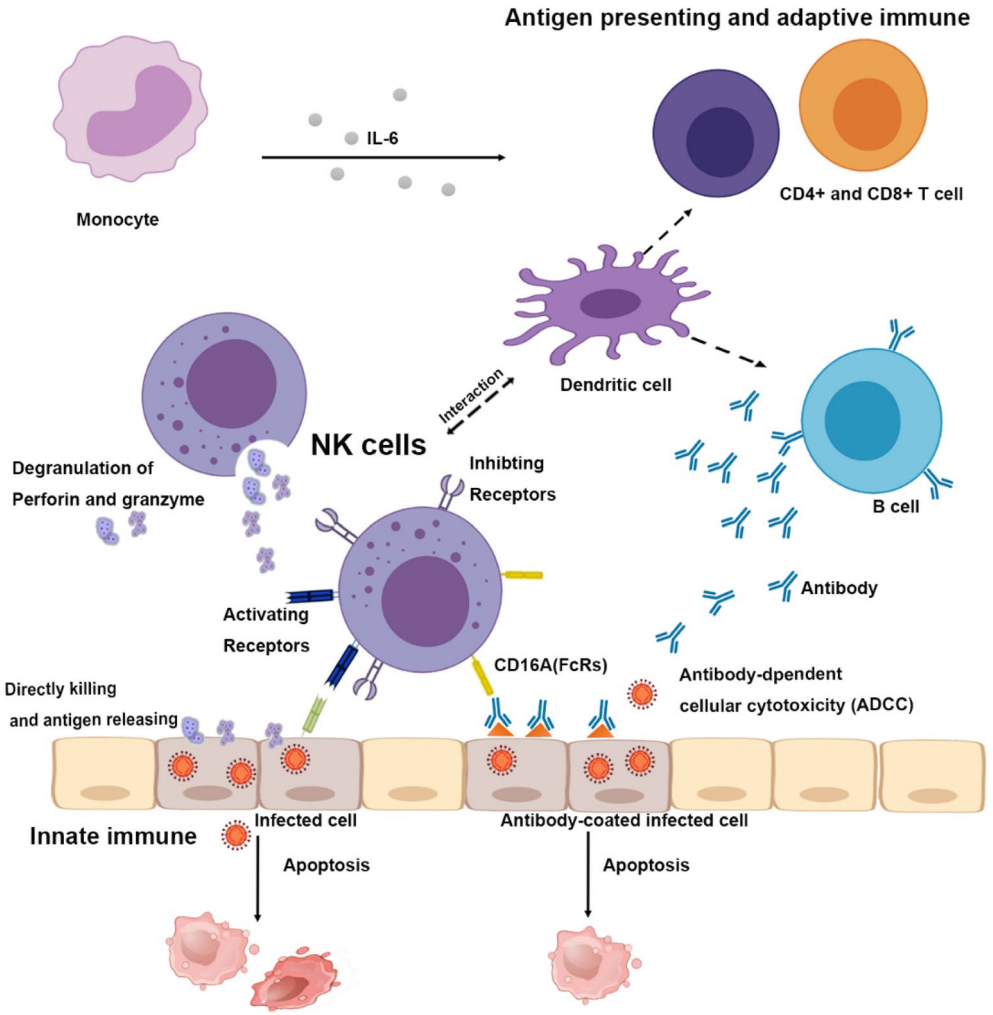

Fig. 2 The innate and adaptive immune response to SARS-CoV-2 infection. Hypothesis of the innate and adaptive immune response to SARS-CoV-2 infection. NK cells have direct killing effect on SARS-CoV-2 infected cells, as well as participate in antibody-dependent cellular cytotoxicity. Through inhibiting and activating receptors, NK cells recognize infected cells. Once activated, the degranulation of NK cells induces the releasing of perforin and granzyme which directly lyse infected cells. NK cells express CD16A, which participates in antibody-dependent cellular cytotoxicity. Meanwhile, NK cells also interact with dendritic cells in antigen presenting process and affect adaptive immune response. Monocytes can secret IL-6 that acts as inflammatory cytokine, resulting in the activation of other immune cells

of specific cytokines [12]. This may explain why the NK cell count is related to a specific IgM/IgG antibody response. So far, there are four COVID-19 related NK cell clinical trials registered on Clinicaltrials.gov, including hematopoietic stem cell derived NK cells infusion (NCT04365101), NK Cells in combination with standard therapy (NCT04280224), IL-15 superagonist- and GM-CSF neutralizing scFv-secreting NKG2D-ACE2 CAR-NK cell therapy for COVID-19 patients (NCT04324996). Natural Killer cells do not cause GVHD, which provides an off-shelf anti-viral cell therapy. This study suggests the potential of Natural Killer cells as an universal COVID-19 treatment.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40164-021-00199-1.

Additional file 1. Additional material-Methods.

Additional file 2: Table S1. Clinical characteristics of patients.

Additional file 3: Table S2. Laboratoryexamination at admission.

Additional file 4: Table S3. Time to achieve the first SARS-CoV-2 nucleic acid negative test and the first positiveIgM/IgG test.

Additional file 5: Table S4. Correlation analysis.

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Authors' contributions
CB conceived the study, analyzed data, and drafted the manuscript. XT and WC collected and analyzed data. YH, SZ, BY, and TP reviewed the manuscript. KY and WQ supported the study technically and reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declared no competing interests.

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