EXPANSION SYSTEM FOR PRODUCING A LARGE AMOUNT (>10^9 CELLS) AND HIGH PURITY (>90%) OF HUMAN CD3$^-$CD56$^+$NK CELLS FROM PBMCs AND THEIR THERAPEUTIC APPLICATION DUE TO ADCC ACTIVITY IN XENOGENIC MOUSE MODEL

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Abstract

Human natural killer (NK) cells are one of attractive candidates for cell-based therapy against any cancers due to their strong cytotoxicity. However, there are few convenient and efficient method to obtain a large amount and high purity of functional NK cells from peripheral blood mononuclear cells (PBMCs) derived from a small amount of blood. Thus, we have developed a robust NK-cell expansion method using OK-432, IL-2 and RetroNectin® induced T (RN-T) cells as a stimulator. RN-T cells were prepared by previously established co-stimulation method using anti-CD3mAb and RetroNectin®, and treated to suppress the growth potential (modified RN-T cells). NK cells could be expanded from PBMCs stimulated with modified RN-T cells, OK-432 and IL-2, then cultured for more than 16 days. In our large-scale culture system using gas-permeable culture bag (CultiLife™215 and CultiLife™Eva), we could obtain 10^9 – 10^10 cells containing a high proportion (>90%) of CD3-CD56+ NK cells from 50mL of peripheral blood. Furthermore, almost all cells displayed functional cell surface molecules such as NKG2D and CD16 implicated in cytotoxicity and antigen dependent cell cytotoxicity (ADCC). Thus, we investigated the antitumor effect of the expanded NK cells combined with Trastuzumab against HER2-positive human gastric cancer cell line NCI-N87 in hIL-2 Tg NOG mice (hIL-2-NOG mice; Central Institute for Experimental Animals). In this experiment, we used purified NK cells to reduce GVHD risk caused by human CD3+ cells including in the expanded cells. As a result, the combination of the NK cells and Trastuzumab dramatically enhanced the antitumor activity compared with each treatment alone. The chimerism of human NK cells in mouse peripheral blood was observed during the observation period without any GVHD symptoms and functional NK-cell surface markers such as CD16 and NKG2D also expressed in human NK cells. Furthermore, human NK cells were observed into tumor tissue even in 3 months after administration. Overall, we have established a robust NK-cell expansion system and the expanded cells showed strong antitumor activity in a xenogenic mouse model. It is considered that our expansion system could be used for chimeric antigen receptor (CAR)-NK cell processing or pluripotent stem cell derived NK-cell manufacture for future application.

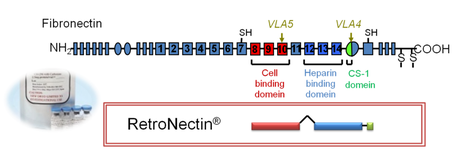
The issues of NK-cell expansion for therapeutic application

✓ Low purity of NK cells after expansion

✓ Low proliferation of NK cells

The important factors for NK-cell expansion are ・・・・

- High purity
- High fold expansion
- High functionality
- Reproducibility



We developed a large-scale NK-cell expansion method using RetroNectin® induced T-cells

Procedure for NK-cell expansion

1) Culture of RN-T (RetroNectin® induced T-cells)

10mL peripheral blood → PBMC

Day-7 → D0 → D7

・Anti-CD3mAb
・RetroNectin®
・IL-2

Modified RN-T

TaKaRa GMP grade

Anti-CD3mAb (GMP) #T210

RetroNectin® (GMP) #T201

2) Culture of NK cells

20~40mL peripheral blood → PBMC

Day0 → Day17-21

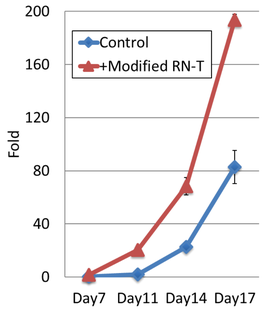
・OK-432
・IL-2

Culture bag CultiLife® 215

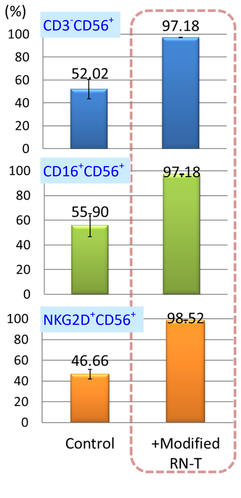
Culture bag CultiLife® Eva

NK-cell expansion using modified RN-T

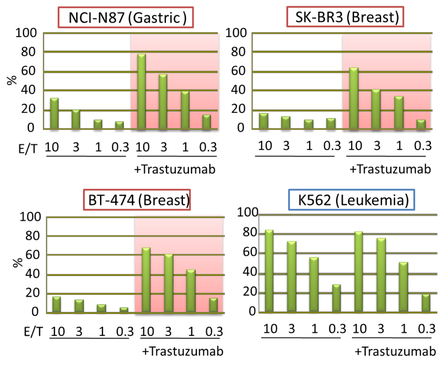
Fold expansion



We could obtain high purity (>90%) NK cells by combination stimuli of OK-432 and modified RN-T.



ADCC activity with Trastuzumab (anti-Her2Ab)



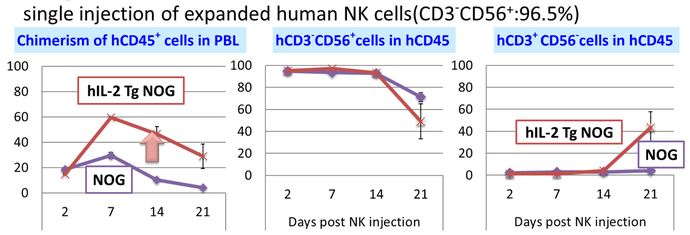
In combination with Trastuzumab, expanded NK cells showed potent ADCC-activity against Her2 positive cell lines, even in NK cell-resistant tumor cells.

hIL-2 Tg NOG mice model and study design for evaluating in vivo ADCC activity

hIL-2 Tg NOG mice model

hIL-2 Tg NOG mice: NOG sub-strain expressing human IL-2
(Central Institute for Experimental Animals)

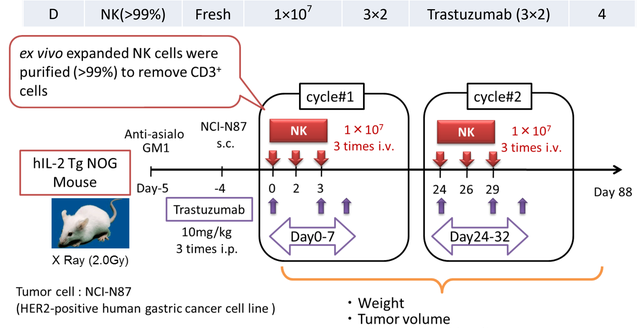
single injection of expanded human NK cells(CD3$^-$CD56$^+$:96.5%)



The persistence of *ex vivo* expanded NK cells was remarkably improved by using hIL-2 Tg NOG mice. CD3$^+$ cell proportion also increased at day21 and it caused the development of GVHD.

Study design

Group	NK				mAb	n
A	-	-	-	-	-	4
B	-	-	-	-	Trastuzumab (3×2)	4
C	NK(>99%)	Fresh	1×10^7	3×2	-	4
D	NK(>99%)	Fresh	1×10^7	3×2	Trastuzumab (3×2)	4

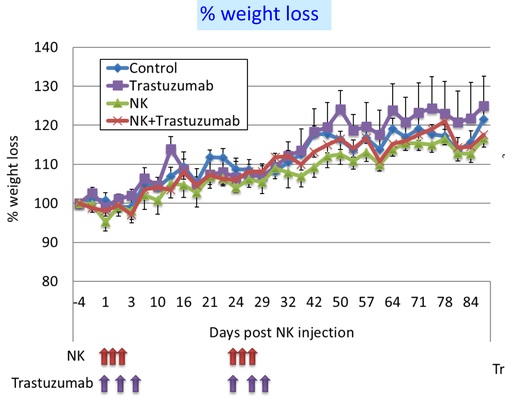


Tumor cell : NCI-N87
(HER2-positive human gastric cancer cell line)

- Weight
- Tumor volume
- FCM analysis (blood)
- *in vitro* ADCC assay (spleen cell)
- Immunohistochemistry

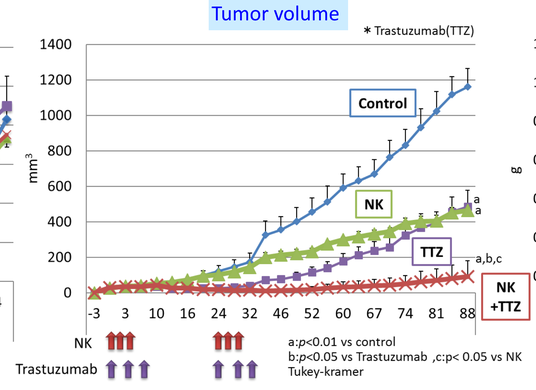
Anti-tumor effect of combination therapy with ex vivo expanded human NK cells and Trastuzumab in hIL-2 Tg NOG mice

% weight loss

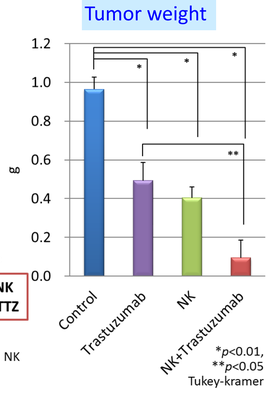


No significant differences in weight loss were observed among the groups.

Tumor volume

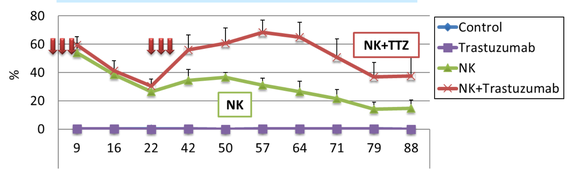


Tumor weight

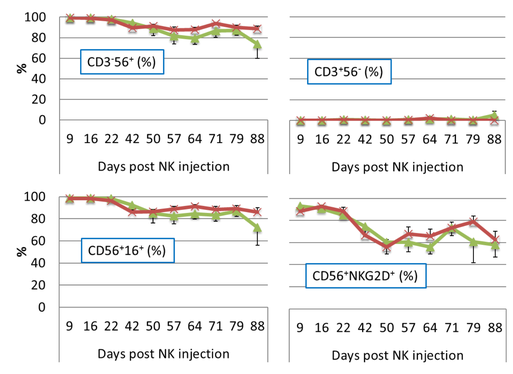


The moderate effects were observed in monotherapies.
NK-cell treatments with Trastuzumab significantly reduced tumor volume and led to complete tumor regression in 2 out of 4 animals.

Chimerism of human CD45$^+$ cells in PBL

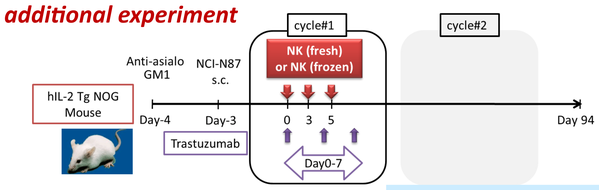


NK-cell surface maker in human CD45$^+$ gate (%)

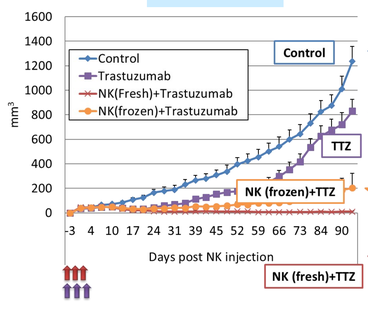


Chimerism of human CD45$^+$ in PBL was observed for long period.
Functional NK-cell surface markers (CD16, NKG2D) were maintained in human CD45$^+$ cells.

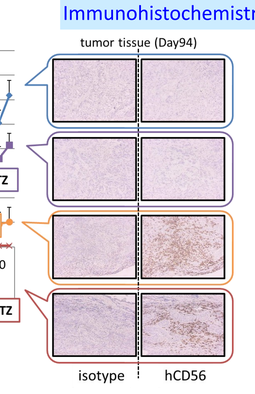
additional experiment



Tumor volume



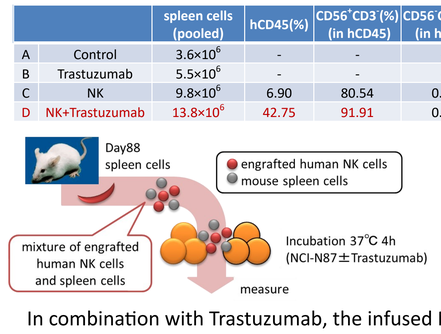
Immunohistochemistry

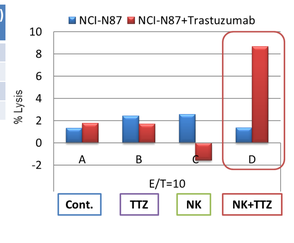


Even in only single cycle of treatment, NK-cell with Trastuzumab significantly reduced tumor volume.
Cryopreserved NK cells also showed anti-tumor effect in combination with Trastuzumab.
Ex vivo expanded human NK cells infiltrated in tumor tissue and they were observed even at 3 months after injection.

in vitro ADCC activity of human NK cells from spleen cells

		spleen cells (pooled)	hCD45(%)	CD56$^+$CD3$^-$(%) (in hCD45)	CD56$^-$CD3$^+$(%) (in hCD45)
A	Control	3.6×10^6	-	-	-
B	Trastuzumab	5.5×10^6	-	-	-
C	NK	9.8×10^6	6.90	80.54	0.60
D	NK+Trastuzumab	13.8×10^6	42.75	91.91	0.09





In combination with Trastuzumab, the infused NK cells were kept potent ADCC activity.

Summary

- ☑ High purity of NK cells can be obtained from small amount of peripheral blood by using our expansion system.
- ☑ The expanded NK cells showed *in vivo* as well as *in vitro* anti-tumor activity.
- ☑ Our NK-cell expansion system is highly potent tool to produce a large amount of functional NK cells.

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