Natural killer cells are specific innate lymphoid cells which have therapeutic ability in adoptive cell transfer—primarily based on most cancers immunotherapy that has been installed throughout a variety of early-phase clinical trials. Natural killer cells to be used in adoptive transfer treatments are received from diverse resources. These cells have attracted widespread interest as adoptive immunotherapy for most cancers because of their preventive antitumor properties. In order to study the efficacy of adoptive natural killer cell immunotherapy in a preclinical manner with clinical translation potential, a reliable \textit{ex vivo} natural killer cell expansion platform may be required. As a preclinical study, we have designed a humanized mouse model using NOD scid gamma mice, human leukaemia cells and expanded natural killer cells. At the beginning, peripheral blood mononuclear cells from patients with different thalassemia subtypes were co-cultured with irradiated, genetically engineered K562-mb15-41BBL cells in the presence of interleukin-2 for 14 d. Humanized NOD scid gamma mice with human leukaemia were treated with different approaches of natural killer cells, gamma delta cells and clusters of differentiation 20 immunoglobulin G1 antibody. \textit{In vivo} results show that our strategy of immunotherapy with expanded natural killer cells has extended survival of mice. Flow cytometry results from the peripheral blood mononuclear cells of the treated mice showed that the expanded natural killer cells have potential ability to effectively kill the leukaemia cells. Based on these findings, adoptive transfer of expanded and activated natural killer cells \textit{ex vivo} is gaining similar clinical evaluation as a potential new therapeutic alternative for patients with tumors and other immunological diseases.

Key words: Thalassemia, immunotherapy, natural killer cells, NOD scid gamma mice

During the last decade, there was excellent interest in making use of Natural Killer (NK) cells adaptive immune treatment for numerous tumors\cite{1-5}. The natural potential of NK cells to kill tumor cells without prior sensitization\cite{6}, their central role in anticancer reaction and immune monitoring \textit{vs.} most cancers\cite{7}, in conjunction with the findings that NK cells did not induce Graft Versus Host Disease (GVHD) after Hematopoietic Cell Transplantation (HCT)\cite{8}, at the same time as preservation transplant \textit{vs.} tumor impact have all been critical to the general entanglement of NK cells for adaptive immune treatment for most cancers\cite{9}. Even so, the development of NK cell immunotherapy for most cancers has been sluggish, especially because of the challenge in acquiring enough quantity of NK cells (NKs) for strong preclinical and medical critiques. In regular human Peripheral Blood Mononuclear Cells (PBMCs), the NK cells include 1\%-32.6\% of PBMCs\cite{10}. Using immunotherapy has extended extensively during the last 25 y. The new findings in fundamental biology of NKs, collectively with developing scientific experience inside the establishment of HCT, have positioned NKs alongside a direction of translational refinement. A better knowledge of the mutual influences among stimulated and suppressive immune cells that respond to infected cells have antitumor roles in establishing NK therapy for cancer, essential clinical advances encompass using cytokines to prompt NKs \textit{in vivo}\cite{11-13}, redirecting or modifying NK cell signatures using monoclonal antibodies\cite{4-6}, adoptive transfer of T cells or NK cells with anti-tumor interest\cite{14,15} and crucially NKs are engineered to engraft with synthetic receptors specific for cancer cells unique antigens\cite{16,17}. In addition to NKs there are the gamma delta cells (\(\gamma\delta\) cells), \(\gamma\delta\) cells are a
little part of lymphocytes cytotoxic T cells. The γδ cells do not request antigenic presentation by Major Histocompatibility Complex (MHC) molecules for recognition and activation\textsuperscript{18,19}. Many studies showed that the expansion of γδ cells \textit{in vitro} or \textit{in vivo} is realistic and functional\textsuperscript{20,21}.

**MATERIALS AND METHODS**

*Ex vivo expansion of NK cells:*

Peripheral blood was obtained from patients and healthy donors after informed written consent. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were incubated with irradiated (100 Gy) artificial antigen expressing K562 modified to express a membrane-bound form of Interleukin (IL)-15 and 4-1BB Ligand (K562-mb15-41BBL) cells at a ratio of 1:1.5 (PBMC:irradiated feeder cells) in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10 % human AB serum (Department of Transfusion Medicine, University Hospital Tuebingen, Germany), L-glutamine (Life Technologies) and 100 IU/ml IL-2 (Proleukin) (Novartis, Basel, Suisse). The K526-mb15-41BBL cell line which was genetically modified to express membrane-bound IL-15 and 4-1BB (Cluster of Differentiation (CD) 137) ligand was kindly provided by Dario Campana, Department of Paediatrics, Center for Translational Medicine, National University of Singapore, Singapore. Half of the medium changes were done every 2-3 d with fresh IL-2 containing medium. Cell culture was maintained under the above described conditions for 14 d. Expanded NK cells were purified by CD56 positive cell selection with antibody-conjugated immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by CD3 positive cell depletion using Dynabeads (Life Technologies, Darmstadt, Germany). Phenotyping of PBMCs of treated NOD Scid Gamma (NSG) mice cells was performed using directly conjugated monoclonal antibodies or appropriate isotype controls against: CD16 (clone 3G8), CD25 (clone M-A251) FITC, CD56 (clone B159) PE-Cy7, CD19 (clone B19 RUO), CD10 (clone H110a) (Becton Dickinson, Heidelberg, Germany). CD3 (clone BW264/56) VioBlue (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were analyzed on a LSRII flow cytometer (Becton-Dickinson, Heidelberg) and FCS Express software (de novo Software, Los Angeles, USA). Mean Fluorescence Intensity (MFI) ratios and percent positive cells were calculated for each cell surface antigen mice.

**NSG mice:**

NSG mice (JAX mouse strain name NOD. Cg-PrkdcscidIl2rgtm1Wjl/SzJ; Jackson Laboratory, USA) were housed in air-flow cages under special pathogen-free conditions at the Laboratory Animal Facility of the University Children’s Hospital Tubingen, Germany. All animal procedures were evaluated by the Animal Care Committee of the University of Tubingen (No. K06/11).

**Humanization of NSG mice:**

Human CD34 (HuCD34) stem cells were obtained from peripheral blood stem cells mobilized with excess Granulocyte-Colony Stimulating Factor (G-CSF) from parental donors which were deficient in T-cells due to CD34 selection (CliniMACS, Miltenyi, Germany). The cells were added in a proportion of 1:2 to a solution of Dimethyl Sulfoxide (DMSO)/5 % Human Serum Albumin (HSA) (20 %/80 %) and a SY-LAB Ice cube device and a regulated freezing rate was used to cytopreserve them. Cells were stained using Trypan blue following thawing and a Neubauer cell count chamber was used to determine their numbers. All donors provided their informed consent to scientifically use surplus cells according to the Declaration of Helsinki. There was an additional increase in the purity of the CD34 Complex (C) population to more than 99.99 % through a second round of CD3C depletion following thawing (LS Magnetic-activated cell sorting (MACS), Miltenyi). All of the stem cell donors were Human Leukocyte Antigen (HLA) mismatched to the human Rhabdomyosarcoma cell lines (RMS A204). The HuCD34C cells (1 \times 10^6 cells in 100 ml prewarmed Phosphate Buffered Saline (PBS)) were added to the tail vein of sublethally irradiated (250 centigray (cGy)) NSG mice. Engraftment was supported by applications of 20 mg FcIL-7 (Merck, Germany) on a weekly basis. In every Nance-Horan syndrome (NHS)-IL 12 (IL-12 was complexed with NHS76) treatment group, long-term NHS-IL 12 cytokine treatment was given to 4 animals with FcIL-7 or IL-2MAB602 for 15 w at most (100 d).

**Tumor implantation and measurement:**

Human leukaemia cells were obtained from patient...
blood and implantation was done into engrafted NSG mice. For each mouse, $1 \times 10^6$ leukaemia cells were transplanted directly into the tail vein of NSG mice.

**Treatment with expanded NK cells:**

Two times every week for each mouse $5 \times 10^6$ expanded NKs or $2.5 \times 10^6$ expanded NKs + $2.5 \times 10^6$ expanded γδ cells with and without using antibody (concentrations of 0.4 µg/ml of CD20 Immunoglobulin G 1 (IgG1) antibody; Mabthera; Roche, Basel, Switzerland) were injected in the tail vein of mice. Treatment was started 24 h after tumor implantation. The control group was injected with Primary Biliary Cholangitis (PBC). Treatment was terminated after 3 w.

**Statistical analysis:**

GraphPad Prism version 5 (GraphPad Software Inc, La Jolla, United States of America (USA)) was used to carry out statistical analysis. Data expression was either in the form of median and range or as mean±Standard Error of the Mean (SEM). Two-tailed t-test was carried out to find out the significance levels and p values of 0.05 or lower was deemed to be statistically significant.

**RESULTS AND DISCUSSION**

Treatment using *ex vivo* expanded NK cells increased the survival rate of mice. To confirm the cytotoxicity of NK cells after *ex vivo* expansion against human leukaemia cells, we tested this toxicity *in vivo* using humanized NSG mice. 24 h after injection of leukaemia cells we started the treatment with *ex vivo* expanded NK cells alone or with γδ cells and CD20 IgG1 antibody, we analyzed the effect of treatment on the survival of mice. The survival curves are shown in fig. 1. The cumulative percentage survival was significantly higher after treatment with NK cells, γδ cells and CD20 IgG1 antibody in comparison with the untreated mice (67.1 %). In general, treatment with NK cells increased survival rate of mice (fig. 1).

Treatment using *ex vivo* expanded NK cells reduced the proliferation rate of leukaemia in PBMCs of mice. To understand the effect of the treatment using *ex vivo* expanded NK cells on the survival rate of mice, we analysed the quantity of leukaemia cells in PBMCs from mice after treatment. The flow cytometry rate shows that the proliferation rate of leukaemia cells was reduced by 10 % after the treatment with expanded NK cells alone, while the treatment in combination between expanded NK cells and γδ cells and CD20 IgG1 has significantly reduced the proliferation rate of leukaemia cells by 20 % (fig. 2).

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**Fig. 1:** Effect of *ex vivo* expanded NK cells on NSG mice survival after tumour injection and effects of treatment using expanded NK cells alone or with expanded γδ cells and CD20 IgG1 antibody on survival. For the 5 mice in every group, the treatment was to be started 24 h after tumour injection, 2 times every week. The treatment was to be terminated after 3 w. Treatment groups include untreated; PBS, NKs: Only with expanded NKs; NKs+Ab: NKs+CD20 IgG1 antibody; γδs+NKS: γδ cells and NKs; γδs+NKS+Ab: γδ cells and NKs and CD20 IgG1 antibody, Note: ( ) γδs+NKS+Ab; ( ) γδs+NKS; ( ) NKs+Ab; ( ) NKs; ( ) untreated
The results of this study indicate that NK cells can be efficiently *ex vivo* expanded and activated despite functional impairment. Co-culture with irradiated K562 cells genetically modified to express membrane-bound IL-15 and 4-1BB ligand shifted the NK cell receptor balance towards activation, resulting in enhanced cytotoxicity against tumor leukemia cells. A main fence in cancer immunotherapy has been the several mechanisms by which cancer induce malfunction or tolerance of immune cells\(^{[22]}\). The application of NK cells as therapeutic beginning for the treatment of cancer, an inversion of phenotypic and functional failure is of utmost importance. Before *ex vivo* expansion, NK cells from patients showed reduced expression of activating receptors and no cytotoxic activity against tumor cell lines. This tumor-associated NK cell phenotype is a common phenomenon in patients with different kinds of malignancies. Moreover, in recent decades, the monoclonal antibodies have been better used to carry out the increase in recognition of the cancer cells by immune cells\(^{[23-25]}\). There are several mechanisms of monoclonal antibodies functions which enclose supplement fixation, induction of Antibody-Dependent Cellular Cytotoxicity (ADCC) and induction of aberrant signaling. The isotype IgG1 subclass is best often used for antibody treatment since it has approved and extremely forceful at stimulating and activating Fragment crystallizable (Fc) receptors on NK cells, neutrophils and macrophages\(^{[26]}\). Medical application of NK cells has been inspired by acceptance of their powerful antitumor activity. Many studies at present shown a respectable foundation for evolution of future NK cell trials for immunotherapy as reduction risks and toxicities\(^{[14,20,27-29]}\). For the improvement of NK cell treatments, either additional study of basic NK biology as a further insight of interactions with other immune cells will be necessary. The aim of developing clinical studies in the future will be to overcome the patient’s immune barriers. In addition, new strategies should be designed to achieve better *ex vivo* expansion rates. Based on the data presented, *ex vivo* expansion and activation of NK cells deserves further clinical

**Fig. 2:** Treatment effect of *ex vivo* expanded NK cells on quantity of leukemia in PBMCs. (A) Representative phenotype of leukemia cells in PBMCs from mice after treatment. Phenotype analysis of quantity of leukemia cells in PBMNCS of mice after treatment with expanded NK cells was performed by flow cytometry; (B) Significant effect of treatment with expanded NK cells and γδ cells on reducing the percent of leukemia cells in the lymphocytes. Treatment groups include untreated; PBS, NKs: Only with expanded NKs; NKs+Ab: NKs+CD20 IgG1 antibody, γδs+NKs: γδ cells and NKs, γδs+NKs+Ab: γδ cells and NKs and CD20 IgG1 antibody, (■) leukemia cells.
evaluation as a possible new treatment approach for patients with soft tissue leukaemia.

Author’s contributions:
All authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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