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Technical note

Retroviral gene transfer into primary human NK cells activated by IL-2 and K562 feeder cells expressing membrane-bound IL-21

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ABSTRACT

Natural killer (NK) cells are capable of rapidly recognizing and efficiently killing tumor cells. This makes them a potentially promising agent for cancer immunotherapy. Additional genetic modifications of NK cells may further improve their anti-tumor efficacy. Numerous technical challenges associated with gene delivery into NK cells have significantly tempered this approach. We achieved efficient retroviral vector transduction of primary human NK cells that were stimulated by a combination of IL-2 and engineered K562 cells expressing membrane-bound IL-21. The activated NK cells were in less differentiated state and expressed NK cell activation receptors NKG2D, NKp30, CD16, and were highly HLA-DR-positive. This NK cell population was highly susceptible to the transduction by both GFP- and NGFR-expressing retroviral vectors, with transduction efficiency exceeding 50%. More mature CD57$^+$ NK cell population was generally resistant to retroviral vector transduction because of poor response to the stimulation. Our findings may facilitate retroviral vector-mediated genetic engineering of human primary NK cells for future immunotherapies.

1. Introduction

NK cells can recognize and destroy stressed and virus-infected cells, and tumor cells. Both stem-cell-derived and mature allogeneic NK cells are effective in the adoptive immunotherapy of several hematological malignancies and are now being considered as a treatment of some solid tumors (Miller et al. 2005; Yang et al. 2016). For successful cancer immunotherapy, adoptively transferred NK cells must be capable of successfully engrafting and persisting in the host, and should be able to selectively target and kill tumor cells. At least some of highly desirable properties of therapeutic NK cells (such as the expression of native or chimeric surface receptors, or the increase of their proliferative potential) could be realized by their specific genetic modifications. In contrast to T lymphocytes, the genetic engineering of the NK cells is often challenged by generally low efficiency of viral vector transduction. However, several reports recently demonstrated successful gene transfer into mature NK cells by viral vectors, which varied in efficiency (Alici et al. 2009; Suerth et al. 2016). Based on previous published work on human T cells, we speculated that activating NK cells before attempting retroviral vector-mediated transduction could improve the efficiency of gene delivery. Here, we used murine leukemia virus (MLV)-based vectors expressing green fluorescent protein (GFP) or C-terminally truncated nerve growth factor receptor (NGFR) reporter genes to test genetic modifications of human lymphocytic leukemia cell line Jurkat, and primary human NK cells activated by combining interleukin 2 (IL-2) with genetically engineered K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21). This combination of NK cell-activating factors has been found to induce a vigorous expansion of functional NK cells capable of enhanced killing of several tumor targets (Denman et al. 2012). Synergistic actions of IL-2 and soluble IL-21 were shown to up-regulate several activation markers in NK cells. These markers included HLA-DR, which marks a subset of activated human NK cells that proliferate vigorously in culture and are capable of degranulation (Skak et al. 2008). In one clinical trial, the levels of HLA-DR$^+$ NK cells correlated with progression-free and overall survival of patients with treatment-refractory solid tumors (Chaput et al. 2013). Here, we show that *ex vivo*-activated less differentiated NK cells characterized by high expression of HLA-DR and low expression of CD57 are highly susceptible to transduction with retroviral vectors.

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; GFP, green fluorescent protein; IL-2, interleukin 2; K562-mbIL21, K562 expressing membrane-bound IL-21; NGFR, nerve growth factor receptor; NK cells, natural killer cells; PBMC, peripheral blood mononuclear cells; PI, propidium iodide

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2. Materials and methods

2.1. Primary cells and cell lines

Human primary NK cells and Jurkat cells were cultured in the complete medium: RPMI-1640 medium containing 2 mM L-glutamine (PanEco, Russian Federation), 1% antibiotic-antimycotic (Sigma Aldrich, USA), and 10% fetal calf serum (FCS, HyClone, USA). Retroviral packaging cell line GP2-293 (Thermo Fisher Scientific, USA) was maintained in DMEM (PanEco, Russian Federation) supplemented with 10% FCS and 1% antibiotic-antimycotic. K562-mbIL21 feeder cells (Denman et al. 2012) were kindly provided by Dr. D. Lee (MD Anderson Cancer Center, USA). The cells were grown in the complete RPMI-1640 medium, then γ-irradiated at 100 Gy, frozen and stored in liquid nitrogen.

2.2. Isolation, activation, and phenotypic characterization of primary NK cells

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers who provided their informed consent (approved by the local research ethics committee) prior to the study. NK cells were isolated from PBMC by immunomagnetic separation using human NK Cell Isolation Kit (Miltenyi Biotech, Bergisch-Gladbach, Germany). To induce activation, NK cells (5×10^5 cells/well) were cultured in 24 well-plates in a complete RPMI-1640 medium containing IL-2 (100 U/ml) and feeder K562-mbIL21 cells (4×10^5 cells/well), for one week. On day three, half of the culture medium in each well was replaced with fresh complete medium containing IL-2. Surface marker expression was analyzed in NK cells by FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with 488 and 640 nm lasers. Immunostaining of NK cells with fluorochrome-labeled antibodies CD56-PE, CD56-APC (clone N901, Beckman Coulter, USA), CD57-FITC, CD57-APC (clone TB03, Miltenyi Biotec,), CD57-PE (clone TB01, eBioscience, USA), KIR2DL2/DL3-PE (clone DX27, Miltenyi Biotech), HLA-DR-FITC (clone Immu-357, Beckman Coulter, USA), CD16-PE (Sorbent, Russian Federation), CD16-APC (clone 3g8, Sony Biotechnology, USA), NKG2D-PE (clone BAT221, Miltenyi Biotec), NKp30-PE (clone P30-15, Biolegend, USA), NKG2C-PE (clone 134,522, R & D Systems, USA), NKG2A-PE (clone REA110, Miltenei Biotec, Germany), or appropriate isotypic controls (all Miltenyi Biotec) was performed as described (Kovalenko et al. 2017). Sorting of NK cell subsets was performed using FACSVantage DiVa fluorescence-activated cell sorter (Becton Dickinson, USA) equipped with 405, 488 and 643 nm lasers.

2.3. Transfection, transduction, and flow cytometry analysis

GP2-293 packaging cells were co-transfected with xlox-GFP or xlox-NGFR vectors expressing GFP or NGFR reporter gene under the control of human PGK promoter, and the pseudotyping construct expressing RD114 envelope glycoprotein, using calcium phosphate transfection kit (Thermo Fisher Scientific). The transfection efficiency was estimated by measuring fluorescence intensity of intracellular GFP or surface NGFR stained with CD271-PE (clone ME20.4-1.H4, Miltenyi Biotec, Germany) using confocal microscopy or flow cytometry, respectively. Retroviral particles were concentrated using the Amicon Ultra-15 Centrifugal Filter Unit cones with 100 nm pores (Millipore, USA). Transduction of Jurkat or *ex vivo*-cultured human primary NK cells was performed in 24-well plates pretreated with RetroNectin, a recombinant fragment of human fibronectin (Takara, USA) as described (Suerth et al. 2016). Transduced cells were enumerated by detecting expression of GFP (or NGFR after CD271-PE staining) in 2, 3 and 5 days post-transduction by flow cytometry. A minimum of 10′000 gated events was collected for each sample. Flow cytometry data were analyzed using FlowJo software version 7.6 (TreeStar, USA). Statistical significance of the differences was determined by Student's *t*-test or Mann-Whitney *U* test for normally or non-normally distributed data, respectively. P-values of < 0.05 (*) were considered significant. Throughout the text the data are presented as mean ± S.E.

2.4. Proliferation and cell cycle assays

For proliferation assessment, NK cells (10^7 or less) were washed in PBS and labeled with 5 mM CFSE (eBioscience, USA) in 100 µl of serum-free medium for 10 min at 25°C in the dark. Then 15 ml completed medium was added. The cells were incubated for 5 min on ice, washed in complete medium twice and then cultivated as described. The samples were analyzed by flow cytometry on days 4 and 7 of incubation. For cell cycle analysis, IL-2/K562-mbIL21-stimulated NK cells were labeled with CD56-PE and CD57-FITC, washed with PBS and stained with 10 µg/ml Hoechst 33,342 (Life Technologies, USA) for 45 min at 37 °C in the dark. Propidium iodide (5 µg/ml) (PI, Invitrogen, USA) was added 5 min before acquiring the data.

3. Results and discussion

3.1. Retroviral transduction of Jurkat cell line

Initially, the retroviral vector system was validated using Jurkat T cell line. GFP- and NGFR-expressing vectors were pseudotyped with RD114 retroviral envelope glycoprotein to enable the transduction of human lymphocytes. The optimal numbers of retroviral particles in concentrated vector stocks mediating effective transduction were determined using Jurkat cells known to be easily transduced by retroviral vectors. We achieved the levels of Jurkat cell transduction between 44 and 66% with GFP vector. The transduction efficiency of NGFR vector

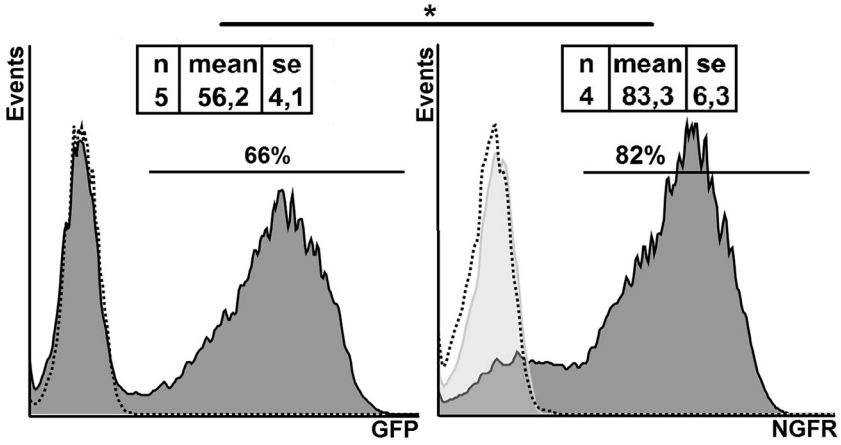

Fig. 1. Transduction of Jurkat T cells (3 days of incubation) using xlox-GFP (left) or xlox-NGFR (right) vectors measured by flow cytometry. Representative results are shown. Autofluorescence controls are shown in dotted line. Isotype-matched control for CD271-PE is shown in light gray. Here and after: n - number of independent experiments, mean - average percentage of positive cells, se - standard error.