Immunobiology 219 (2014) 547–553

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

hIL-15 gene-modified human natural killer cells (NKL-IL15) augments the anti-human hepatocellular carcinoma effect *in vivo*

Wen Jiang, Cai Zhang, Zhigang Tian, Jian Zhang*

Institute of Immunopharmacology & Immunotherapy, School of Pharmaceutical Sciences, Shandong University, 44 Wenhua West Road, Jinan 250012, PR China

ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 25 July 2013
Accepted 12 March 2014
Available online 20 March 2014

Keywords:
Interleukin-15
Gene transfer
Natural killer cells
Natural killer cell line

ABSTRACT

Genetic modification of NK cells may provide new possibilities for developing effective cancer immunotherapy by improving NK cell function and specificity. We previously established human interleukin-15 (hIL-15) gene-modified NKL cells (NKL-IL15) and demonstrated their therapeutic efficiency against human hepatocellular carcinoma (HCC) *in vitro*. To further assess the applicability of NKL-IL15 cells in adoptive cellular immunotherapy, we further investigated their natural cytotoxicity against HCC *in vivo* in the present study. NKL-IL15 cells exhibited strong inhibition on the growth of transplanted human HCC tumors in xenograft nude mouse models. Further investigation showed that NKL-IL15 cells expressed much higher levels of cytolysis-related molecules, including NKp80, TRAIL, granzyme B, IFN-γ, and TNF-α, than parental NKL cells in response to HCC stimulation. Moreover, soluble mediators secreted by NKL-IL15 cells decreased HCC cell proliferation; in particular, NKL-IL15-derived TNF-α and IFN-γ induced higher NKG2D ligand expression on target cells and resulted in the increased susceptibility of HCCs to NKL-mediated cytolysis. These results show that hIL-15 gene-modified human NK cells can augment the anti-tumor effect of NK cells on human HCC *in vivo* and suggest their promising applicability as a new candidate for adoptive immunotherapy against HCCs in the future.

Introduction

Natural killer (NK) cells are key components of the innate immune system that play important roles in the first line of defense against various types of tumors and virus-infected cells independent of pre-sensitization or major histocompatibility (MHC) restriction (Cerwenka and Lanier, 2001; Miller, 2001; Roder and Pross, 1982). Various studies demonstrate that NK cells are involved in eradicating experimentally induced and spontaneous tumors in mice by releasing cytolysis-associated molecules and cytokines (Smyth et al., 1998, 2001; van den Broek et al., 1995). The ability of NK cells to effectively respond to tumor cells makes them promising effectors for immunotherapeutic strategies (Kärre et al., 1986), and their application in immunotherapy against cancer has recently entered clinical trials (Ljunggren and Malmberg, 2007).

Although using highly purified primary NK cells would be ideal for immunotherapy, these cells present technical limitations, such as generating a large enough number of highly purified NK cells to meet clinical requirements. Thus, NK cell lines were established under GMP conditions to take advantage of their unlimited proliferation potential (Tonn et al., 2001). The NKL cell line, for instance, is a well-characterized and currently established malignant NK cell line from the peripheral blood of a patient with $CD3^{-}CD16^{+}CD56^{+}$ large granular lymphocyte (LGL) leukemia; as these cells maintain the most original NK cell features among all NK cell lines, they have strong potential for use in adoptive immunotherapy as an effector cell population (Maasho et al., 2004; Robertson et al., 1996).

Another distinct advantage of using NK cells lines in cancer immunotherapy is the relative ease of genetically modifying cell lines, which may provide new possibilities for improving NK cell function or endowing these cells with additional functions. Until now, several cytokine genes, including IL-2, IL-15, and stem cell factor (SCF) (Nagashima et al., 1998; Zhang et al., 2004a,b; Jiang et al., 2008), have been used to modify NK cell lines in order to augment NK cell activation and/or cytotoxicity against tumor cells. In particular, IL-15 plays various important roles in regulating NK cells,

Abbreviations: CIK, cytokine-induced killer; DC, dendritic cells; ELISA, enzyme-linked immunosorbent assay; GMP, Good Manufacturing Practice; HCC, hepatocellular carcinoma; IFN-γ, interferon gamma; IL, interleukin; LAK, lymphokine activated killer; MHC, major histocompatibility; OD, optical density; PBMC, peripheral blood monocyte cell; SCF, stem cell factor; TNF-α, tumor necrosis factor-alpha.

* Corresponding author. Tel.: +86 531 8838 3781; fax: +86 531 8838 3782.
E-mail addresses: zhangj65@sdu.edu.cn, zj5995@sina.com (J. Zhang).

http://dx.doi.org/10.1016/j.imbio.2014.03.007
0171-2985/

which is demonstrated best in IL-15Rα$^{-/-}$ and IL-15$^{-/-}$ mice that do not develop any functional NK cells (Kennedy et al., 2000; Lodolce et al., 1998). Indeed, IL-15 is the major physiologic growth factor responsible for NK cell ontogeny (Fehniger and Caligiuri, 2001) and is also a potent regulator of NK cell proliferation, survival, and cytolytic activity (Grund et al., 2004; He et al., 2004).

In a previous study, we successfully established an hIL-15 gene-modified NKL cell line (NKL-IL15) and confirmed that the addition of hIL-15 augmented the NKL cell-mediated anti-hepatocellular carcinoma (HCC) effect *in vitro* (Jiang et al., 2008). However, whether this anti-HCC effect could also be improved in an *in vivo* setting was unknown. Therefore, to further confirm the applicability of NKL-IL15 cells against HCC cells and HCC tumors *in vivo*, we evaluated the anti-HCC efficacy of NKL-IL15 cells in xenograft tumor models in the present study. Moreover, we identified the mechanisms underlying hIL-15 gene modification-induced enhancement of NKL cell activation.

Materials and methods

Cell culture and cell lines

The human hepatocellular carcinoma cell line HepG2 was maintained in our laboratory and grown in RPMI-1640 medium (GIBCO/BRL, Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS). The NKL cell line was a gift from Professor Jin BQ (Department of Immunology, Fourth Military Medical University, Xi'an, PR China); it was maintained in our laboratory and cultured in complete IL-2-containing (100 U/mL) RPMI-1640 medium. NKL-IL15 and NKL-vec cells were established in our laboratory as described previously (Jiang et al., 2008) and cultured in the same conditions as NKL cells. All cells were incubated at 37 °C and 5% CO_2.

Animals

Female nude BALB/c mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and kept under specific pathogen-free conditions. All experimental procedures involving animals were conducted in accordance with the experimental animal guidelines approved by the State Science and Technology Commission, PR China. The animal study proposal and protocol were approved by the Ethical Committee of Shandong University.

Tumor challenge and treatment

To evaluate local tumor growth, HepG2 cells (5×10^6) were injected subcutaneously (s.c.) into the right flank of nude mice, or were administered intraperitoneally (i.p.). The mice were then randomly assigned to 3 experimental groups (PBS control, NKL-vec, and NKL-IL15) with 6 mice in each group. After 800 cGy (200 cGy/min) irradiation, 5×10^7 NKL-IL15 or NKL-vec cells were harvested and injected intravenously (i.v.) in a 200 μL volume on days 7 and 14 after tumor inoculation, while 200 μL of PBS was injected into the control mice. Four weeks later, the mice were sacrificed, and tumors, spleens, and livers were collected and weighed. Splenic lymphocytes were isolated by density gradient centrifugation and enumerated. To evaluate survival of xenograft mice, mice in each experimental group ($n = 6$/group) received 5×10^6 HepG2 cells (200 μL) i.p., followed by treatment with PBS control or 5×10^7 irradiated NKL-IL15 or NKL-vec cells on days 7 and 14 after tumor inoculation; mouse survival was evaluated twice a day.

Analysis of NKL cells in the tumor infiltrate

To evaluate NKL cells in the tumor infiltrate, HepG2 cells (5×10^6) were injected s.c. into the right flank of nude mice. After 7 days, 5×10^7 NKL-IL15 or NKL-vec cells were labeled with CFSE at 37 °C for 10 min and then inoculated i.v. into these tumor-bearing nude mice. Tumors were isolated 12 h later from xenograft mice, dissected into small pieces, and incubated in 0.25% trypsin at 37 °C for 20 min. Then, the upper portion of the cell suspension was carefully recovered and passed through a 70-μm cell sieve. After the cells were washed 3 times with PBS, the cells in the suspension were evaluated by FACS analysis.

Co-culture assay

HepG2 plus NKL-IL15 or NKL-vec cells were co-cultured using a 0.4 μm porous Transwell system in 12-well plates (Corning Costar, Tewksbury, MA, USA) in a 1:1 ratio for 24 h; NKL cells cultured in complete RPMI 1640 medium served as a control. To analyze their NK cell features, NKL-IL15 or NKL-vec cells were plated into the wells, while HepG2 cells were plated into the Transwell insert. To analyze HepG2 cell features, HepG2 cells were plated into the wells, while NKL-IL15 or NKL-vec cells were plated into the Transwell insert.

Cytokine ELISA

NKL cells (3×10^5/well) were plated in triplicate in 12-well plates with or without HepG2 cells. After 24 h of co-culture, TNF-α and IFN-γ levels in cell culture supernatants were evaluated by commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following the manufacturer's instructions.

Flow cytometry analysis

To detect the expression levels of the NKG2D ligands (ULBP1-3, MICA/B), HepG2 cells were stained with PE-conjugated ULBP1-3 (R&D Systems), PE-conjugated MICA/B (eBioscience, San Diego, CA, USA), or isotype control (eBioscience) at 4 °C for 45 min. To detect NKp80 and TRAIL expression levels, NKL-IL15 and NKL-vec cells were stained with PE-conjugated NKp80 (R&D Systems) or PE-conjugated TRAIL (eBioscience) at 4 °C for 45 min. For intracellular staining of CD107a, perforin, and granzyme B, NKL-IL15 and NKL-vec cells were treated with monensin (Sigma) for 4 h to inhibit the secretion pathway, and then these NKL cells were harvested and labeled with PE-conjugated CD107a (eBioscience), FITC-conjugated perforin (eBioscience), FITC-conjugated granzyme B (eBioscience), or isotype control (eBioscience) at 4 °C for 45 min. All stained cells were analyzed using a flow cytometer (FACSCalibur, USA), and the data were processed using WinMDI 2.9 software (Scripps Research Institute).

Proliferation assay

HepG2 cells (3×10^5/well) were plated in 12-well plates at 37 °C in a 5% CO_2 incubator and cultured alone or co-cultured with NKL-IL15 cells or NKL-vec cells in a Transwell plate for 24 h. Viable HepG2 cells in each well were counted after trypan blue staining. Each sample was performed in triplicate, and each well was counted 4 times.

Cytokine neutralization and receptor blockade

The following antibodies were used to neutralize cytokines in culture: anti-IFN-γ mAb (R&D Systems) was used at a final concentration of 5 ng/mL, and anti-TNF-α mAb (R&D Systems) was used