Cytotherapy, 2014; 16: 1419–1430

International Society for Cellular Therapy
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Effect of exposure to interleukin-21 at various time points on human natural killer cell culture

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Abstract

Background aims. Interleukin-21 (IL-21) can enhance the effector function of natural killer (NK) cells but also limits their proliferation when continuously combined with IL-2/IL-15. Paradoxically, membrane-bound (mb)-IL-21 has been shown to improve human NK cell proliferation when cultured with IL-2/mb-IL-15. To clarify the role of IL-21, we investigated the effect of the timing of IL-21 addition to NK cell culture. *Methods.* IL-2/IL-15–activated NK cells were additionally treated with IL-21 according to the following schedules; (i) control (without IL-21); (ii) first week (day 0 to day 7); (iii) intermittent (the first 3 days of each week for 7 weeks); (iv) after 1 week (day 8 to day 14); and (v) continuous (day 0 to day 49). The expression of NK receptors, granzyme B, perforin, CD107a, interferon-γ, telomere length and NK cell death were measured by flow cytometry. *Results.* Compared with the control (2004.2-fold; $n = 10$ healthy donors) and intermittent groups (2063.9-fold), a strong proliferative response of the NK cells on day 42 was identified in the "first week" group (3743.8-fold) ($P < 0.05$). NK cells treated with IL-21 in the "first week" group showed cytotoxicity similar to that in control cells. On day 28, there was a significant increase in cytotoxicity of "first week" NK cells that received IL-21 treatment for an additional 2 days compared with the "first week" NK cells ($P < 0.05$). *Conclusions.* These data suggest that controlling temporal exposure of IL-21 during NK cell proliferation can be a critical consideration to improve the yields and cytotoxicity of NK cells.

Key Words: *IL-2, IL-15, IL-21, natural killer cells*

Introduction

Natural killer (NK) cells are known to play a cytotoxic role in killing a variety of human cancer cells as well as an immunoregulatory role by secreting cytokines (1). The anti-tumor effect of NK cells is enhanced on exposure to cytokines such as interleukin (IL)-2, IL-15 and IL-21 (2,3). It has also been reported that IL-2 and IL-15 enhance the proliferation of NK cells *in vitro* and *in vivo* (4–7). On the basis of this understanding, the therapeutic potential of adoptive transfer of *ex vivo*–expanded NK cells has been explored in preclinical studies and clinical trials for the treatment of solid tumors as well as hematological malignancies (7–10).

To maximize the therapeutic effect of NK cell–based immunotherapy, several protocols that use cytokines, stimulants, feeder cells such as K562 and HFWT and combinations of two or three of these approaches have been developed for the expansion and activation of NK cells (11–13). Recently, effective expansion and activation of NK

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(Received 2 August 2013; accepted 29 April 2014)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2014.04.008

cells was achieved with the use of genetically engineered feeder K562 cells expressing a combination of several molecules: 4-1BBL, membrane-bound (mb) IL-15, major histocompatibility complex class I–related chain A, tCD19, CD64, CD86, and mbIL-21 (11,12,14).

Because enhanced activation of NK cells has been reported through the use of a combination of various cytokines, such as IL-2, IL-15 and IL-21, Park *et al.* (15) applied IL-21 to enhance the function of expanded NK cells, which had been activated by K562-mb15-41BBL cells plus IL-2/IL-15 provided in the culture medium during the expansion. However, culture of mouse NK cells with the use of IL-15 alone resulted in pronounced proliferation and an increased cumulative number of cells, whereas IL-21 antagonized the proliferative effect of IL-15 (16). These results suggested that continuous exposure of NK cells during expansion to IL-21 combined with IL-15 could result in decreased proliferation; thus, the authors used IL-21 to stimulate NK cells for the final 96 h of culture and not continuously throughout the expansion period (15). They showed that IL-21 significantly enhanced the cytolytic activity and interferon (IFN)-γ production of *ex vivo*–expanded NK cells in response to trastuzumab-coated breast cancer cells; however, no beneficial effect on expansion rate was observed (15). It was also reported that IL-21 increased expression of perforin in NK cells, enhanced degranulation and induced NK cell cytotoxicity without augmenting NK cell proliferation (17). Notably, Denman *et al.* (14) demonstrated that genetically engineered K562 cells expressing mbIL-21 greatly improve NK cell expansion. Therefore, there is ambiguity in the effect of IL-21 on NK cell expansion, depending on the timing of treatments.

It is necessary to develop additional protocols for the combination of IL-21 with IL-2/IL-15 to enhance the anti-tumor effect of expanded NK cells as well as to maintain their proliferation rate and/or to avoid NK cell apoptosis. In contrast to a common protocol that involves continuous exposure of multiple cytokines to NK cells, in this study, we evaluated the effects of exposure to IL-21 according to different schedules on *ex vivo* NK cell expansion with the use of K562-mb15-41BBL cells in the presence of IL-2/IL-15.

Methods

Cells and culture

Human breast cancer cell lines (SKBR3 and MDA-MB-231) and K562 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified 5% CO_2 incubator.

Cytokines and antibodies

Recombinant human IL-2 and IL-15 (PeproTech, Rocky Hill, NJ, USA) were used to expand NK cells, and IL-21 (PeproTech) was used to stimulate NK cells at various time points. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 antibody (Ab) and phycoerythrin (PE)-Cy5–conjugated anti-human CD56 Ab were used to evaluate the purity of expanded NK cells. PE-conjugated anti-mouse immunoglobulin G1 and PE-conjugated anti-human CD16, CD69, NKG2D, NKp30, NKp44, NKp46 and CD158b Abs were used to examine the surface expression of NK cell receptors. PE-conjugated anti-human IFN-γ, perforin and granzyme B Abs were used for intracellular staining, and PE-conjugated anti-human CD107a Ab was used as a surrogate marker of degranulation. All fluorescent Abs were from BD Biosciences (San Jose, CA, USA).

Isolation of human peripheral blood mononuclear cells and ex vivo *expansion of NK cells*

NK cells were expanded by co-culture with K562-mb15-41BBL cells (gift of Dr D. Campana, National University of Singapore) as described in Imai *et al.* (12) and Fujisaki *et al.* (18), with a slight modification. Peripheral blood mononuclear cells (PBMCs) were isolated with the use of Ficoll-Hypaque (d = 1.077 g/mL; Lymphoprep; Axis-Shield, Oslo, Norway) from healthy adult donors. PBMCs (3×10^6/mL/well) were co-cultured with 100 Gy gamma ray–irradiated K562-mb15-41BBL cells (0.5×10^6/mL/well) in a 24-well tissue culture plate in the presence of 10 U/mL recombinant human IL-2 in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin and 4 mmol/L of L-glutamine). The medium was replaced every 2–3 days with fresh medium containing 10 U/mL IL-2. After 1 week, the concentration of IL-2 was increased to 100 U/mL, and 5 ng/mL of soluble IL-15 was added to the medium. The medium was replaced every 2–3 days.

To generate five different groups, PBMCs were co-cultured with irradiated K562-mb15-41BBL cells in the presence of IL-2/IL-15, and 5 ng/mL IL-21 (PeproTech) was added every 2–3 days at varying time points (Figure 1A): (i) control (without IL-21