International Immunopharmacology 22 (2014) 486–491

Contents lists available at ScienceDirect

International Immunopharmacology

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

Ex vivo-expanded natural killer cells kill cancer cells more effectively than *ex vivo*-expanded γδ T cells or αβ T cells

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ARTICLE INFO

Article history:
Received 30 November 2013
Received in revised form 22 July 2014
Accepted 31 July 2014
Available online 11 August 2014

Keywords:
Natural killer cells
γδ T cells
αβ T cells
Cytotoxicity
Cancer patients
Rituximab

ABSTRACT

Adoptive immunotherapy of cancer is evolving with the development of novel technologies for generating a large number of activated killer cells such as natural killer (NK) cells, γδ T cells, and αβ T cells. We have recently established large-scale culture methods to generate activated NK cells from human peripheral blood, and demonstrated that expanded NK cells have higher cytotoxicity against cancer cells than freshly isolated NK cells. In this study, we compared cultured NK cells with cultured γδ T and αβ T cells that were prepared by conventional culture methods regarding the expression of cytotoxic molecules and cytotoxicity against cancer cells. Natural cytotoxicity receptors such as NKp30, NKp44 and NKp46, and perforin were expressed most exclusively on NK cells. Granzyme A, NKG2D, and interferon-γ were dominantly expressed in NK cells and γδ T cells but not in αβ T cells. Consistent with the expression profiles of the cytotoxic molecules, cultured NK cells from both healthy volunteers and cancer patients demonstrated significantly higher cytotoxicity against cancer cell lines, including MHC class I-positive cell lines, compared with cultured γδ T cells and cultured αβ T cells. Additionally, NK cells, unlike γδ T cells or αβ T cells, expressed high levels of CD16, and showed augmented cytotoxicity when co-administered with an anti-CD20 monoclonal antibody drug, rituximab. These results suggest the excellent efficacy of expanded NK cells for cancer treatment.

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1. Introduction

NK cells, γδ T cells, and αβ T cells kill cancer cells in a major histocompatibility complex (MHC)-unrestricted manner [1]. With advancements in technology, these killer cells are becoming available for adoptive immunotherapy as *ex vivo*-expanded killer cells. *Ex vivo*-expanded αβ T cells have been studied since the 1980s [2], and have been used to treat cancer such as hepatocellular carcinoma [3] and lung cancer [4,5]. Adoptive transfer of autologous γδ T cells is a newer promising approach for cancer immunotherapy [6,7]. NK cells are yet another population of effectors for adoptive immunotherapy [8,9]. Expansion and activation of NK cells *ex vivo*, however, is challenging and currently under intensive investigation [8,10]. We have recently developed a large-scale *ex vivo* expansion protocol for generating NK cells with highly augmented cytotoxicity suitable for adoptive transfer therapy [11,12]. In addition to the direct cytotoxicity against cancer cells, NK cells have also been suggested to potentiate the therapeutic efficacy of monoclonal antibody (mAb) drugs [12]. In most instances, antibody-dependent cellular cytotoxicity (ADCC) is one of the critical mechanisms underlying the clinical efficacy of anticancer antibodies [13], as exemplified by the treatment of adult T-cell leukemia/lymphoma with Potelligent [14]. Because both NK cells and γδ T cells express CD16 (FcγRIII), a receptor that binds to the Fc region of IgG1 and IgG3, they are expected to function as major effector cells in ADCC [15].

In this study, to deepen the understanding on the *ex vivo*-expanded killer cells, we compared cultured NK cells with γδ T cells and αβ T cells regarding the expression of cytotoxic molecules and the cytotoxicity against cancer cells with or without coadministering a mAb drug, rituximab.

2. Materials and methods

2.1. Cells and cell fraction

Human peripheral blood samples were obtained from healthy volunteers and cancer patients with written informed consent. Peripheral

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; E/T ratio, effector-to-target ratio; FcγR, Fcγ receptor; mAb, monoclonal antibody; MHC, major histocompatibility complex; NCR, natural cytotoxicity receptor; NK cell, natural killer cell; PBMCs, peripheral blood mononuclear cell; rhIL-2, recombinant human IL-2.

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http://dx.doi.org/10.1016/j.intimp.2014.07.036
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blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation.

The K562, Raji, and Daudi cell lines were obtained from the American Type Culture Collection (Manassas, VA). The UB2MT and C1AK cell lines were established in our laboratory as described previously [12]. All these tumor cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

2.2. Ex vivo expansion of killer cells

NK cells, γδ T cells, and αβ T cells were expanded from PBMCs as described previously [6,11,12,16]. In brief, cultured NK cells were prepared using BINKIT® (Biotherapy Institute of Japan, Japan) on the basis of a protocol using an anti-CD16 monoclonal antibody-immobilized culture flask as described previously [12]. Cultured γδ T cells were prepared by culturing PBMCs in ALyS medium (Cell Science & Technology Institute, Japan) supplemented with 700 IU/ml recombinant human IL-2 (rhIL-2) (Chiron, Netherlands) and 5 μM of zoledronate (Novartis, Switzerland). Cultured αβ T cells were prepared by culturing PBMCs in ALyS medium supplemented with 700 IU/ml rhIL-2 in an anti-CD3 antibody-immobilized flask. Between days 3 and 14, all cell cultures were maintained in ALyS medium supplemented with 350 IU/ml rhIL-2 in a culture flask or a culture bag (NIPRO, Japan).

2.3. Flow cytometry

Cells were stained with directly conjugated mouse anti-human mAbs against CD3, CD16, CD56, Vγ9 TCR, NKG2D (CD314) (Beckman Coulter, CA), NKp30 (CD337), NKp44 (CD336), and NKp46 (CD335) (BD Bioscience, CA). For intracellular IFN-γ staining, cells were stimulated with PMA/ionomycin (Sigma Chemical, St. Louis, MO) for 4 h in the presence of Brefeldin A (Sigma Chemical, St. Louis, MO) and stained for CD3, CD56, and Vγ9 TCR to identify cell populations. Subsequently, cells were fixed and permeabilized with Intraprep (Beckman Coulter, CA), and stained with anti-IFN-γ mAb (Beckman Coulter, CA). To detect perforin- or granzyme-positive cells, cultured cells were stained for cell surface markers and fixed as described above, and then stained with anti-perforin or anti-granzyme A mAb (BD Bioscience, CA). Stained cells were analyzed using CYTOMICS FC500 cytometer equipped with CXP software (Beckman Coulter, CA).

2.4. Cytotoxicity assay

Cytotoxicity was measured by Calcein-AM-release assay using TERASCAN VP (Minerva Tech., Japan) as previously described [12]. In brief, target cells were labeled with the immunofluorescent dye Calcein-AM (Dojindo Lab., Japan) and incubated with effector cells at various effector-to-target (E/T) ratios for 2 h (K562), 4 h (Daudi and Raji), or 6 h (UB2MT and C1AK). To determine ADCC, cells were incubated with 10 μg/ml human IgG1 (Sigma-Aldrich Corp., MO) or rituximab (Roche, Switzerland) in the presence or absence of 10 μg/ml anti-CD16 mAb (Beckman Coulter, CA). Cytotoxicity was evaluated *via* the fluorescence intensity of target cells measured using TERASCAN VP before and after the incubation.

2.5. Statistical analysis

The Welch test was used to determine statistical significance of difference.

3. Results

3.1. Ex vivo expansion of killer cells from PBMCs

Ex vivo-expanded killer cells were prepared from aliquots of PBMCs of healthy donors (n = 4). After 14 days of culture using BINKIT®, the number of NK cells ($CD3^-CD56^+$) increased by 732-fold (229–1201-fold, n = 4), accounting for 65.0% (31.4%–84.1%, n = 4) of the cultured cells (Fig. 1). After 14 days of culture by conventionally optimized methods for each cell type, the number of γδ T cells ($CD3^+V\gamma9^+$) increased by 6677-fold (2527–16,332-fold, n = 4), accounting for 73.5% (24.7–95.1%, n = 4) of the culture cells, and that of αβ T cells ($CD3^+V\gamma9^-$) increased by 3970-fold (483–13,912-fold, n = 4), accounting for 78.3% (61.0–87.8%, n = 4).

3.2. Expression of effector molecules on cultured killer cells

Expression of NK receptors and cytotoxic molecules on cultured killer cells were determined by flow cytometry. Each cell population was gated on the basis of the expression of cell surface markers such as CD3, CD56, and Vγ9. Natural cytotoxicity receptors (NCRs), including NKp30, NKp44, NKp46, and perforin were expressed predominantly on NK cells, whereas an activating NK receptor, NKG2D, and IFN-γ were expressed in all three cell types. Granzyme A-positive cells were observed mostly among NK cells and γδ T cells (Fig. 2).

3.3. Cytotoxicity against cancer cell lines

Cytotoxicity against various cancer cell lines was determined using cultured NK, γδ T, and αβ T cells shown in Fig. 1 as effectors. The cultured NK cells showed the highest cytotoxicity against all five cancer cell lines including MHC class I-positive cell lines (Fig. 3). Less potent cytotoxicity was observed with cultured γδ T cells or αβ T cells. The cytotoxicity showed an inverse correlation with the positivity of MHC class I molecules; higher cytotoxicity was observed against MHC class I-negative K562 and Daudi cell lines compared with lower cytotoxicity against MHC class I-positive Raji, UB2MT and C1AK cell lines (Fig. 3).

3.4. ADCC

Cultured NK cells, when administered with rituximab, show a higher ADCC than freshly isolated NK cells [12]. When ADCC against Daudi cells was compared among the three cell types, cultured NK cells showed a much higher ADCC than cultured γδ T or αβ T cells (Fig. 4A). Consistent with this observation, CD16 (FcγRIII), the most common Fc receptor, was present almost exclusively on NK cells (Fig. 4B).

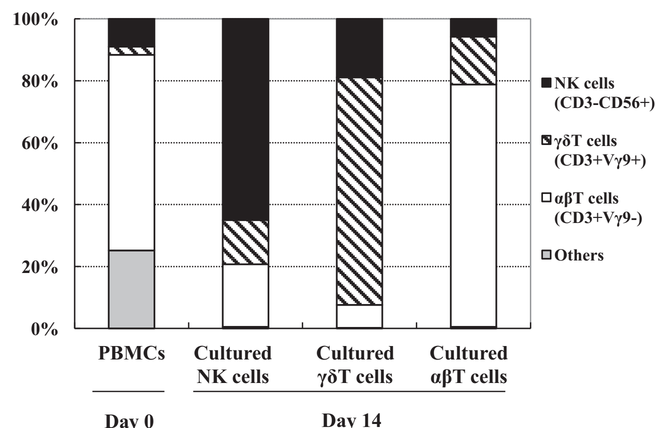

Fig. 1. Cell types on days 0 and 14 of cell culture. Cells were prepared from PBMCs of healthy donors (n = 4). Cell types were determined based on the expression of cell surface markers: $CD3^-CD56^+$ for NK cells, $CD3^+V\gamma9^+$ for γδ T cells, and $CD3^+V\gamma9^-$ for αβ T cells. Cultured NK cells were prepared using BINKIT® (Biotherapy Institute of Japan, Japan). Cultured γδ T and αβ T cells were prepared using conventional culture methods. Mean values for four donors are shown.

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