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In Vivo Inverse Correlation in the Activation of Natural Killer T Cells Through Dual-Signal Stimulation via a Combination of α-Galactosylceramide–Loaded Liposomes and Interleukin-12

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ABSTRACT

Alpha-galactosylceramide (GC) represents a potentially new class of adjuvant because GC strongly induces interferon (IFN) gamma production from natural killer T (NKT) cells, leading to the induction of strong antitumor immunity. Interleukin (IL)-12 is another stimulating signal that induces IFN-γ production by NKT cells. We report herein on an investigation of the effect of recombinant IL-12 on NKT cell activation, when used in combination with GC-loaded octaarginine modified liposomes (GC-Lip). IFN-γ production from splenocytes simulated with GC-Lip was dose dependently enhanced in the presence of IL-12 *in vitro*. In contrast, IFN-γ production *in vivo* was enhanced at a low dose of IL-12. Enhanced IFN-γ production was observed in the case of low doses (0.5 μg and 2.5 μg) of GC-Lip but not a high dose (5 μg), that is, the IL-12 combination enhanced NKT cell activation at a 10-fold lower GC dose. The use of the above combination also enhanced the expansion of the NKT cell population. These findings indicate that *in vivo* IFN-γ production is inversely correlated with the dose of IL-12 during dual signal stimulation of NKT cells via both GC-Lip and IL-12, indicating that the dose of GC-Lip can be reduced without weakening NKT cell activation.

Introduction

Natural killer T (NKT) cell–based immunotherapy represents a powerful therapy for treating cancer. NKT cells are narrowly defined as a T-cell lineage that express NK lineage receptors and are characterized by the expression of an invariant T-cell receptor (TCR) encoded by Vα14Jα18 in mice and Vα24Jα18 in humans.[1] Activated NKT cells trigger antitumor responses by inducing the production of large amounts of interferon (IFN) gamma, which acts on NK cells and CD8 cytotoxic T cells to eliminate major histocompatibility complex class I negative and positive tumors, respectively. Thus, the activation of NKT cells can be considered to represent a new adjuvant in cancer therapy that could be applied to a wide range of tumors. Alpha-galactosylceramide (GC), a synthetic glycolipid, functions as a ligand for the activation of NKT cells.[2-4] GC is presented by CD1d molecules, lipid antigen-presenting molecules, on antigen-presenting cells (APCs).[2-4] NKT cells recognize GC presented on a CD1d molecule via TCR. The administration of GC-pulsed dendritic cells (DCs) has been shown to effectively induce antitumor activity in therapeutic settings.[5] Moreover, in clinical studies, the adaptive transfer of GC-pulsed DCs resulted in clinical benefits in some patients due to enhancement in NKT cell activation.[6-8] Therefore, NKT cell–based immunotherapy has the potential to function as a powerful and attractive therapy against cancer.

In contrast to the treatment with GC-pulsed DCs, when free soluble GC was administered, no or low clinical outcomes were reported among patients.[9] One of the causes for such disappointing results appears to be the insufficient activation of NKT cells due to the uncontrolled delivery of GC to APCs. To overcome this problem, the use of delivery systems, particularly nanoparticles, promises to be of great value. The incorporation of GC into nanoparticles possibly offers several advantages compared with the use of free soluble GC, including, for example, enhanced cellular uptake by APCs and minimal side-effects thanks to the lower doses needed to achieve a biological effect.[10] In a previous study, we succeeded in incorporating GC into a stearylated octaarginine (STR-R8) modified liposome (R8-Lip) and in inducing the activation of NKT cells.[11]

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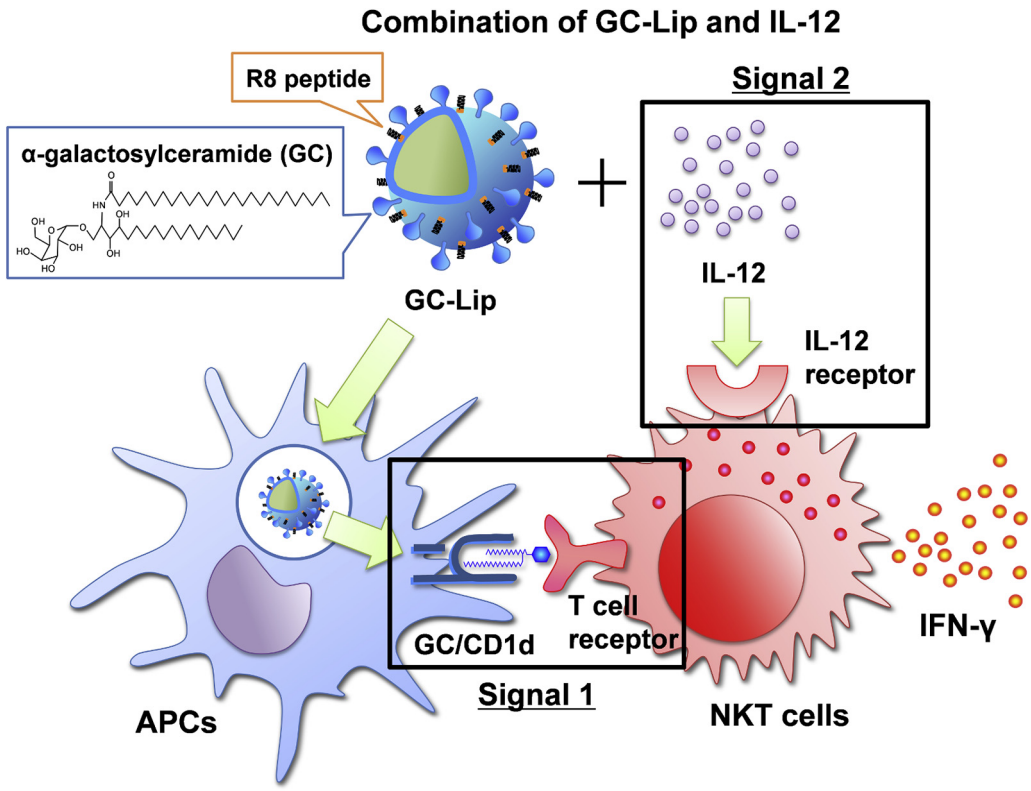

Figure 1. Conceptual image showing the dual-signal stimulation strategy. The first stimulating signal is a CD1d-mediated signal through GC that is present on the surface of APCs. The second activation signal is an IL-12–mediated signal. These stimulating signals activate NKT cells and induce them to produce IFN-γ which consequently activates other cells, such as NK cells and T cells.

The R8 peptide is a cell-penetrating peptide which can be used to deliver various molecules to cells; hence, the R8-Lip was used as a vaccine delivery system.[12-17] The intravenous administration of GC-loaded R8-Lip (GC-Lip) at a GC dose of 5 μg to mice resulted in a significant enhancement in NKT cell expansion, IFN-γ production, and anti-tumor effects against a metastatic B16-F10 melanoma in lungs, compared with the use of the free, soluble form of GC.[11] The enhancement in GC-mediated antitumor immunity can be attributed to the control of GC biodistribution by the developed liposomes, that is, GC was efficiently delivered to APCs.[11] However, high doses of GC (2-5 μg) induced an anergic state in NKT cells,[18] resulting in the suppression of further NKT cell-mediated antitumor immunity. On account of this, it was necessary to decrease the injected dose of GC.

In addition to NKT cell activation via a TCR signal provided by a GC/CD1d complex, a cytokine signal that depends on the constitutive expression of certain cytokine receptors on NKT cells also induces NKT cell activation.[19] The combination of TCR and cytokine stimuli act in concert, resulting in robust NKT cell activation, and interleukin (IL)-12 is the best-described cytokine mediator of NKT cell activation.[20,21] Hence, we hypothesized that a therapy involving a combination of GC-Lip and IL-12 could reduce the dose of GC-Lip needed and augment NKT cell activation compared with a single signal-mediated activation (Fig. 1). It is noteworthy that the effect of IL-12 on NKT cell activation mediated by GC-loaded nanoparticles has not been examined so far. In this study, we investigated the impact of the use of a combination of IL-12 and GC-Lip treatment *in vitro* and *in vivo*. Interestingly, the effect of this combination was inconsistent between *in vitro* and *in vivo* conditions. An inverse correlation between the dose of IL-12 and IFN-γ levels was observed *in vivo*, that is, IFN-γ production was enhanced when a low dose of IL-12 was used. Consequently, the combination with IL-12 resulted in enhancing the NKT cell activation mediated by the GC-Lip at a 10-fold lower dose of GC.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC) and N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG2000) were purchased from NOF Corporation (Tokyo, Japan). Cholesterol (Chol) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). STR-R8 was synthesized by Kurabo (Osaka, Japan). GC (KRN7000) was obtained from Funakoshi Co. Ltd. (Tokyo, Japan). Recombinant IL-12 was purchased from R&D Systems, Inc. (Minneapolis, MN). PE/Cy7 anti-mouse CD19, FITC anti-mouse CD3, and each of the isotype controls were obtained from BioLegend (San Diego, CA). Mouse GC/CD1d Tetramer-SA-PE was purchased from MBL International (Woburn, MA).

Animals

C57BL/6 female mice (6 to 8 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Experiments using mice were approved by the Pharmaceutical Science Animal Committee of Hokkaido University.

Preparation of GC-Lip

The GC-Lip was prepared as reported previously.[11] The GC-Lip was composed of EPC, Chol, DSPE-PEG2000, and STR-R8 (70:30:2:5 molar ratio). A total of 560 nmol of EPC, 240 nmol of