International Immunopharmacology 39 (2016) 353–358

Contents lists available at ScienceDirect

International Immunopharmacology

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

Novel immunostimulators with a thiazolidin-4-one ring promote the immunostimulatory effect of human iNKT cells on the stimulation of Th2-like immune responsiveness *via* GATA3 activation *in vitro*

Ming Meng [a], Chunxiao Li [a], Fei Yang [a], Hua Chen [b], Xiaoliu Li [b], Yongbin Yang [a], Dongzhi Chen [a,*]

[a] Department of Immunology, School of Medicine, Hebei University, Baoding 071000, China
[b] Key Laboratory of Chemical Biology of Hebei Province, College of Chemistry & Environmental Science, Hebei University, Baoding 071002, China

ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form 10 August 2016
Accepted 11 August 2016
Available online 18 August 2016

Keywords:
Invariant natural killer T cells
Immunostimulators
α-Galcer
IFN-γ/IL-4

ABSTRACT

Invariant natural killer T cells (iNKTs) are important innate immune cells which get involved in various immune responses in both mice and humans. These immune reactions range from self-tolerance to development of autoimmunity and responses to pathogens and tumor development. In this study, we aimed to explore the effects of the novel immunostimulators (CH1b and CH2b) containing thiazolidin-4-one on the functions of human invariant natural killer T cells (iNKTs). First of all, iNKTs in peripheral blood mononuclear cells were expanded with α-Galactosylceramide (α-Galcer) *in vitro*. Then, the highly purified iNKTs were isolated from PBMCs using magnetic cells sorting (MACS). Next, we investigated the impacts of CH1b and CH2b on proliferation, cytokines production, cytotoxicity, and the associated signaling pathways in iNKT cells. Finally, we found that CH2b could significantly promote the activated iNKTs proliferation, increase the production of Th2 cytokines, and induce Th0 differentiation into Th2 subset *via* GATA 3 signaling pathway. Besides, CH2b could markedly enhance the cytotoxic ability of the activated iNKTs. Therefore, we concluded that CH2b, a promising candidate immunostimulator, might be used for the treatment of infections, tumors, autoimmune and allergic diseases, and for the correction of Th1/Th2 balance disorders in future.

1. Introduction

Invariant natural killer T cells (iNKTs) are a distinctive subset of T lymphocytes, which can express invariant Vα24-Jα18 α-chains paired with Vβ11 in humans [1]. iNKTs are involved in various immune responses in both mice and humans, ranging from self-tolerance to development of autoimmunity and responses to pathogens and tumors [2–4]. As a consequence of cytokine- and cell-mediated contacts, other immune cells can also be regulated. These iNKTs recognize lipid antigens and glycolipid antigens presented by CD1d molecules, suggesting that the activation of iNKTs is CD1d-restricted [5]. α-Galcer is a type of synthetic glycolipid antigen which can be presented to iNKTs by antigen-presenting cells (APCs) through CD1d molecules [6], inducing activation of iNKTs. Once activated, iNKTs can induce an antitumor Th1 response (IFN-γ) or an immunosuppressive Th2 response (IL-4) [7,8] and promote the production of pro-inflammatory cytokines, including Th1-type cytokines (IFN-γ), and immunoregulatory cytokines, including Th2-type cytokines (IL-4), which can be regulated by T-Bet and GATA3, respectively. Th1- and Th2-defining cytokines as well as other cytokines (IL-2, IL-5, IL-9, IL-10, IL-17, IL-21, TGFß, and GMCSF) are massively and concurrently produced upon α-GalCer stimulation. Therefore, through the action of immunostimulators, iNKTs can play significant roles in the regulation and further correction of the Th1/Th2 imbalance.

The thiazolidin-4-one ring is a core substructure in various synthetic pharmaceuticals which are associated with diverse biological activities, such as anticancer, antiviral, and anti-inflammatory [9] effects. In an earlier study, certain thiazolidine derivatives, such as levamisole, pidotimod, and CGP 52608, exhibited potent immunostimulatory activities [10]. Previously, we found that thiazolidin-4-one-linked pseudodisaccharides showed excellent immunostimulatory activities [11], which prompted us to develop novel *C*-pseudonucleosides having a thiazolidin-4-one moiety to investigate their action on the human immune system. In our study, we synthesized large amounts of *C*-pseudonucleosides containing thiazolidin-4-one. After extensive screening, we ultimately found that CH1b and CH2b exerted the optimal immunomodulatory activity of all C-pseudonucleosides examined (Fig. 1), and found that they promoted LPS-treated B-cell proliferation, induced the differentiation of Th0 into the Th2 subset, and might have a bias towards Th2 type immune responses [12]. The activation of iNKTs is CD1d-restricted, indicating that iNKTs recognize lipid antigens and glycolipid antigens presented by CD1d molecules. However, unlike α-Galcer, our novel immunostimulators CH1b and CH2b are exceedingly

* Corresponding author.
E-mail address: chenddzz@163.com (D. Chen).

http://dx.doi.org/10.1016/j.intimp.2016.08.010
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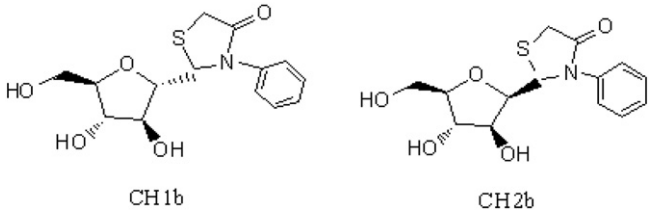

Fig. 1. The structures of CH1b and CH2b, which are *C*-pseudonucleosides bearing a thiazolidin-4-one ring.

small molecular saccharide derivatives. Thus, they cannot directly stimulate the non-activated iNKTs through CD1d molecules to elevate their functions. Consequently, we initially used α-Galcer to activate iNKTs when expanded *in vitro*. Then, we further investigated the effect of CH1b and CH2b on the function of the activated iNKTs. In addition, based on findings of preliminary experiments, we optimized the experimental methods and observed that CH2b showed the best effect when the concentration of α-Galcer was 5 μM. Ultimately, on the basis of the results of a preliminary experiment, we chose 100 ng/ml as the optimal concentration of α-Galcer. In the present study, we explored the effect of CH1b and CH2b on the proliferation, cytokine secretion, and cytotoxicity of α-Galcer-activated human iNKTs. The final results demonstrated that CH2b promoted the activated iNKTs proliferation and significantly enhanced the cytotoxic ability of iNKTs. Furthermore, CH2b increased the production of Th2-like cytokines by iNKTs, and probably induced further the differentiation of Th0 into a Th2 subset.

2. Material and methods

2.1. Cells

PBMCs were obtained in samples of peripheral blood of healthy donors. The mycoplasma-free cell line K562 was purchased from Shanghai Cell Bank, Chinese Academy of Science (Shanghai, China).

2.2. Materials

RPMI1640, Fetal Bovine Serum (FBS), PBS, and CyQuant ® cell proliferation assay kit were obtained from Life Technologies Corporation (Grand Island, NE, USA). Ficoll-Paque™ Plus was obtained from Sigma Aldrich Chemical Company (St. Louis, MO, USA). ELISA kit for IL-4 and IFN-γ were produced by R&D Systems (Minneapolis, MN, USA). KRN7000 (α-Galcer) was manufactured by ENZO Life Sciences (Farmingdale, NY, USA). Anti-iNKT microbeads (Human), anti-iNKT-PE antibody (anti-Vα24-PE antibody), and Human IL-2 were purchased from Miltenyi Biotech (Miltenyi Biotec, GmbH, Germany). CD3-FITC and IgG1 isotype were acquired from BD Pharmingen (San Diego, CA, USA).

2.3. Methods

2.3.1. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from whole blood collected in heparinized tubes. Blood samples were diluted with an equal volume of PBS and layered onto Ficoll-Paque™ Plus (Sigma, USA) according to the recommendations in the manufacturer's manual [13]. The tubes were centrifuged for 15 min at 800g, and the buffy-coat layers were collected and washed twice with equal volumes of PBS. The remaining cells were resuspended in RPMI 1640 medium supplemented with 10% FBS.

2.3.2. In vitro expansion of iNKTs

We improved the method reported by Kuwatani [14] to achieve better expansion [15]. PBMCs (2×10^6/ml) were cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and IL-2 (100 IU/ml) in a 75-cm^3 cell culture flask at 37 °C and 5% CO_2. Then, 100 ng/ml of α-Galcer was added to the flask, and the samples were cultured for four days. On day 4, the media was removed to collect the expanded PBMCs. Further, cell density (2×10^6/ml) was adjusted in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, IL-2 (100 IU/ml), and α-Galcer (100 ng/ml) for culturing for another four days. On day 8, fresh complete culture medium was added to activate iNKT cells for three days, and then cells were collected for purification and obtaining the α-Galcer-activated iNKTs for a subsequent study.

2.3.3. Purification of iNKTs

The iNKTs were purified from the expanded PBMCs above through an iNKTs isolation kit according to the recommendations of the manufacturer's manual described previously [16]. Briefly, iNKTs were labeled with anti-iNKT microbeads. After washing, the labeled iNKTs were resuspended in cold PBS and separated through AutoMACS Pro Separator (Miltenyi Biotec, GmbH, Germany). The purity of the obtained iNKTs was analyzed through flow cytometry.

2.3.4. Cell viability assay

The α-Galcer-activated iNKTs (5×10^6/ml) suspended in RPMI 1640 containing 10% FBS were seeded in 96-well flat-bottom culture plates and incubated with several concentrations (0, 1, 3, 5, 7, 10, 25, 50, and 100 μM) of CH1b and CH2b, as well as with α-Galcer (100 ng/ml), for 24 h. Afterwards, 10 μl of 1 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromides) dissolved in PBS was added into each well, and the culture continued for another 4 h at 37 °C. Then, 100 μl of 0.1 N acidified isopropanol was added into each well. The absorbance at 570 nm was measured. Cell viability was obtained by the formula below:

$$\%\ \text{increased viability} = [OD_{sample} - OD_{untreated}] / OD_{untreated} \times 100\%.$$

2.3.5. Cell proliferation

The α-Galcer-activated iNKTs (2×10^6) in 500 μl RPMI 1640/well containing 10% FBS, and IL-2 (100 IU/ml) were seeded in 24-well plates and incubated with 100 ng/ml α-Galcer and/or 5 μM CH1b and 5 μM CH2b for 72 h. In all groups, the experiments with each concentration were performed in triplicates. Further, after the supernatant was removed, all cells were washed twice with PBS or RPMI 1640, and then stored at −80 °C until processed with CyQUANT® Cell Proliferation Assay Kit as described previously [17].

2.3.6. Elisa

The culture supernatants from the cultured iNKTs treated with 100 ng/ml α-Galcer and/or CH1b and CH2b at 5 μM and 5 μM for 48 h were subjected to detection of the concentrations of cytokines IL-4 and IFN-γ using ELISA. The assay was conducted according to the procedures recommended in the manufacturer's instructions of ELISA kits [18].

2.3.7. Flow cytometry analysis

Flow cytometry was used to examine the purity of iNKT after purification. iNKTs were blocked with 1% BSA in cold PBS for 5 min at room temperature. Afterward, iNKTs were labeled with CD3-FITC (clone UCHT1, BD Pharmingen, CA, USA), iNKT-PE (Miltenyi Biotec, GmbH, Germany), and IgG1 isotype (clone MOPC-21, BD Pharmingen, CA, USA) antibodies for 30 min at 4 °C in the dark. Further, the labeled cells were washed with PBS containing 0.05% sodium azide and 1% BSA and were analyzed through flow cytometry (Becton Dickinson FACSCalibur, USA) [19].

2.3.8. Cytotoxicity assays

^{51}Cr-release assay was carried out to detect the cytotoxic ability of iNKTs to K562 cells as described previously [14]. The K562 cells used as a target were labeled with $Na^{51}CrO_4$ for 1 h. Then, the labeled K562 cells were washed twice with PBS, suspended in RPMI-1640, and plated in 96-well U-plates at 5×10^5/well. iNKTs were added as effector cells at