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Activation of human invariant natural killer T cells with a thioglycoside analogue of α-galactosylceramide

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Abstract Activation of CD1d-restricted invariant NKT (iNKT) cells with the glycolipid α-galactosylceramide (α-GalCer) confers protection against disease in murine models, however, clinical trials in humans have had limited impact. We synthesized a novel thioglycoside analogue of α-GalCer, denoted α-S-GalCer, and tested its efficacy for stimulating human iNKT cells in vitro. α-S-GalCer stimulated cytokine release by iNKT cells in a CD1d-dependent manner and primed $CD1d^+$ target cells for lysis. α-S-GalCer-stimulated iNKT cells induced maturation of monocyte-derived dendritic cells into antigen-presenting cells that released IL-12 and small amounts of IL-10. The nature and potency of α-S-GalCer and α-GalCer in human iNKT cell activation were similar. However, in contrast to α-GalCer, α-S-GalCer did not activate murine iNKT cells in vivo. Because of its enhanced stability in biological systems, α-S-GalCer may be superior to α-GalCer as a parent compound for developing adjuvant therapies for humans.

Abbreviations: 7-AAD, 7-aminoactinomycin D; APC, antigen-presenting cell; CFSE, carboxyfluorescein succinimidyl ester; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; α-GalCer, αlpha-galactosylceramide, α-S-GalCer, thioglycoside analogue of α-GalCer; GM-CSF, granulocyte-monocyte colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; iNKT cell, invariant natural killer T cell; LPS, lipopolysaccharide; mAb, monoclonal antibody; mfi, mean fluorescence intensity; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PHA, phytohaemagglutinin; poly I:C, polyinosinic:polycytidylic acid; Th, helper T.

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1. Introduction

Invariant natural killer T (iNKT) cells are unconventional regulatory and effector T cells that recognize glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule CD1d [1,2]. They can be activated by a number of endogenous and bacterial glycosphingolipids [3,4], but the xenogeneic glycolipid, α-galactosylceramide (α-GalCer), a potent agonist for murine and human iNKT cells [5] has been used as the prototype antigen for most studies on iNKT cells. Upon *in vitro* activation with α-GalCer, iNKT cells kill a range of tumor cell lines [6], rapidly release cytokines [2,7,8], promote maturation of dendritic cells (DC) into antigen-presenting cells (APC) [9–12] and promote maturation of B cells into antibody-secreting plasma cells [13,14]. Studies in murine models of disease have demonstrated critical roles for iNKT cells in the prevention and reversal of tumor growth, prevention of autoimmune disease, and immunity against a diversity of microbial pathogens [15–18]. Numerical and functional deficiencies of iNKT cells have been reported in humans with various diseases [19,20] but clinical trials involving α-GalCer stimulation of iNKT cells have yielded disappointing results [21,22].

A notable feature of iNKT cells is their ability to rapidly release cytokines that exert major effects on early and delayed adaptive immunity against tumors and infectious pathogens [2,7,8]. α-GalCer stimulation of murine and human iNKT cells results in the simultaneous production of the Th1 cytokine interferon-γ (IFN-γ) and the Th2 cytokines IL-4 and IL-13 [7,8]. Th1 cytokines are thought to be critical for the antitumor effects of α-GalCer, while the Th2 cytokines can attenuate antitumor immunity and autoimmune disease pathology [17,23,24]. Therefore, the efficacy of α-GalCer may be limited by reciprocal inhibition exhibited by Th1 and Th2 cytokines and this may be reflected in the low efficacy of α-GalCer for the treatment of solid tumors in a phase I study [25]. Hence, a number of α-GalCer derivatives and analogues have been synthesized to develop compounds which can selectively induce particular responses in iNKT cells. Modifications to the galactose residue revealed that the α-anomeric configuration is a key pharmacophoric element of α-GalCer [26,27]. Modifications to the acyl chains, including shortening [28–30] and the introduction of double bonds [31] resulted in iNKT cell ligands that preferentially stimulated Th2 cytokine secretion, while the addition of aromatic groups resulted in Th1-inducing ligands [32,33]. Substitution of the O-glycosidic linkage in α-GalCer with a C-glycosidic linkage resulted in a Th1-inducing compound with 100-fold greater antimetastatic activity in mice [34]. Silk and co-workers [35] identified threitol ceramide as a weak iNKT cell agonist, which led to the advantage for some applications of less consequent killing of $CD1d^+$ APC by iNKT cells and lowered anergic effects on repeated exposure.

We recently synthesized a thioglycoside analogue of α-GalCer (α-S-GalCer; Fig. 1) using a novel approach that resulted in the galactosyl thiol being produced exclusively as α-anomers [36]. Here we show that this α-S-GalCer compound binds CD1d and stimulates cytotoxicity and cytokine secretion by human peripheral blood iNKT cell lines and renders them capable of inducing maturation of DCs into APCs. This is the first demonstration of a thioglycoside analogue of α-GalCer that activates iNKT cells. Its increased stability in biological systems and enhanced flexibility around the anomeric linkage places α-S-GalCer and its substituted derivatives as a potentially superior family of ligands, compared to α-GalCer, for therapeutic activation of human iNKT cells.

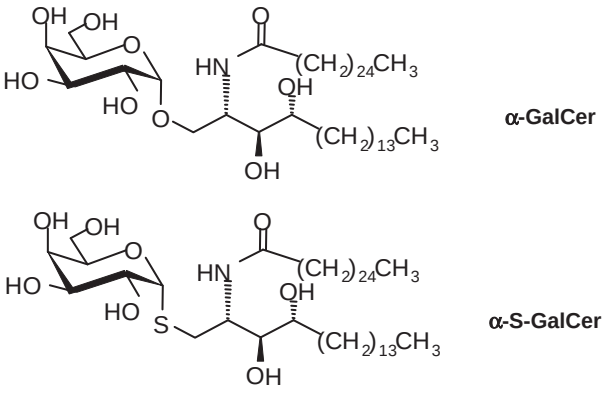

Figure 1 Molecular structures of α-GalCer and the thioglycoside analogue (α-S-GalCer) used in the present investigation.

2. Materials and methods

2.1. Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CCR7, CD1d, CD3, CD4, CD8, CD11c, CD14, CD40, CD54, CD80, CD83, CD86, CD107a, HLA-DR, the Vα24 and Vβ11 chains that form the T cell receptor (TCR) present on iNKT cells, the complementarity-determining region-3 of the invariant Vα24Jα18 TCR chain (6B11) and isotype control mAbs were obtained from BD Biosciences (Oxford, UK), Immunotools (Friesoythe, Germany), R&D Systems (Abingdon, UK), eBioscience (Hatfield, UK) or Coulter Immunotech (Galway, Ireland). Following staining, cells were analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

2.2. Generation of iNKT cell lines

Peripheral blood mononuclear cells (PBMC) were prepared from healthy blood donors by standard density gradient centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway). iNKT cells were sorted from PBMC by staining with a phycoerythrin (PE)-conjugated anti-iNKT cell mAb (clone 6B11) followed by positive selection of the PE-positive cells by magnetic bead separation using anti-PE magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). In some cases iNKT cells were enriched by flow cytometric sorting of 6B11-positive cells using a MoFlo™ XDP Cell Sorter (Beckman Coulter).

Sorted iNKT cells were expanded *in vitro* using two different methods that were developed in our laboratory and yielded sufficient numbers of iNKT cells for functional studies. Method 1 involved culturing 1,000 iNKT cells in complete RPMI medium (RPMI 1640 containing 0.05 mM