

Immunology

Novel thioglycoside analogs of α -galactosylceramide stimulate cytotoxicity and preferential Th1 cytokine production by human invariant natural killer T cells

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Abstract

Invariant natural killer T (iNKT) cells recognize glycolipid antigens bound to CD1d molecules on antigen-presenting cells. Therapeutic activation of iNKT cells with the xenogeneic glycolipid α -galactosylceramide (α -GalCer) can prevent and reverse tumor growth in murine models, but clinical trials using α -GalCer-stimulated human iNKT cells have shown limited efficacy. We synthesized a series of thioglycoside analogs of α -GalCer with different substituents to the galactose residue and found that two of these compounds, XZ7 and XZ11, bound to CD1d-transfected HeLa cells and activated lines of expanded human iNKT cells. Both compounds stimulated cytolytic degranulation by iNKT cells and while XZ7 preferentially stimulated the production of the antitumor cytokine interferon- γ (IFN- γ), XZ11 preferentially stimulated interleukin-4 (IL-4) production. This biased T helper type 1 effector profile of XZ7 was also evident when iNKT were stimulated with dendritic cells presenting this glycolipid. Separate analysis of the responses of CD4⁺, CD8 α ⁺ and CD4⁺CD8⁺ iNKT cells indicated that XZ7 preferentially activated CD8 α ⁺ iNKT cells, and to a lesser degree, CD4⁺CD8⁺ iNKT cells. The partial agonist effect of glycolipid XZ7, inducing cytotoxicity and IFN- γ production but not IL-4 production, indicates that specific protumour activities of iNKT cells can be abolished, while preserving their antitumor activities, by introducing structural modifications to α -GalCer. Since XZ7 was much less potent than α -GalCer as an iNKT cell agonist, it is unlikely to be superior to α -GalCer as a therapeutic agent for cancer, but may serve as a parent compound for developing more potent structural analogs.

Key words: α -galactosylceramide, antitumor immunity, CD1d, iNKT cells, thioglycoside analogs

Introduction

T lymphocytes orchestrate adaptive immunity by directly killing pathogen-infected and tumor cells and by releasing soluble factors (cytokines) that selectively activate and regulate other cells of the immune system. They can detect specific antigens using clonogenic antigen receptors (TCR). Most TCRs display specificity for peptide antigens presented on major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC). However, some T cells respond to lipid-based antigens presented by the CD1 family of MHC-like molecules, which are typically expressed by APC (Bendelac et al. 2007; Salio et al. 2014). The most extensively studied lipid-reactive T cell is the invariant natural killer T (iNKT) cell, which expresses a semi-invariant TCR α -chain (V α 24J α 18 in humans and V α 14J α 18 in mice) paired with one of a limited number of β -chains, which recognizes glycolipid antigens bound to CD1d. A number of self (Brennan et al. 2011; Facciotti et al. 2012) and microbial (Kinjo et al. 2005; Mattner et al. 2005) glycosphingolipids have been shown to bind to CD1d and stimulate iNKT cells, but most of our understanding of iNKT cells comes from studies using the xenogeneic glycolipid, α -galactosylceramide (α -GalCer). Upon activation with α -GalCer, iNKT cells kill target cells and secrete a diverse range of growth factors and cytokines, allowing them to contribute to the activation of T cells (Gumperz et al. 2002; O'Reilly et al. 2011), natural killer cells (Carnaud et al. 1999) and macrophages (Lynch et al. 2015). Activated iNKT cells can also interact directly with other cells of the immune system and can induce the maturation of dendritic cells (DC) into APC (Kitamura et al. 1999; Vincent et al. 2002) and of B cells into antibody-secreting plasma cells (Galli et al. 2003; Zeng et al. 2013). Thus, immune recognition of glycolipids plays a central role in the activation and regulation of innate and adaptive immune responses.

iNKT cells are thought to play a central role in immunity against tumors. Mice lacking CD1d or iNKT cells are predisposed to developing cancer (Cui et al. 1997) and therapeutic activation of iNKT cells can prevent and reverse tumor growth in murine models (Kawano et al. 1997; Crowe et al. 2005). iNKT cells can directly kill human tumor cell lines (Metelitsa et al. 2001; Bassiri et al. 2013), with CD8⁺ iNKT cells exhibiting superior cytotoxicity when compared to CD4⁺ and CD4⁺CD8⁺ iNKT cells (O'Reilly et al. 2011). Numerical and functional iNKT cell deficiencies have been reported in a number of human cancers (Kenna et al. 2003; Molling et al. 2005; Berzins et al. 2011) and clinical trials involving the adoptive transfer of α -GalCer-pulsed autologous DC and/or ex vivo expanded iNKT cells are ongoing for a number of human cancer types (Nieda et al. 2004; Chang et al. 2005; Kunii et al. 2009). However, these trials have to date shown limited clinical efficacy.

Critical to the antitumor potential of iNKT cells is their ability to release the Th1 cytokine interferon- γ (IFN- γ). However, human iNKT cells activated with α -GalCer have a unique ability to simultaneously release Th1 and Th2 cytokines (Gumperz et al. 2002; O'Reilly et al. 2011), including the Th2 cytokines IL-4, IL-10 and IL-13, which can attenuate antitumor immunity and promote tumor growth (Terabe et al. 2005; Bricard et al. 2009; Lynch et al. 2015). Th1/Th2 cytokine production differs among subsets of human iNKT cells, with CD8⁺ iNKT cells being the predominant producers of Th1 cytokines and CD4⁺ iNKT cells predominantly producing Th2 cytokines (Gumperz et al. 2002; O'Reilly et al. 2011). The efficacy of α -GalCer as an antitumor drug may be limited by inhibition of antitumor immune responses exhibited by Th2 cytokines.

To optimize the antitumor properties of human iNKT cells, 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 antigenic property of α -GalCer (Xing et al. 2005). Modifications to the acyl chains, including shortening (Miyamoto et al. 2001; Goff et al. 2004) and the introduction of double bonds (Yu et al. 2005), resulted in iNKT cell ligands that preferentially stimulated Th2 cytokine secretion, while the addition of aromatic groups resulted in Th1-inducing ligands (Fujio et al. 2006; Chang et al. 2007). 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 (Schmieg et al. 2003). We synthesized a thioglycoside analogue of α -GalCer (α -S-GalCer) and found that it was similar to α -GalCer in its ability to bind to CD1d and stimulate human iNKT cells, but had increased stability in biological systems and enhanced flexibility around the anomeric linkage (Hogan et al. 2011). In the present study, we have designed and biologically tested a number of analogs of α -S-GalCer.

The CD1d binding cleft is characterized by two hydrophobic pockets, the A'-pocket and the F'-pocket. The crystal structure of CD1d/ α -GalCer/iNKT TCR interaction shows that both the mouse and human iNKT TCRs adopt a tilted and parallel docking mode over CD1d, with the acyl chain of α -GalCer buried in the A'-pocket and the phytosphingosine chain buried in the F'-pocket (Borg et al. 2007; Pellicci et al. 2009). In contrast, the α -galactosyl head group protrudes from the cleft, such that it is directly available to make contact with the iNKT TCR. The 2-hydroxyl (2-OH) of the galactose ring, the 3-OH of the sphingosine chain, and the anomeric oxygen of α -GalCer form hydrogen bonds with residues on CD1d, which serve to anchor α -GalCer in the lipid-binding groove of CD1d. The 2-, 3- and 4-OH groups of the galactose ring interact with the invariant TCR α -chain making hydrogen bonds with the corresponding amino acids, which are responsible for the fine specificity that the NKT TCR exhibits for α -GalCer. Only the 6-OH group of the sugar ring is not involved in any hydrogen bond formation during α -GalCer binding and recognition and modifications of the 6-OH group can be made without losing antigenicity (Kawano et al. 1997; Prigozy et al. 2001; Zhou et al. 2002). Based on this premise, a number of groups have synthesized novel α -GalCer analogs with substitutions in the 6-OH position of the galactose, which resulted in ligands that could bind to CD1d and stimulate iNKT cells (Liu et al. 2006; Trappeniers et al. 2008).

Based on our recent work on α -S-GalCer (Hogan et al. 2011; Murphy et al. 2013) and the above literature, glycolipids XZ7 and XZ11 were designed and chemically synthesized. Glucosylation of 6-OH of α -S-GalCer gives disaccharide ceramide XZ7 (Zhang et al. 2017), while thioglucosylation of the same group gives target molecule XZ11 (Figure 1). Their ability to bind to human CD1d and activate human iNKT cells was tested using set of iNKT cell lines generated from blood samples from healthy donors.

Results

Phenotypic analysis of iNKT cell lines

iNKT cell lines were sorted from human blood and expanded in the presence of α -GalCer, IL-2 and irradiated feeders and phenotypically examined by flow cytometry. Purity of iNKT cell lines was at least 92% and frequently higher than 99% (Figure 2A). The distributions

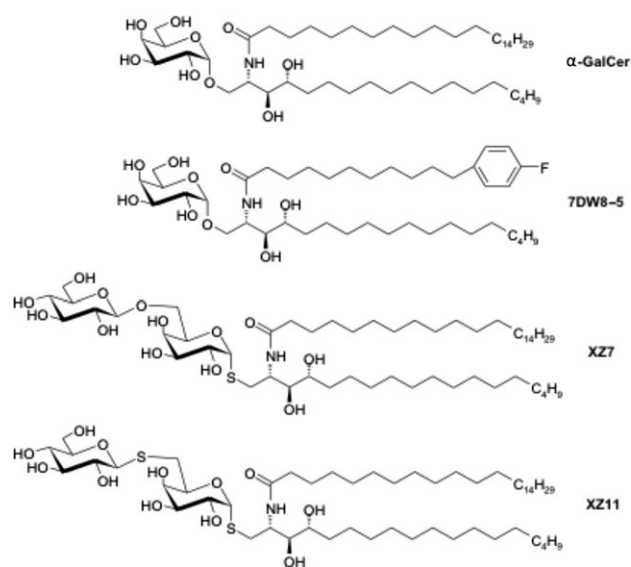


Fig. 1. Chemical structures of the glycolipids used in the present study. α -GalCer, α -galactosylceramide; 7DW8-5, α -GalCer analog with shorter fatty acyl chain and fluorinated benzene ring at the end of fatty acyl chain; XZ7, α -S-GalCer analog with 6-O-glucosylated; XZ11, α -S-GalCer analog with 6-deoxy-6-thioglucosylated.

of CD4⁺, CD8⁺ and CD4[−]CD8[−] (double negative; DN) iNKT cells varied considerably in the seven lines tested (Figure 2B), and included one (line 2) which comprised CD8⁺ and DN but not CD4⁺ iNKT cells (Figure 2C). Since, line 2 is representative of the variation in the repertoires of iNKT cells seen in humans (O'Reilly et al. 2011), and of the methods used to expand iNKT cells for clinical applications, we did not exclude this line from subsequent analyses.

The novel glycolipids XZ7 and XZ11 stimulate cytolytic degranulation by iNKT cells in vitro

Expanded iNKT cell lines were cocultured for 4 h with equal numbers of CD1d-transfected or mock-transfected HeLa cells (hereafter referred to as HeLa-CD1d and HeLa-mock) that were previously loaded with medium, 100 ng/mL α -GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7 or XZ11. Cytolytic degranulation was assessed by flow cytometric measurement of cell-surface CD107a expression (Figure 3A). iNKT cells exhibited significant CD107a expression after stimulation with α -GalCer and 7DW8-5. A weaker but dose-dependent response to XZ7 (Figure 3B) and XZ11 (Figure 3C) was also observed. Little or no degranulation occurred in the absence of glycolipids.

The novel glycolipids XZ7 and XZ11 stimulate intracellular cytokine production by iNKT cells

Expanded iNKT cell lines were cocultured for 4 h with equal numbers of CD1d-transfected or mock-transfected HeLa cells that were previously loaded with medium, 100 ng/mL α -GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7 or XZ11. The proportions of iNKT cells that produced intracellular IFN- γ and IL-4 were quantified by flow cytometry (Figure 4A). Up to 2% of iNKT cells expressed IFN- γ and 12% produced IL-4 in response to CD1d-transfected HeLa cells in the absence of stimulation, presumably due to residual activation following expansion. CD1d-transfected HeLa cells pulsed with α -GalCer or 7DW8-5 induced significant increases

in IFN- γ and IL-4 positivity by iNKT cells. Results from seven different iNKT cell lines showed that XZ7 presented by CD1d induced weak but significant IFN- γ but not IL-4 production, compared to unstimulated controls when used at 10,000 ng/mL (Figure 4B). In contrast, XZ11 induced IL-4, but not IFN- γ , production by a minority of iNKT cells (Figure 4B). We also quantified cytokine release by the cells, stimulated as above, using ELISA (Figure 5). The results show again that iNKT cells stimulated with 10,000 ng/mL XZ7 produced IFN- γ and cells stimulated with similar amounts of XZ11 produced IL-4. Of note, the amounts of both cytokines were 3–4-folds lower than were produced by the same cells in response to stimulation with 100 ng/mL α -GalCer or 10 ng/mL 7DW8-5, presumably reflecting the low frequencies of cells that produced these cytokines (Figure 4).

Activation of iNKT cells by XZ7 presented by DC

Since XZ7, presented by HeLa-CD1d cells, was capable of stimulating the antitumor functions (cytolytic degranulation and IFN- γ production) of iNKT cells without inducing IL-4 production, thought to have protumor effects (Terabe et al. 2005; Bricard et al. 2009), we next investigated if this glycolipid had similar effects when presented by DCs, which naturally express CD1d (Gerlini et al. 2001). Expanded iNKT cell lines were cocultured for 4 h with equal numbers of monocyte-derived DC that were previously loaded with medium, 100 ng/mL α -GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7. Cells were stained with mAbs specific for cell surface CD107a, CD3 and the V α 24J α 18 TCR (Figure 6A) or mAbs specific for cell-surface CD3 and V α 24J α 18 and intracellular IFN- γ and IL-4 (Figure 6B and C) and analyzed by flow cytometry. DC pulsed with α -GalCer and 7DW8-5 induced significant CD107a, IFN- γ and IL-4 expression by iNKT cells, whereas DC pulsed with medium alone did not activate iNKT cells, confirming the absence of endogenous iNKT cell stimulatory glycolipids. In contrast, DC pulsed with XZ7 induced a dose-dependent induction of CD107a (Figure 6A) and IFN- γ (Figure 6B), but not IL-4 (Figure 6C), expression by iNKT cells. Thus, glycolipid XZ7 appears to stimulate cytotoxicity and Th1 cytokine production, but not Th2 cytokine production by iNKT cells.

Cytolytic degranulation by iNKT cells in response to XZ7 is restricted to the CD4-negative subset

XZ7, presented by HeLa-CD1d cells or monocyte-derived DC, induced cytolytic degradation and Th1 but not Th2 cytokine production by iNKT cells. Since CD4⁺, CD8⁺ and DN iNKT cells differentially exhibit cytotoxicity, Th1 and Th2 cytokine production (Gumperz et al. 2002; O'Reilly et al. 2011), we next investigated if XZ7 preferentially activates subsets of iNKT cells. Expanded iNKT cell lines were cocultured for 4 h with equal numbers of HeLa-CD1d cells (Figure 7A) or monocyte-derived DC (Figure 7B) that were previously loaded with medium, 100 ng/mL α -GalCer, 10 ng/mL 7DW8-5 or 10–10,000 ng/mL of XZ7. Cytolytic degranulation was measured by flow cytometric analysis of CD107a expression. CD4⁺, CD8⁺ and DN iNKT cells exhibited significant CD107a expression after stimulation with the agonist glycolipids α -GalCer or 7DW8-5 when presented with CD1d-transfected HeLa cells or DC. However, XZ7 presented by CD1d-transfected HeLa cells stimulated CD107a expression by CD8⁺ and DN iNKT cells, in a dose-dependent manner, but not by CD4⁺ iNKT cells (Figure 7A). When DC were used as APCs, XZ7 induced degranulation by CD8⁺ iNKT cells in a dose-dependent manner, but not by CD4⁺ or DN iNKT cells (Figure 7B).

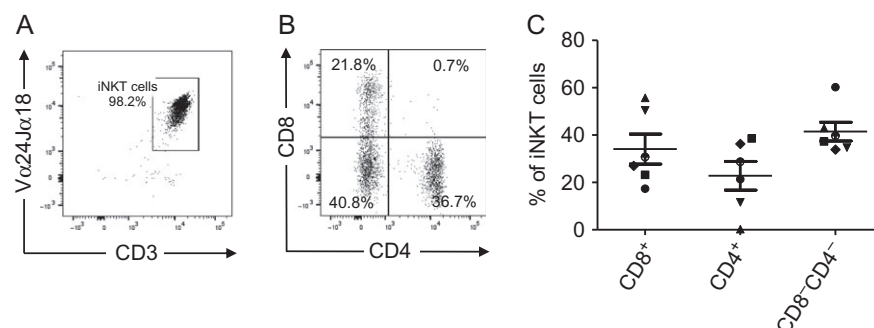


Fig. 2. Purity and phenotype of iNKT cell lines. Flow cytometry plots showing expression of CD3 and the iNKT cell receptor ($V\alpha 24J\alpha 18$) (A) and CD4 and CD8 (B) by an expanded iNKT cell line. (C) Scatter plot showing the frequencies of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ iNKT cells within lines of iNKT cells generated from seven healthy donors (distinguished by symbols). Horizontal lines show means.

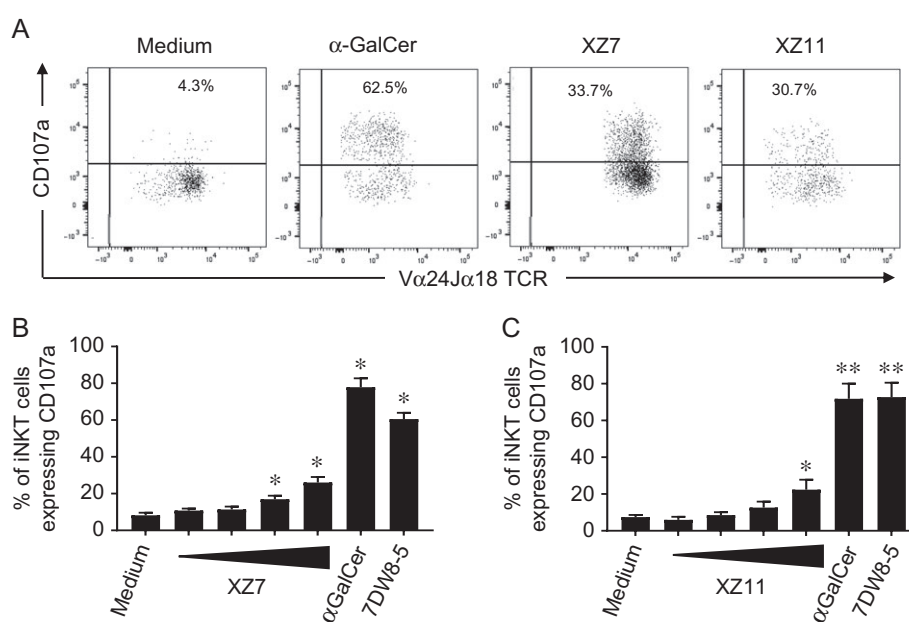


Fig. 3. Cytolytic degranulation by iNKT cells in response to the novel glycolipids XZ7 and XZ11. Lines of expanded iNKT cells were cocultured with CD1d-transfected HeLa cells, previously pulsed with medium alone, 100 ng/mL of α -GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ7 or XZ11 (summarized by black triangles). Cells were stained with antibodies specific for CD107a, CD3 and the $V\alpha 24J\alpha 18$ T cell receptor (TCR), and analyzed by flow cytometry. (A) Flow cytometry dot plots showing the expression of cell-surface CD107a by iNKT cells after stimulation with medium, α -GalCer, XZ7 and XZ11. (B and C) Graphs showing mean ($\pm$ SEM) percentages of iNKT cell lines generated from seven donors that expressed CD107a after stimulation with XZ7 (B) and XZ11 (C). * $P < 0.05$, ** $P < 0.01$ compared to medium-only controls using the Mann-Whitney U test.

Discussion

iNKT cells play critical roles in antitumor immunity in murine models and can be activated therapeutically to prevent or reverse tumor growth (Cui et al. 1997; Kawano et al. 1997; Crowe et al. 2005). These observations have led to a number of clinical trials of the utility of α -GalCer, α -GalCer-pulsed DC or α -GalCer-expanded iNKT cells as cellular therapies in humans (Nieda et al. 2004; Chang et al. 2005; Kunii et al. 2009). The results have shown that iNKT cell-directed therapies were well-tolerated and that they resulted in elevated serum levels of proinflammatory cytokines and persistent expansions of IFN- γ -producing cells. However, in all studies, the clinical efficacies were modest.

It is possible that the iNKT cell-based immunotherapy trials in humans failed because their disease is too advanced, or because their cellular immune responses are impaired by previous or ongoing use of conventional cytotoxic chemotherapies or radiotherapies. Indeed, high pretreatment numbers of iNKT cells are associated with better responses in clinical trials (Giaccone et al. 2002). The divergent responses to iNKT cell-based therapies in mice and humans may also reflect numerical or functional differences in murine and human iNKT cells. iNKT cells are found at 100-fold lower frequencies in humans compared to mice at most body locations tested (Kenna et al. 2003; Berzins et al. 2011). Furthermore, mice and humans have different subset distributions of iNKT cells, with most murine iNKT cells expressing $CD4^+$ or DN phenotypes, whereas humans

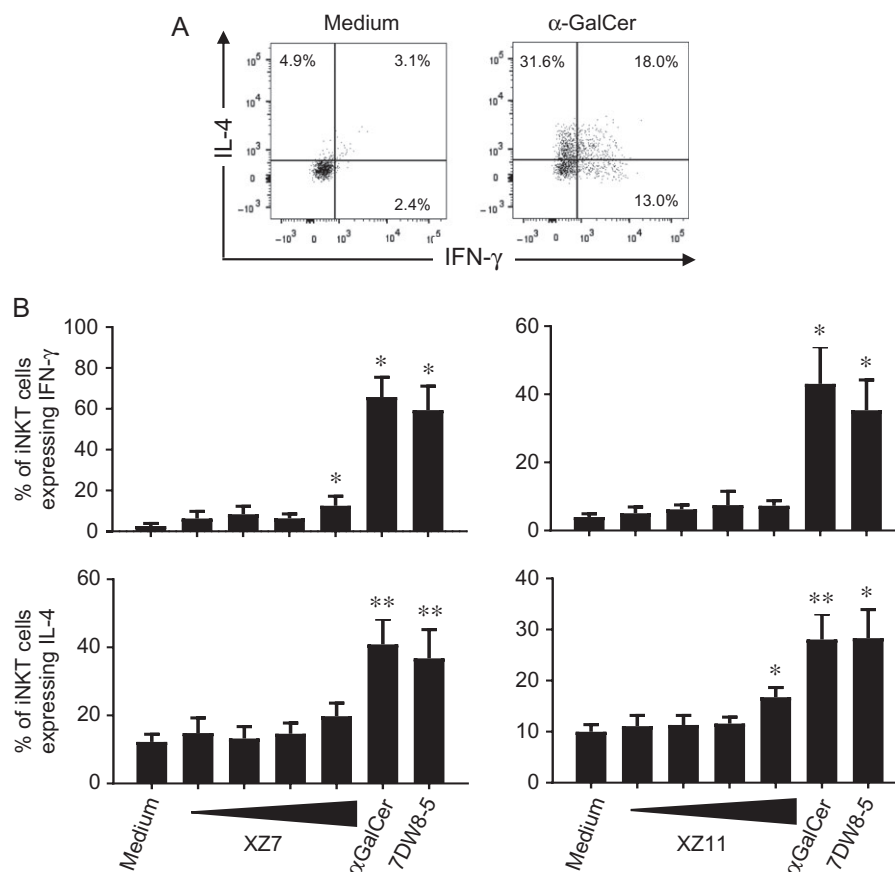


Fig. 4. Cytokine production by iNKT cells in response to the novel glycolipids XZ7 and XZ11. Expanded iNKT cells were cocultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 100 ng/mL of α -GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ7 or XZ11 (summarized by black triangles). Cells were stained with mAbs specific for cell-surface CD3 and V α 24J α 18 and intracellular IFN- γ and IL-4 and analyzed by flow cytometry (A). (B) Graphs showing mean ($\pm$ SEM) percentages of iNKT cells producing IFN- γ (upper panels) or IL-4 (lower panels) after stimulation with XZ7 (left panels; $n = 4$) or XZ11 (right panels; $n = 7$). * $P < 0.05$, ** $P < 0.01$ compared to medium-only controls using the Mann-Whitney U test.

have significant populations of CD4⁺, CD8⁺ and DN iNKT cells which differ in their effector activities (Gumperz et al. 2002; O'Reilly et al. 2011). Given the pleiotropy of iNKT cell functions, it is likely that differences in iNKT cell subset numbers and distributions between mice and humans and between different humans may underlie the divergent outcomes seen after therapeutic activation. Additionally, differences in the fine specificities of TCR recognition of CD1d/glycolipid complexes by murine and human iNKT cells (Wun et al. 2012) will also contribute to divergent outcomes of iNKT cell activation, and in this regard, α -GalCer may not be the ideal iNKT cell agonist for therapeutic use in humans.

In the present study, we have synthesized and biologically tested a number of novel glycolipid analogues of α -GalCer that we predicted would bind to CD1d and activate human iNKT cells. We previously showed that replacement of the glycosidic oxygen atom in α -GalCer with a sulfur atom created a glycolipid that could bind to CD1d and induce simultaneous Th1 and Th2 cytokine production by iNKT cells (Hogan et al. 2011). Since thioglycosides have more flexibility around the glycosidic linkage compared to the corresponding O-glycosides, owing to the longer C-S bond and weaker stereoelectronic effects (Witczak 1999), we predicted that, after binding to CD1d, the sugar head of α -S-GalCer may orientate in a different angle from that of α -GalCer, which could result in differential recognition by TCR of iNKT cells. We synthesized analogs of α -

S-GalCer with an extra glucose or thioglucose connected to the hydroxyl groups of the galactose moiety. We found that two of these compounds, in which the 6-OH group of the galactose moiety of α -S-GalCer was substituted with a glucose group, one with a 6-O-glucosyl group (XZ7) and the other with a 6-thio-glucosyl group (XZ11), could bind to CD1d and displayed agonist activity for human iNKT cells. Both glycolipids, in the presence of CD1d⁺ APC, were capable of inducing cytolytic degranulation by iNKT cells, albeit with weaker potency than α -GalCer and 7DW8-5, requiring 100-fold higher concentrations. Both glycolipids activated cytokine production by proportions of iNKT cells, but while XZ7 predominantly stimulated intracellular IFN- γ production and secretion, XZ11 stimulated IL-4 production and secretion. Again, the stimulatory capacities of XZ7 and XZ11 were weaker than those of α -GalCer and 7DW8-5, which at 100–1000-folds lower concentrations activated higher proportions of iNKT cells resulting in the secretion of 3–4-folds higher amounts of cytokines than XZ7 and XZ11.

Since glycolipid XZ7 was capable of stimulating antitumor activities of iNKT cells (cytolytic degranulation and IFN- γ production) without stimulating IL-4 production, this compound was selected for further analysis. We first tested if iNKT cells stimulated with XZ7 presented by DC, which constitutively express CD1d (Bendelac et al. 2007; Salio et al. 2014), have the same functional profile as iNKT cells stimulated with XZ7 presented by HeLa-CD1d cells.

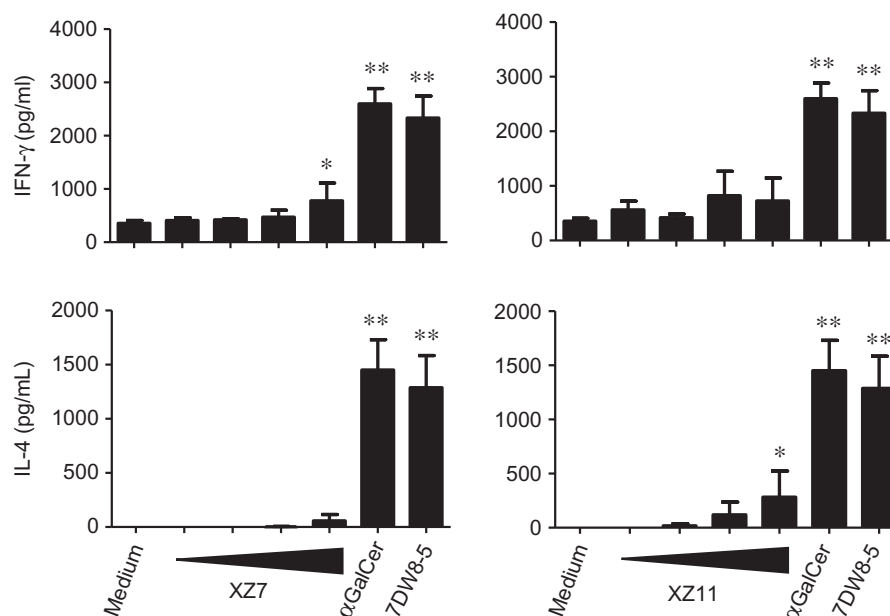


Fig. 5. Cytokine release by iNKT cells in response to the novel glycolipids XZ7 and XZ11. Expanded iNKT cells were cocultured with CD1d-transfected HeLa cells, previously loaded with medium alone, 100 ng/mL of α -GalCer, 10 ng/mL of 7DW8-5, or 10, 100, 1000 or 10,000 ng/mL of XZ7 or XZ11 (summarized by black triangles). The release of IFN- γ and IL-4 into the cell supernatants was measured by ELISA. Graphs show mean ($\pm$ SEM) levels of iNKT cells producing IFN- γ (upper panels) or IL-4 (lower panels) after stimulation with XZ7 (left panels; $n = 4$) or XZ11 (right panels; $n = 4$). * $P < 0.05$, ** $P < 0.01$ compared to medium-only controls using the Mann-Whitney U test.

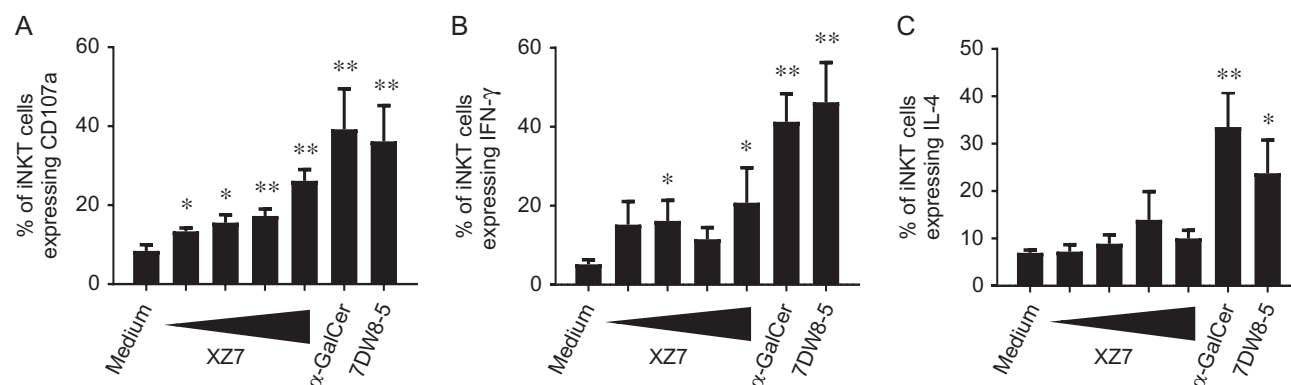


Fig. 6. Cytolytic degranulation and cytokine production by iNKT cells in response to XZ7 presented by dendritic cells. iNKT cells were stimulated by monocyte-derived dendritic cells previously pulsed with medium alone, 100 ng/mL of α -GalCer, 10 ng/mL of 7DW8-5 or 10, 100, 1000 or 10,000 ng/mL of XZ7 (summarized by black triangles). Cells were stained with mAbs specific for CD107a, CD3 and the V α 24J α 18 TCR (A) or mAbs specific for cell-surface CD3 and V α 24J α 18 and intracellular IFN- γ and IL-4 (B and C) and analyzed by flow cytometry. (A–C) Graphs showing mean ($\pm$ SEM) percentages of total iNKT cells that expressed CD107a (A, $n = 5$), IFN- γ (B, $n = 6$) and IL-4 (C, $n = 6$) after stimulation with the glycolipids. * $P < 0.05$, ** $P < 0.01$ compared to medium-only controls using the Mann-Whitney U test.

The results showed that XZ7 presented by DC induced degranulation and IFN- γ production, but not IL-4 production, by iNKT cells, confirming the potential antitumor activity of this novel glycolipid *in vitro*. Since, only a small fraction of iNKT cells were activated by XZ7 in all experiments, we next investigated if CD4 $^{+}$, CD8 α^{+} and DN iNKT cells were similarly activated. The results indicate that, while α -GalCer and 7DW8-5 stimulated degranulation by all subsets of iNKT cells, XZ7 only stimulated CD8 α^{+} iNKT cells when DC were used as APC and CD8 α^{+} and DN iNKT cells when HeLa-CD1d cells were used. No degranulation was observed in CD4 $^{+}$

iNKT cells within any of six lines of iNKT cells that were stimulated with HeLa-CD1d or DC presenting XZ7. The failure of XZ7 to activate CD4 $^{+}$ iNKT cells may explain why only a small proportion of total iNKT cells were activated and may underlie the inability to of this glycolipid to stimulate IL-4 production, an effector function that is associated with CD4 $^{+}$ iNKT cells (Gumperz et al. 2002; O'Reilly et al. 2011).

IFN- γ and other Th1 cytokines promote host immunity to tumors by stimulating tumor antigen presentation and promoting tumor cytotoxicity by innate and adaptive lymphocytes. They also

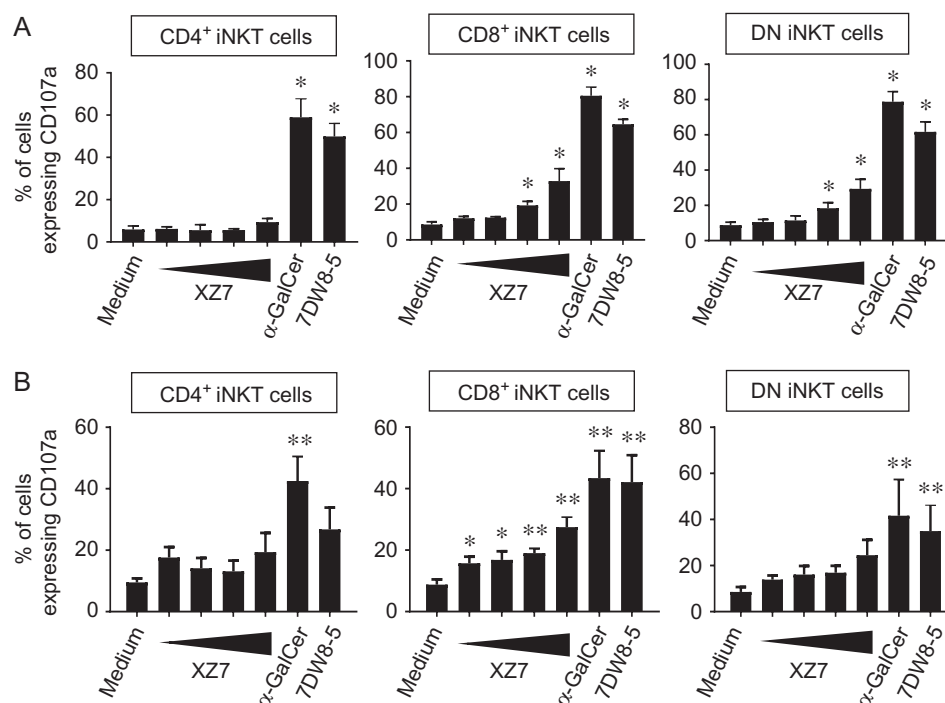


Fig. 7. Cytolytic degranulation by iNKT cells in response to XZ7 is restricted to the CD4-negative subset. iNKT cells were stimulated by HeLa-CD1d cells (A) or monocyte-derived dendritic cells (B) that were previously pulsed with medium alone, 100 ng/mL of α -GalCer, 10 ng/mL of 7DW8-5 or 10, 100, 1000 or 10,000 ng/mL of XZ7. Cells were stained with mAbs specific for CD107a, CD3, CD4, CD8 and the V α 24J α 18 TCR, and analyzed by flow cytometry. Graphs show mean ($\pm$ SEM) percentages of CD4⁺ (left panels), CD8⁺ (center panels) and CD4⁺CD8⁺ (DN; right panels) iNKT cells that expressed cell-surface CD107a after stimulation with XZ7 presented by HeLa-CD1d cells (A, $n = 4$) and dendritic cells (B, $n = 6$) after stimulation with the glycolipids. * $P < 0.05$, ** $P < 0.01$ compared to medium-only controls using the Mann–Whitney U test.

inhibit proliferation and modulate apoptosis, differentiation and migration of tumor cells (Ikeda et al. 2002; Parker et al. 2016). On the contrary, IL-4 and Th2 cytokines are regulatory cytokines for cytotoxic T cells and NK cells and they can permit tumor growth through the inhibition of cell-mediated immunity (Brown and Hural 1997). Production of the Th2 cytokines IL-4, IL-10 and IL-13 by NKT cells has been reported to inhibit antitumor immunity and promote tumor growth (Terabe et al. 2005; Bricard et al. 2009; Lynch et al. 2015). Since human iNKT cells simultaneously produce Th1 and Th2 cytokines upon activation with α -GalCer, this glycolipid may not be an optimal ligand for stimulating the antitumor immune responses of iNKT cells. The partial agonist effect of glycolipid XZ7, inducing cytotoxicity and IFN- γ production, but not IL-4 production, indicates that the protumour activities of iNKT cells can be prevented, while preserving their antitumor activities, by introducing a second glycosyl group to α -S-GalCer, which we previously found to have similar activity to that of α -GalCer. XZ7 is unlikely to be superior to α -GalCer as a therapeutic agent for cancer because its potency as an iNKT cell agonist is much lower than that of that of α -GalCer. However, it may serve as a parent compound for the development of structural analogs that display similar cytokine profiles but with greater potency.

Materials and methods

Glycolipids

α -GalCer or 7DW8-5 were purchased from Funakoshi Co. Ltd (Tokyo Japan). The chemical synthesis of XZ7 and XZ11 has previously been described (Zhang et al. 2017).

Antibodies and flow cytometry

Fluorochrome-conjugated monoclonal antibodies (mAb) specific for human CD3, V α 24J α 18 (clone 6B11), CD8, CD4, IL-4 (BioLegend, London, UK), CD107a, (BD Bioscience, Oxford, UK), IFN- γ (Miltenyi Biotec, Germany) were used. Flow cytometry was performed using a FACSCanto flow cytometer (BD Bioscience, Oxford, UK), and FlowJo Version 10 (Tree Star, Ashland, OR) software was used for analysis.

Generation of iNKT cell lines

Peripheral blood mononuclear cells (PBMCs) were prepared from buffy packs kindly provided by the Irish Blood Transfusion Service, Dublin, by standard density gradient centrifugation over LymphoprepTM (Stemcell Technologies, Cambridge, UK). iNKT cells were enriched from total human PBMC by magnetic bead separation using anti-iNKT cell Microbeads (Miltenyi Biotec, Gladbach Bergische, Germany) followed by sorting of V α 24J α 18⁺ CD3⁺ cells using a MoFlo XDP Cell Sorter (Beckman Coulter, Galway, Ireland). Highly purified iNKT cells were expanded by culturing 1000 iNKT cells in the wells of a 96-well round bottom microtitre plate, in iNKT cell medium (RPMI 1640 containing 0.05 mM L-glutamine, 10% HyClone FBS, 100 mg/mL streptomycin, 100 U/mL penicillin, 2.5 μ g/mL amphotericin B—fungizone, 25 mM HEPES, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 1% non-essential amino acids mixture and 1% essential amino acids mixture; Gibco-BRL, Paisley, UK and Thermo-Scientific, Logan, UT) and stimulating them with the iNKT cell glycolipid ligand α -GalCer (100 ng/mL) and 250 U/mL IL-2 (Miltenyi Biotec) in the presence of an excess (2×10^5) irradiated allogeneic PBMC prepared from two donors. Stock α -GalCer was thawed followed by heating to 80°C for

2 min, sonication for 10 min and vortexing for 1 min before diluting to the required concentration in iNKT cell medium followed by heating to 80°C for 2 min, sonication for 5 min and vortexing for 1 min before each further dilution. After 24 h and again after 48 h and subsequently every 3–4 days, medium was replaced with fresh iNKT cell medium containing 250 U/mL IL-2. Cells were expanded for a minimum of 3 weeks before being used in experiments. iNKT cells were kept at high cell densities and split 1 in 2 in fresh 96-well plates when multiple cell layers were observed using an inverted microscope. Cells were restimulated as above every 4–6 weeks. The purities of iNKT cells were >98% as determined by flow cytometry.

Generation of DC

Monocytes were enriched to >90% purity from PBMC by positive selection using CD14 microbeads (Miltenyi Biotec). The monocytes were allowed to differentiate into immature DC by culturing them for 6 days in the presence of GM-CSF and IL-4 as described previously (Hogan et al. 2011). Flow cytometry was used to verify that differentiation into iDC had taken place and cells expressed HLA-DR and CD11c but not CD14.

Glycolipid activation of iNKT cells

iNKT cells were stimulated *in vitro* with α -GalCer or 7DW8-5, or XZ7 and XZ11, which were synthesized as described above. The structures of the glycolipids used are shown in Figure 1. HeLa-mock and HeLa-CD1d cells or DC were used as APCs. HeLa-mock and HeLa-CD1d cells were a gift from Dr Steven Porcelli, Albert Einstein College of Medicine, New York. Cells were cultured in complete DMEM (cDMEM) medium (DMEM containing 0.05 mM L-glutamine, 10% HyClone fetal calf serum, 100 units/mL penicillin, 100 μ g/mL streptomycin, 2.5 μ g/mL amphotericin B Fungizone and 25 mM HEPES) in T75 flasks and split twice per week in a 1:5 ratio.

The 0.1×10^6 APCs were cultured for 18 h with glycolipids in 96-well round bottom microtitre plates. Glycolipid stocks were thawed followed by heating to 80°C for 2 min, sonication for 10 min and vortexing for 1 min before diluting to the required concentration in cDMEM medium. After 18 h, the medium was then aspirated from the wells and replaced with iNKT cell medium containing equal numbers of iNKT cells and an anti-CD107a mAb. Monensin (25 μ M; Biolegend) was added after 1 h to prevent proteolysis of the mAb conjugate upon reinternalization of CD107a. After a further 3 h, cells were stained with mAbs specific for CD3, V α 24J α 18, CD4 and CD8 and analyzed by flow cytometry. Cytolytic degranulation by iNKT cells was examined by flow cytometric analysis of CD107a expression. We previously have shown that CD107a expression by human iNKT cells directly correlates closely with target cell death (Hogan et al., 2011; O'Reilly et al. 2011).

Measurement of cytokine production

IL-4 and IFN- γ production by iNKT cells were assessed by flow cytometric analysis. The 0.1×10^6 iNKT cells were stimulated by CD1d-transfected HeLa cells or DC, previously pulsed with different concentrations of glycolipids, as described above. Stimulations were carried out for 4 h in the presence of brefeldin A, to prevent cytokine release from the cells. After stimulation, the cells were stained with mAb specific for cell surface CD3, V α 24J α 18, CD8 and CD4, fixed with 4% paraformaldehyde, permeabilized with 0.2% saponin (Sigma-Aldrich, Poole, UK) and stained with fluorochrome-labeled antibodies specific for IL-4 and IFN- γ (BioLegend). The expression

of IL-4 and IFN- γ by iNKT cell subsets was then analyzed by flow cytometry. Amounts of IL-4 and IFN- γ released by iNKT cells, similarly stimulated but in the absence of brefeldin A, were measured by enzyme-linked immunosorbent assays (ELISA) using antibody pairs purchased from R&D Systems (Abingdon, UK).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 6.0 (GraphPad Prism, San Diego, CA). *P* values between groups were obtained using the unpaired Mann–Whitney *U* test, and paired *t* test. *P* values of <0.05 (*), *P* < 0.01(**) and *P* < 0.001 (***) were considered statistically significant.

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Conflict of interest statement

None declared.

Abbreviations

α -GalCer, α -galactosylceramide; α -S-GalCer, thioglycoside analog of α -galactosylceramide; APC, antigen-presenting cell; DC, dendritic cell; DN, double negative for CD4 and CD8; IFN- γ , interferon- γ ; IL-4, interleukin-4; iNKT cell invariant natural killer T cell; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; TCR, T cell antigen receptor.

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