

CD1d-restricted Recognition of Synthetic Glycolipid Antigen by Human Natural Killer T Cells

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Summary

A conserved subset of mature circulating T cells in humans expresses an invariant V α 24-J α Q T cell receptor (TCR)- α chain rearrangement and several natural killer (NK) locus-encoded C-type lectins. These human T cells appear to be precise homologues of the subset of NK1.1⁺ TCR- α / β ⁺ T cells, often referred to as NK T cells, which was initially identified in mice. Here we show that human NK T cell clones are strongly and specifically activated by the same synthetic glycolipid antigens as have been shown recently to stimulate murine NK T cells. Responses of human NK T cells to these synthetic glycolipids, consisting of certain α -anomeric sugars conjugated to an acylated phytosphingosine base, required presentation by antigen-presenting cells expressing the major histocompatibility complex class I-like CD1d protein. Presentation of synthetic glycolipid antigens to human NK T cells required internalization of the glycolipids by the antigen-presenting cell and normal endosomal targeting of CD1d. Recognition of these compounds by human NK T cells triggered proliferation, cytokine release, and cytotoxic activity. These results demonstrate a striking parallel in the specificity of NK T cells in humans and mice, thus providing further insight into the potential mechanisms of immune recognition by NK T cells and the immunological function of this unique T cell subset.

Key words: natural killer T cell • human • CD1 • antigen presentation • glycolipid

Natural killer (NK) T cells are a recently described subpopulation of TCR- α / β ⁺ CD4⁺ or CD4⁻8⁻ T lymphocytes that have distinctive phenotypic and functional properties (1). These T cells were first identified in mice, where they can be distinguished from conventional T cells by their expression of the NK locus-encoded C-type lectin molecule NK1. Another hallmark of murine NK T cells is their extremely restricted TCR repertoire, with the great majority expressing an invariant TCR- α chain structure (V α 14-J α 281) paired preferentially with V β 8, 7, or 2 (2, 3). Recently, it has been demonstrated that the human immune system contains a population of T cells that show striking conservation of most of the key features of murine NK T cells (4–6). These human NK T cells express an invariant V α 24-J α Q TCR- α chain, which is highly homologous to the invariant TCR- α chain of murine NK T cells (2). In addition, human NK T cells express a restricted V β repertoire (7, 8) and several NK locus-encoded C-type lectins, including NKR-P1A (CD161), which is the homologue of murine NK1 (4).

At present, the function of NK T cells in the immune response remains incompletely resolved. However, much interest has been generated by the finding that murine and human NK T cells are able to produce large amounts of

both IL-4 and IFN- γ rapidly upon activation, and may represent a major source of early IL-4 in certain immune responses (4, 9). Recent results also suggest major roles for murine NK T cells in the rejection of malignant tumors (10) and in regulating autoimmunity (11–13). Given these potentially important activities of NK T cells, it has become of great interest to resolve the question of what foreign or self-antigens these T cells recognize. Initial insight into this question came with the finding that both murine and human NK T cells are capable of specifically recognizing the MHC class I-like CD1d protein (4, 14). As other members of the CD1 family have recently been shown to mediate the presentation of foreign lipids and glycolipids to T cells (15–17), this suggested the possibility that the actual target ligands of NK T cells may be CD1 proteins that have bound foreign or endogenous lipids.

Recently, Kawano et al. reported the CD1-dependent recognition of a family of synthetic glycolipid antigens by murine NK T cells, thus providing the first insight into the structure of foreign antigens recognized by this T cell subset (18). The glycolipids that were active in their experiments were composed of an α -anomeric sugar linked to a ceramide type acylphytosphingosine (APS) lipid, and are structurally related but distinct from the abundant ceramide-

containing glycolipids (e.g., gangliosides) found in normal mammalian tissues (19). In this study, we show that human CD1d-restricted T cells also respond to these α -glycosyl-APS, and have a fine specificity for different compounds in this family that is remarkably similar to that shown for mouse NK T cells. Recognition of these synthetic glycolipids was restricted exclusively by human CD1d, as other members of the human CD1 family (CD1a, -b, and -c) were unable to present these antigens to human NK T cells. Our results provide further evidence for the conservation of a functional NK T cell subset in humans, and support the hypothesis that the immunological role of these T cells involves recognition of foreign or endogenous glycolipids.

Materials and Methods

Human NK T Cell Clones and CD1 Transfectants. Human NK T cell clones DN2.B9, DN2.C7, and DN2.D5 were derived, maintained, and phenotypically analyzed as previously described (4, 8). The cervical carcinoma cell line HeLa was transfected with the expression vector pSR α -Neo containing cDNA inserts encoding CD1a, CD1b, CD1c, or CD1d according to previously described methods (20). The HeLa CD1d/a transfectant expressed a chimeric form of CD1d with the extracellular domains of CD1d fused to the transmembrane plus cytoplasmic domains of CD1a. This stable transfectant was produced by the introduction into HeLa cells of a previously described expression construct encoding this chimeric protein (4).

T Cell Proliferation Assays. 5×10^4 T cells were plated in triplicate in flat-bottomed 96-well plate wells with 10^4 mitomycin C-treated APCs (0.1 mg/ml for 1 h at 37°C; Sigma Chemical Co., St. Louis, MO). Media for all T cell cultures was RPMI 1640 (GIBCO BRL, Gaithersburg, MD) plus 10% fetal bovine serum (HyClone Labs., Logan, UT) and other additives as previously detailed (21). The synthetic glycolipid antigens used in this study were provided by the Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd. (Gunma, Japan), and have been described in detail previously (18). Although these were previously designated as glycosyl-ceramides, here we have referred to them as glycosyl-APS derivatives because of the hydroxylations of the third and fourth carbons in their sphingosine base that are characteristic of phytosphingosines (19). Synthetic glycolipids were dissolved at 100 μ g/ml in DMSO, and diluted into culture medium to the indicated final concentrations. For mAb blocking experiments, the following mAbs were added as purified IgG to a final concentration of 20 μ g/ml: W6/32 (anti-HLA-A, -B, and -C; reference 22); CD1d51 (anti-CD1d; reference 4); and P3 (non-binding IgG1 control; reference 23). For light fixation of the APC surface, CD1d-transfected HeLa cells were incubated for 1 h on ice in 2 ml of 0.9% NaCl containing 75 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (ECDI; Pierce Chemical Co., Rockford, IL), followed by extensive washing in RPMI 1640 medium. Cultures were incubated at 37°C in a 5% CO₂ incubator, pulsed with 1 μ Ci of [³H]thymidine (2 Ci/mmol) on day 2 and harvested 16 h later for β scintillation counting using a Tomtec harvester and a Betaplate scintillation counter (Wallac, Gaithersburg, MD).

Cytokine and Cytotoxic T Cell Assays. 5×10^4 T cells were cultured in triplicate wells with 10^4 mitomycin C-treated CD1d-transfected HeLa cells in the absence or presence of 100 ng/ml

of α -galactosyl-APS (α GalAPS). Phytohemagglutinin (PHA-P; Difco, Detroit, MI) was added to some cultures (1:2,000 final dilution) as a positive control. Supernatants were harvested after 48 h, and cytokine levels determined by capture ELISA as described (4). T cell cytotoxic activity was measured using a standard 4-h ⁵¹Cr-release assay (21). Mock-transfected or CD1d-transfected HeLa cells were labeled with 200 μ Ci of ⁵¹Cr for 2 h at 37°C, followed by incubation for 12 h at 37°C in complete medium with or without 200 ng/ml of α GalAPS, and then washed and used as target cells. Assays were performed in triplicate and results were expressed as percentage of maximum specific ⁵¹Cr release.

Results and Discussion

Specific Recognition of Synthetic Glycolipid Antigens by Human NK T Cell Clones. Three previously characterized human NK T clones (DN2.B9, DN2.C7, and DN2.D5; reference 4) bearing the invariant V α 24-J α Q TCR- α chain paired with different V β 11⁺ TCR- β chains were tested for their ability to respond to synthetic APS-containing glycolipid antigens presented by CD1d-transfected HeLa cells. Because human NK T cells generally have intrinsic autoreactivity to CD1d-expressing cells (4), modifications of the culture conditions were required for optimal demonstration of the response to synthetic glycolipids. Preliminary experiments (data not shown) revealed that CD1d-transfected HeLa cells were particularly suited as APCs for these studies, since they did not require additional exogenous signals such as PMA to support the proliferation of human NK T cells. Using a relatively low APC/T cell ratio (1:5), we observed low to moderate autoreactivity and consistent augmentation of the proliferation of human NK T cell clones when the synthetic glycolipid α GalAPS was added (Fig. 1 a).

The response to α GalAPS appeared to be specific for NK T cell clones, since this compound did not stimulate proliferation of a panel of other human T cell clones that did not express the invariant V α 24-J α Q TCR- α chain (data not shown). The specificity of the response to synthetic glycolipids was also assessed by examining the responses of NK T cell clones to a panel of structurally related glycolipids. These included two previously demonstrated strong stimulators of murine NK T cells (α GalAPS and α GluAPS), as well as four related analogues that were non-stimulatory or weakly stimulatory in the murine system (APS, α ManAPS, β GalAPS, and 3,4-deoxy α GalAPS) (18). NK T cell clones responded strongly to α GalAPS and α GluAPS but not to the other analogues (Fig. 1 b, and additional data not shown), demonstrating a pattern very similar to that observed for murine NK T cells. Thus, the specific recognition of synthetic glycolipids by a human NK T cell clone required an α -anomeric sugar (either galactose or glucose) and the presence of hydroxylations at positions 3 and 4 of the phytosphingosine base, exactly as described for murine NK T cells.

CD1 Restriction of Synthetic Glycolipid Recognition by Human NK T Cells. In mice, two CD1 genes encode extremely homologous proteins, both of which are most

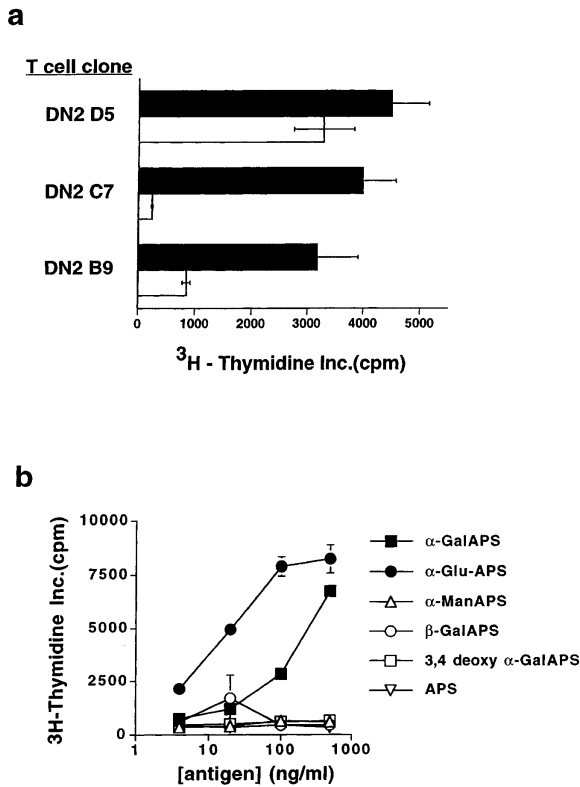


Figure 1. Human NK T cell clones specifically respond to synthetic glycolipid antigens. (a) Proliferative responses of three different human NK T cell clones to CD1d-transfected HeLa cell APCs were significantly augmented by addition of the synthetic glycolipid α GalAPS. White bars show proliferation in response to CD1d⁺ HeLa cells plus vehicle (DMSO) alone; black bars show proliferation in response to CD1d⁺ HeLa cells plus 100 ng/ml α GalAPS. Results are shown as the mean and one standard deviation of triplicate values in this and all subsequent figures. (b) Proliferative responses of human NK T cell clone DN2.C7 to a panel of structurally related synthetic glycolipid antigens. APS is the non-glycosylated acyl-phytosphingosine lipid. α GalAPS, α GluAPS, and α ManAPS indicate compounds in which α -anomeric galactose, glucose, and mannose, respectively are conjugated at position 1 of the hexose ring to the APS moiety. 3,4-deoxy α GalAPS is identical to α GalAPS except for the absence of the hydroxyl groups on carbons 3 and 4 of the phytosphingosine base. For complete structures of the synthetic glycolipids, see Kawano et al. (18). Thymidine incorporation in the absence of synthetic glycolipid antigen was 500 cpm in the experiment shown.

closely related to human CD1d. In contrast, humans have a more diversified family of CD1 proteins, with genes encoding five distinct isoforms designated CD1a, -b, -c, -d, and -e (24). Previous work has shown that the human CD1d protein is a target for direct recognition by human NK T cell clones (4), whereas CD1a, -b, and -c present lipid and glycolipid antigens to other populations of T cells (15–17, 21). To determine if the human NK T cell response to α -glycosyl-APS was restricted only by the CD1d protein, or if other human CD1 isoforms could also support this response, we tested the ability of HeLa cells stably transfected with each of the human CD1 proteins to present α GalAPS. For all three human NK T cell clones tested, the response to α GalAPS was seen only when CD1d⁺ HeLa cells were used as APCs (Fig. 2 a and data

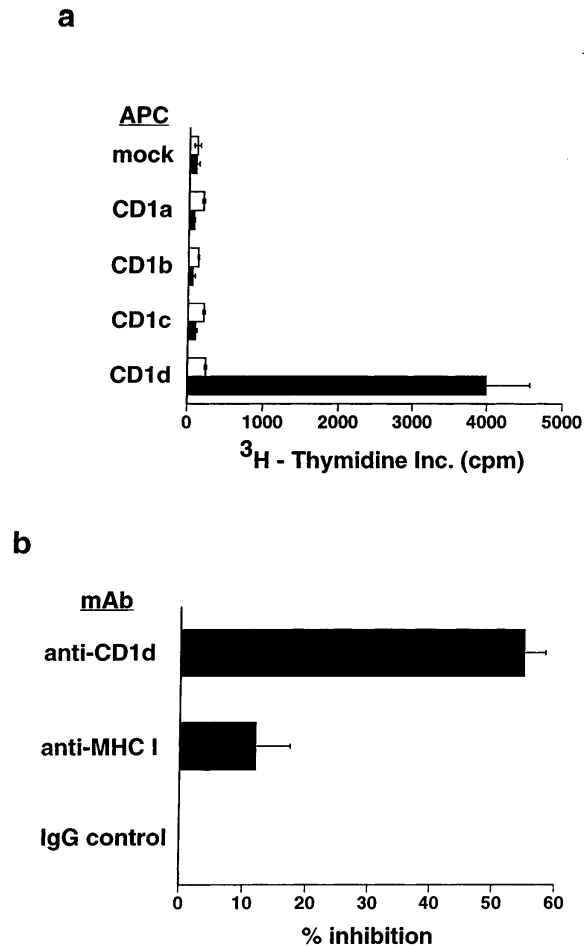


Figure 2. CD1d dependence of synthetic glycolipid antigen recognition by human NK T cells. (a) Response of human NK T cell clone DN2.C7 to α GalAPS (100 ng/ml) in the presence of HeLa cells not expressing CD1 (transfected with vector alone; *mock*) or expressing each of the known protein isoforms of human CD1. T cell proliferation in the absence of α GalAPS is shown by the white bars, and in the presence of α GalAPS by the black bars. Identical results were obtained with two other human NK T cell clones, DN2.B9 and DN2.D5 (data not shown). (b) Inhibition of α GalAPS-stimulated proliferation of human NK T cell clone DN2.B9 by anti-CD1d mAb. Results are shown as the percentage of inhibition of the α GalAPS-dependent proliferation in the presence of CD1d⁺ HeLa cells. With no antibody present, proliferation in the presence of the α GalAPS was 3,900 cpm, and in the absence of α GalAPS was <200 cpm in the experiment shown. Marginal blocking was observed with anti-MHC class I mAb W6/32 in some experiments. This was also seen in parallel experiments using MHC class II-restricted T cell clones (data not shown), and thus most likely represents a nonspecific effect of this antibody.

not shown). Confirmation of this CD1d-restriction was obtained by mAb blocking experiments, which demonstrated that an mAb against the cell surface expressed conformation of CD1d protein (CD1d51) significantly inhibited the response to α GalAPS (Fig. 2 b). Thus, responses of human NK T cells to glycosyl-APS derivatives were dependent on APC expression of CD1, and CD1d was the only human CD1 isoform that presented these glycolipids to NK T cells.

Requirements for Uptake and Endosomal Loading of Synthetic Glycolipid Antigens. To determine if antigen internalization by CD1d⁺ APCs was required for presentation, we examined the effects of blocking plasma membrane trafficking by light chemical fixation of APCs. Because aldehyde fixatives caused strong augmentation of the autoreactivity of human NK T cells to CD1d⁺ APCs (4), we instead used the chemical cross-linker ECDI as a fixative

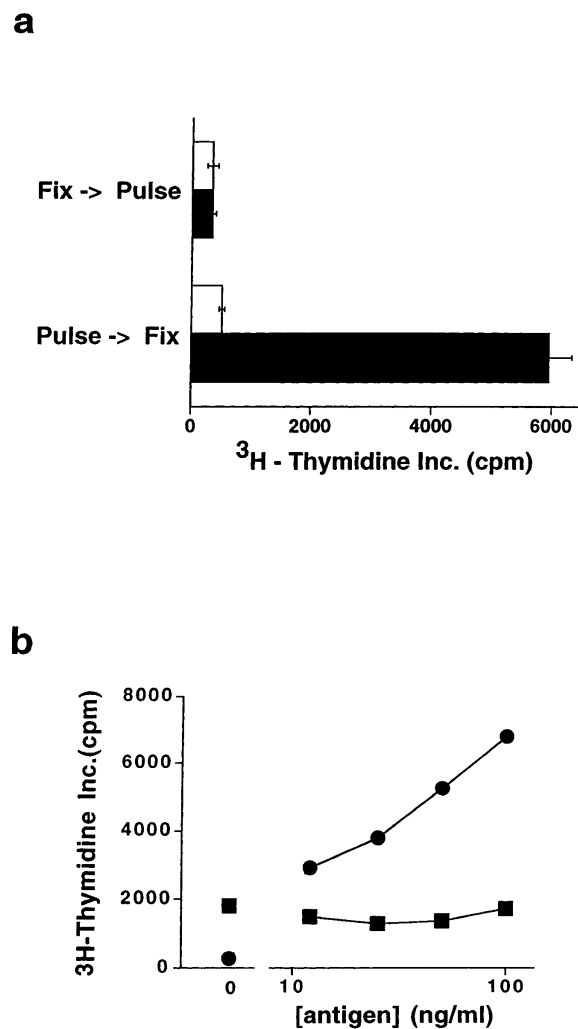


Figure 3. Requirement for antigen uptake and endosomal delivery of CD1d in recognition of synthetic glycolipids by human NK T cells. (a) Proliferative responses of clone DN2.C7 to ECDI-fixed APCs. CD1d⁺ HeLa cells either were pulsed for 12 h with α GalAPS (100 ng/ml) and subsequently fixed by ECDI treatment, or were fixed first followed by α GalAPS pulsing. White bars represent APCs pulsed with vehicle (DMSO) alone, and black bars represent APCs pulsed with α GalAPS. (b) Proliferation of clone DN2.C7 to α GalAPS presented by HeLa cell transfectants expressing wild-type CD1d (circles) versus HeLa transfectants expressing the CD1d/a chimera protein that lacks an endosomal targeting signal (squares). Both transfectants expressed comparable levels of immunoreactive CD1d on the cell surface. Proliferation in the absence of APCs was <200 cpm in this experiment. Note that DN2.C7 showed a weak but significant response to the CD1d/a transfectant in the absence of added antigen, but no augmentation of this response at any concentration of α GalAPS tested.

(25). CD1d⁺ HeLa cells were surface cross-linked by ECDI either before or after a 12-h pulse with α GalAPS (100 ng/ml), and then tested for their ability to stimulate proliferation of clone DN2.C7. Whereas APCs treated with ECDI after the antigen pulse stimulated strong proliferation of DN2.C7, fixation with ECDI before antigen pulsing completely abrogated the response to α GalAPS (Fig. 3 a). This strongly suggested a requirement for antigen uptake by APCs in the response to this synthetic glycolipid.

Consistent with this requirement for antigen uptake, studies of α GalAPS presentation to murine NK T cells have demonstrated that this process is inhibited by chloroquine, and thus may require endosomal acidification (18). To further examine this possibility, we assessed the ability of a modified form of human CD1d that is deficient in endosomal localization to present α GalAPS. Human CD1d, like CD1b and CD1c but not CD1a, has a short cytoplasmic tail containing a tyrosine-based four-amino acid motif (24). For CD1b, this motif has been shown to be critical for endosomal localization and efficient microbial lipid an-

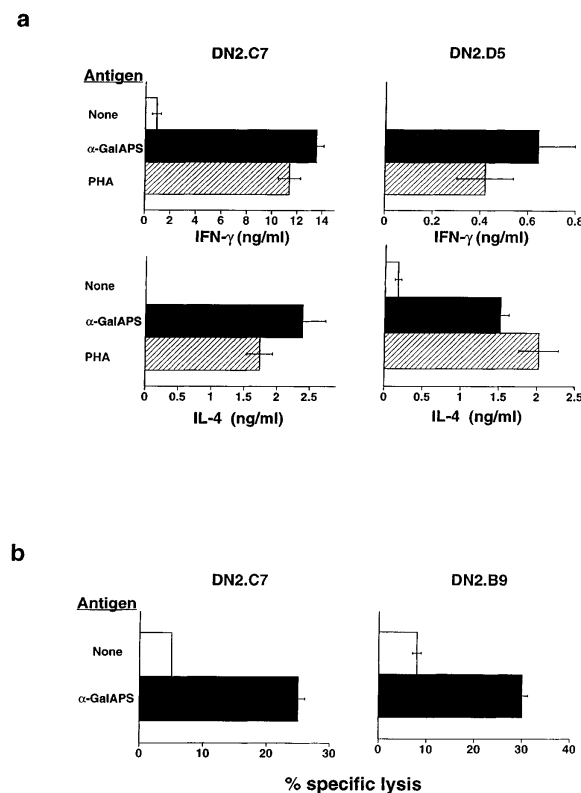


Figure 4. Activation of effector functions of human NK T cells by synthetic glycolipid antigens. (a) Stimulation of IL-4 and IFN- γ secretion by α GalAPS. Human NK T cell clones were cultured with CD1d-expressing HeLa cells in the presence (white bars) or absence (black bars) of α GalAPS (100 ng/ml) or with PHA (hatched bars), and supernatants were harvested after 48 h and assayed for cytokines by ELISA. No cytokine production was detected in cultures containing T cells only or CD1d-transfected HeLa cells only (data not shown). (b) Human NK T cell clones specifically lysed CD1d-transfected HeLa cells pulsed for 12 h with α GalAPS (200 ng/ml). No lysis was observed using mock-transfected HeLa cells as targets (data not shown).

tigen presentation by this molecule (20). Previously, we generated a chimeric form of the human CD1d protein in which the CD1d ectodomains are fused to transmembrane and cytoplasmic domains of CD1a, eliminating the endosomal targeting signal normally present in CD1d. Previous studies with this construct expressed in B lymphoblastoid cells showed that the chimeric CD1d/a protein was defective in endosomal localization, but was well expressed on the cell surface (4). To assess the importance of endosomal delivery of CD1d, we examined the dose response of clone DN2.C7 to α GalAPS presented by HeLa cells expressing either wild-type CD1d or the CD1d/a chimera. This revealed a striking effect of the deletion of the targeting motif, essentially abolishing the ability of CD1d/a to present α GalAPS (Fig. 3 b). This finding, along with the requirement for antigen internalization by the APCs, strongly suggested that synthetic glycolipid antigens colocalize with CD1d in the endocytic system, and that this is required for the generation of a complex that is recognized by NK T cells on the APC surface.

Functional Consequences of CD1d-restricted Presentation of Synthetic Glycolipids. A hallmark of both murine and human NK T cells has been their ability to rapidly produce cytokines associated with both Th1 (IFN- γ) and Th2 (IL-4) responses upon TCR engagement (1, 4, 11). Interestingly, however, it has been noted that several conditions exist in which this apparent Th0 profile of cytokine production may be skewed either toward a predominance of IFN- γ (11, 26) or of IL-4 (14, 27). We found that stimulation with α GalAPS generated substantial levels of both IFN- γ and IL-4 by the three human NK T cell clones tested (Fig. 4 a, and data not shown). Some variation between clones in the relative amounts of IFN- γ and IL-4

production was observed, but in all cases the cytokine production mirrored that observed with activation by PHA. Human NK T cell clones also showed enhanced cytotoxic activity against CD1d⁺ HeLa cells pulsed with α GalAPS (Fig. 4 b). Thus, CD1d-restricted recognition of α GalAPS augmented the known effector functions of human NK T cell clones, and preserved their Th0 pattern of cytokine secretion.

Our results demonstrated CD1d-restricted recognition of synthetic glycosyl-APS by human NK T cells, and confirmed the striking conservation of this T cell subset between humans and mice in both phenotype and function. The presentation of synthetic glycolipids by CD1d also supports the growing body of data demonstrating that the main function of CD1 proteins may be the presentation of foreign microbial lipid and glycolipid antigens (15–17, 21). In spite of this substantial progress, the main immunological functions of NK T cells remain unclear. The synthetic glycolipids used in this study are not yet known to have precise counterparts in relevant infectious agents, and the prototype natural compounds in this family were originally isolated not from microbial pathogens but from marine sponges (18). Nevertheless, given the impressive antitumor effects of NK T cells and a variety of studies indicating that they may play a role in regulating autoimmune inflammatory diseases, the discovery of exogenous ligands that can modulate the activity of this unique T cell subset is of great potential importance. Our results demonstrating that synthetic glycosyl-APS derivatives are active modulators of the human NK T cell system suggests that these compounds may be promising candidates for immunotherapy of human diseases.

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