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Invariant Natural Killer T cells are not affected by lysosomal storage in patients with Niemann-Pick disease type C

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Summary

Invariant Natural Killer T (iNKT) cells are a specialised subset of T cells that are restricted to the MHC class I like molecule, CD1d. The ligands for iNKT cells are lipids, with the canonical superagonist being α -galactosylceramide, a non-mammalian glycosphingolipid. Trafficking of CD1d through the lysosome is required for the development of murine iNKT cells. Niemann-Pick type C (NPC) disease is a lysosomal storage disorder caused by dysfunction in either of two lysosomal proteins, NPC1 or NPC2, resulting in the storage of multiple lipids, including glycosphingolipids. In the NPC1 mouse model iNKT cells are virtually undetectable, which is likely due to the inability of CD1d to be loaded with the selecting ligand due to defective lysosomal function and/or CD1d trafficking. However, in this study we have found that in NPC1 patients iNKT cells are present in normal frequencies phenotype and functional response to stimulation. In addition, antigen-presenting cells derived from NPC1 patients are functionally competent to present several different CD1d/iNKT cell ligands. This further supports the hypothesis that there are different trafficking requirements for the development of murine and human iNKT cells and a functional lysosomal/late-endosomal compartment is not required for human iNKT cell development.

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Keywords

Antigen processing/presentation; CD1 molecules; NKT cell

Introduction

Invariant Natural Killer T (iNKT) cells are defined by their invariant T cell receptor and restriction to the MHC class I like molecule, CD1d. iNKT cells express multiple markers associated with Natural Killer cells and have the ability to rapidly release both T_H1 (e.g. IFN- γ) and T_H2 (e.g. IL-4) cytokines after engagement acting as bridge between innate and adaptive immunity [1]. iNKT cells play important roles in host protection against pathogens, cancer and auto-immunity. iNKT cells are lipid-reactive, with the canonical superagonist being α -galactosylceramide (α -GalCer) a non-mammalian glycosphingolipid. Mammalian glycosphingolipids (GD3 and iGb3), mammalian phospholipids, and pathogen-derived glycolipids (α -galactosyl diacylglycerol, α -glycuronsyl ceramides) have also been shown to activate iNKT cells [2]. iNKT cells develop in the thymus, where they undergo a process of positive selection with double positive thymocytes presenting selecting ligand(s) on CD1d [3].

Rodents only have one member of the CD1 family, CD1d, whereas humans have five members, CD1a to e [4] that have differential intracellular trafficking patterns [5]. Murine CD1d exhibits a broad intracellular trafficking pattern, transiting the early and late endosomes, and also the lysosome, which is necessary for successful thymic selection [6, 7]. In addition functional lysosomes are required for the presentation of activating ligands to murine iNKT cells [8]. In contrast, lysosomal trafficking in human antigen presenting cells does not appear to be required to present self-derived iNKT cell stimulating ligands but may be required for exogenous ligands [9].

Lysosomal storage disorders result from inherited defects in lysosomal proteins [10]. These disorders can be caused either by a primary defect in a catabolic enzyme (e.g. Tay-Sachs and Sandhoff disease) or a defect in a transporter, channel or regulatory protein (e.g. NPC1 disease). Lysosomal storage caused by a deficient lysosomal enzyme has been shown to lead to reduced iNKT cells in murine models of Sandhoff disease [11, 12], Tay-Sachs disease [11], GM1 gangliosidosis [11–13] and Fabry disease [14, 15]. In the NPC1 mouse the numbers of iNKT cells are greatly reduced but this is associated with impaired late-endosome/lysosome fusion in addition to the lysosomal lipid storage [11, 16]. NPC disease can be caused by mutations in one of two genes *NPC1* or *NPC2* [17]. Dysfunction of the NPC1 protein leads to decreased lysosomal calcium content which accounts for the failure of endocytic vesicle fusion and the complex pattern of lipid storage observed [18].

With the differential trafficking of murine and human CD1d for iNKT cell ligand presentation *ex vivo* and the requirement of normal lysosomal CD1d trafficking/function for murine iNKT cell development *in vivo* we reasoned that examining iNKT cells in NPC patients would reveal whether the findings in the murine model extends to humans. It has been reported that iNKT cells are present at normal frequencies in the peripheral blood of Fabry disease patients [19] and are slightly increased in Gaucher disease patients [20]. Here, we have studied iNKT cell frequencies and functional responses in NPC1 disease patients and the ability of patient-derived EBV-B cell lines to stimulate iNKT cells. In contrast to the murine model of NPC1, we found unchanged iNKT cell frequencies in NPC1 patients. In addition, the functional response of NPC1 iNKT cells to stimulation was normal as was the ability of NPC1 antigen presenting cells to present a variety of iNKT cells ligands to control iNKT cells.

Results and discussion

Human NPC1 patients do not have an alteration in iNKT cell number or phenotype

We analysed the frequency of iNKT cells in the peripheral blood of controls, NPC1 patients and NPC1 heterozygote carriers by flow cytometry (gating strategy in supplementary figure 1). As previously reported, the frequencies of iNKT cells are very low in normal human peripheral blood, typically in the range of 0.1 to 1% of total T cells (Fig. 1, [21]). In contrast to the NPC1 mouse where iNKT cells are undetectable, iNKT cells could be identified and were present at normal frequencies in the peripheral blood of NPC1 patients and heterozygotes (Fig. 1). This indicates that fusion of late-endosomes and lysosomes is not required for the generation, delivery or loading of iNKT cell selecting ligand(s) in the thymus or for their maintenance in the periphery. The percentage of iNKT cells expressing the NK cell marker, CD161, was determined and no difference between the groups was observed (Fig. 1). In addition the CD4 and CD8 status of the iNKT cells was checked and there was no difference between the groups (Fig. 1).

Lysosomal storage does not cause increased surface CD1d expression

It has previously been reported that the expression of CD1d on peripheral blood monocytes is increased in Gaucher disease and this was suggested to be due to lysosomal glycosphingolipid storage [20]. We analysed the expression of CD1d on monocytes (CD14⁺) and B cells (CD19⁺) and found no differences between the groups (Fig. 2 and gating strategy supplementary figure 2) suggesting that in NPC1 patients and heterozygote carriers there is no alteration in cell surface CD1d expression.

NPC1 iNKT cells and antigen presenting cells are functionally competent

In order to test the function of iNKT cells derived from NPC1 patients we generated iNKT cell lines from three patients that were co-cultured with human CD1d expressing THP1 cells that had been pulsed with three different exogenous antigen or treated with the TLR 7/8 ligand R848 [22]. The response of the iNKT cells was determined by measuring interferon gamma (IFN- γ), interleukin 4 (IL-4) and granulocyte monocyte colony stimulating factor (GM-CSF) production in the supernatant. The three NPC1 iNKT cell lines responded to both exogenous and endogenous ligands and produced comparable levels of the cytokines (Fig. 3A).

Finally, we investigated the ability of antigen presenting cells derived from NPC1 patients and heterozygotes to stimulate iNKT cell lines by generating EBV transformed B cell lines. Once established these B cell lines down-regulated endogenous CD1d, and we therefore transduced them with a lentiviral human or mouse CD1d construct before use in antigen presentation assays. Expression of human or mouse CD1d was comparable between NPC1 and heterozygote EBV-B cell lines but slightly lower than that of C1R, an EBV-B cell line used as a control (supplementary figure 3). Using the intensity of LysoTracker green staining, which accumulates in acidic intracellular vesicles, as a measure of lysosomal storage, we confirmed that the enhanced lysosomal storage characteristic of NPC1 peripheral blood B cells [23] was retained in the NPC1 EBV-B cell lines (Fig. 3D).

We used three different iNKT cell ligands that have been reported to require different conditions for loading onto CD1d. α GalCer loading has been reported to require access to a functional lysosomal compartment [9], Gal(α 1-2)GalCer requires cleavage of the terminal galactose residue by lysosomal α -galactosidase before it can stimulate iNKT cells [15] and C20:2 can be loaded at the cell surface [24]. We found all three iNKT cell ligands could be presented by the NPC1 and heterozygote EBV-B cell lines transduced with human CD1d and resulted in similar or greater iNKT cell activation compared to control C1R cells as

determined by IFN- γ in the supernatant (Fig. 3B). This may be due to the fact that the block of lysosomal human CD1d trafficking or function is not complete in these B cell lines and/or that some α -galactosidase activity may reside in the late endosome. In contrast, when transduced with mouse CD1d presentation of α GalCer and C20:2 was not affected but the two NPC1 lines with the greatest lysosomal storage as defined by LysoTracker green (Fig. 3D) staining exhibited a significant defect in Gal(α 1–2)GalCer presentation (Fig. 3C) compared to the other NPC1 EBV-B cell line.

Concluding remarks

Our study demonstrates that lysosomal dysfunction does not alter iNKT cell frequencies in the blood of NPC1 patients, implying that this compartment is not required for the generation or loading of iNKT cell selecting ligand in the human thymus. This is consistent with studies using *in vitro* models of human CD1d auto-antigen presentation [9] suggesting that loading occurs in the early endosomes. This is in contrast to the murine model of NPC1 in which peripheral iNKT cells are virtually undetectable, most likely because of impaired selection in the thymus [6, 7]. Antigen presentation by human B cell lines generated from NPC1 patients and heterozygotes and transfected with human CD1d demonstrated normal presentation of three different exogenous antigens, particularly Gal(α 1–2)GalCer [9], which needs the terminal sugar to be cleaved before it is recognised by iNKT cells [15], indicating that unimpaired lysosomal trafficking and/or function is not essential for human CD1d ligand loading. In contrast, and in agreement with the reported requirement for normal lysosomal trafficking/function for murine CD1d ligand loading, the two NPC1 B cell lines that exhibited the greatest lysosomal storage had reduced capacity to stimulate iNKT cells when pulsed with Gal(α 1–2)GalCer. The differences between the murine model of NPC1 and human patients may have wider implications for the validity of using mouse models to define human iNKT cell selecting ligands.

Materials and methods

Human blood samples

Venous blood was collected in EDTA tubes and maintained at room temperature for a maximum of 60 hours prior to cell separation. Control samples were obtained after informed consent/assent and ethical approval from centres in the UK or USA. NPC1 patient samples were obtained from patients from centres in the UK, USA and Germany with informed consent or assent. Heterozygote samples were obtained with informed consent/assent from the parents of affected patients or known carrier siblings.

Cell preparation

Peripheral blood was loaded onto an equal volume of Histopaque 1077 (Sigma-Aldrich) and spun at $400 \times g$ for 30 min at room temperature. The mononuclear cell layer was isolated and washed twice with Dulbecco's phosphate buffered saline (D-PBS), counted and viability determined using Trypan blue.

Cell staining

Antibodies were used according to the manufacturers instructions and the following clones were used CD14 APC-H7 (M Φ P9), CD1d PE (CD1d42), CD19 PE-CyTM7 (SJ25C1), CD161 APC (DX12), CD3 Pacific BlueTM (UCHT1), CD4 APC-H7 (SK3), CD8a PerCP-CyTM5.5 (SK1) Invariant NK T cell PE (6B11) and CD45 FITC (2D1) (all from BD Biosciences). Mononuclear cells (1 million) were stained with antibody cocktails diluted in FACS buffer (BD CellWASHTM supplemented with 2% fetal bovine serum and 5% mouse serum) on ice for 30 min. For iNKT cell identification 3 to 5 million mononuclear cells were

stained. Human CD1d monomers loaded with α GalCer were prepared as previously described [25], with staining performed according to standard protocols with the inclusion of a viability stain (Live/Dead® fixable aqua dye Invitrogen). Cells were washed twice with FACS buffer before being resuspension in BD FACS™ lysing solution. Isotype control antibodies in combination with fluorescence minus one staining protocols were used to establish gating with strategies shown in supplementary figures 1 and 2.

Flow Cytometry

Samples were acquired on a 3 laser BD FACS Canto™ II flow cytometer using BD FACSDiva™ software version 6.1 collecting a minimum of 10,000 gated B cells or monocytes for CD1d expression or a minimum of 1,000,000 viable lymphocytes for iNKT cell frequencies. Data were analysed using FlowJo software v8.6 (TreeStar Inc).

Preparation of iNKT cell lines

B cell depleted mononuclear cells (B cells were collected for biochemical monitoring as part of a biomarker study) were washed 3 times in complete medium (RPMI 1640 containing 5% human AB serum (Sigma-Aldrich, Poole, UK), 10 μ M beta-mercaptoethanol, 20 μ g/ml gentamycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 2 mM glutamine (all media from Invitrogen Paisley, UK)). The cells were counted and plated at 3 to 5 million cells in 2 ml complete medium with 100 ng/ml α GalCer. On day 4 recombinant human IL-2 was added at 20 U/ml (Peprotech EC, London, UK) and on day 6 IL-2 was increased to 500 U/ml. Cells were checked everyday and split as required with fresh medium containing 500 U/ml IL-2 added every 2 days. Once expanded iNKT cell frequency was checked by FACS using human CD1d/ α GalCer tetramers and anti-CD3 as described above. When sufficient numbers of iNKT cells were present in the cultures iNKT cells were sorted by staining with anti-CD3 antibody and CD1d/ α GalCer tetramers using a MoFlo sorter (Dako Cytomation). The purified iNKT cells were then restimulated and expanded with 1 μ g/ml PHA-P (Sigma-Aldrich) and irradiated feeder cells according to standard procedures. Purity of the cell lines was confirmed by FACS staining before use in stimulation assays and was greater than 98% tetramer positive. NPC1 genotypes of the donors used for the generation of the lines are line A not found, line B 3182T>C and 3562G>T and line C I1061T, I1094T.

Generation of EBV transformed B cell lines

Total blood lymphocytes (1 to 3 million cells) were washed twice with RPMI 1640 medium with 20 μ g/ml gentamycin and then resuspended in 1 ml EBV containing supernatant in a T25 flask. After 24 hours 9 ml of RPMI 1640 containing 15 % foetal bovine serum (Biosera UK), 2 mM glutamine and 2 μ g/ml cyclosporin A (New England Biolabs) was added and the cells were passaged as required once the transformed B cells started to grow out. As transformed B cells downregulate surface CD1d, the cells were transduced with a lentiviral construct encoding cyan fluorescent protein tagged to human or untagged mouse CD1d. After transduction, CD1d expression and lysosomal storage (using the fluorescent dye LysoTracker® green DND-26 (Invitrogen), 200 nM in D-PBS for 10 min at room temperature) was assessed by FACS staining and EBV-B cell lines were sorted for CD1d positive cells using a MoFlo sorter. NPC1 genotypes of the donors used for the generation of the lines are NPC1 1920delG, IVS9-1009G>A and data unavailable and for NPC1 heterozygote 1920delG and data unavailable.

iNKT cell stimulation

NPC1 patient derived iNKT cell lines were used at least 14 days after restimulation. Antigen presenting cells (human CD1d cherry lentiviral transfected THP1 cells) were left untreated,

pulsed with α GalCer (100 ng/ml), Gal(α 1–2)GalCer (150 ng/ml) or C20:2 (15 ng/ml) or matured with the Toll like receptor 7/8 agonist R848 (5 μ g/ml Invivogen). THP1 cells were co-cultured with iNKT cells at a 2:1 THP1 to iNKT cell ratio in 96 U bottom wells and supernatant was harvested after 36 hours. IFN- γ (MabTech), IL-4 (BD Pharmingen) and GM-CSF (eBioscience) levels in the supernatant were measured by ELISA according to manufacturers protocols.

Antigen presentation function

NPC1 patient or NPC1 heterozygote human or mouse CD1d lentiviral transduced EBV transformed B cell lines were left untreated or pulsed with α GalCer (50 ng/ml), Gal(α 1–2)GalCer (150 ng/ml) or C20:2 (15 ng/ml) before being used as antigen presenting cells in iNKT cell stimulation assays as described above using iNKT cells prepared from a healthy donor. As we were unable to transduce control blood due to the donors working within the department the control B cell line C1R was transfected with human CD1d cyan fluorescent protein and used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(iNKT cell)	Invariant Natural Killer T cell
(NPC)	Niemann Pick-type C disease

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Figure 1. Frequencies and phenotype of iNKT cells from NPC1 patients and controls
 (A) iNKT cell frequencies, expressed as the percent of viable T cells, was determined by CD1d- α GalCer tetramers and antibody (6B11) staining. For each sample at least one determination of frequency by tetramer staining and at least one by 6B11 antibody was performed and averaged. (B) Percentage of CD161⁺ iNKT cells was determined only on samples that had greater than 500 iNKT cells. (C) Percentage of CD4⁺, CD8⁺ or CD4⁻CD8⁻ iNKT cells were determined on samples which had greater than 500 iNKT cells. Gating strategy is shown in supplementary figure 1 and each symbol represents an individual donor.

Figure 2. Cell surface expression of CD1d on blood monocytes and B cells from NPC1 patients, NPC1 heterozygotes and controls

Intensity of CD1d staining on peripheral blood monocytes (A, upper panel) and B cells (B, lower panel) was determined. Shown is the mean fluorescence intensity (MFI) of CD1d after gating on the appropriate cell subset, each symbol represents an individual donor. Gating strategy is shown in supplementary figure 2.

Figure 3. Functional response of NPC1 iNKT cell lines and NPC1 and NPC1 heterozygote derived antigen-presenting cells

(A) IFN- γ , IL-4 and GM-CSF produced by iNKT cell lines derived from 3 different NPC1 patients in response to exogenous ligands and endogenous ligands (induced by R848) presented by THP1 cells. Data is mean $\pm$ SEM, n=2, representative of 2 independent experiments.

(B) IFN- γ produced by a control iNKT cell line in response to 3 different exogenous ligands (α GalCer at 50 ng/ml, Gal(α 1-2)GalCer at 150 ng/ml and C20:2 at 15 ng/ml) presented by EBV-B cell lines derived from NPC1 patients, NPC1 heterozygotes or control C1R cells transfected with human CD1d. Data is mean $\pm$ SEM, n=2, representative of 2 independent experiments..

(C) IFN- γ produced by a control iNKT cell lines in response to 3 different exogenous ligands (α GalCer at 50 ng/ml, Gal(α 1-2)GalCer at 150 ng/ml and C20:2 at 15 ng/ml) presented by EBV-B cell lines derived from NPC1 patients and NPC1 heterozygotes transfected with mouse CD1d. Data is mean $\pm$ SEM, n=2, representative of 2 independent experiments.. Statistical significance was tested by a one way ANOVA was performed with a Tukey post test using Prism v4 (GraphPad Software Inc, La Jolla, CA, USA), *P<0.05 and **P<0.01

(D) Representative histograms of LysoTracker green staining on EBV-B cell lines; cells were identified on the basis of size (FSC vs SSC) and the MFI value is indicated, from three independent experiments.