nature immunology

Peroxisome-derived lipids are self antigens that stimulate invariant natural killer T cells in the thymus

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The development and maturation of semi-invariant natural killer T cells (*i*NKT cells) rely on the recognition of self antigens presented by CD1d restriction molecules in thymus. The nature of the stimulatory thymic self lipids remains elusive. We isolated lipids from thymocytes and found that ether-bonded mono-alkyl glycerophosphates and the precursors and degradation products of plasmalogens stimulated *i*NKT cells. Synthetic analogs showed high potency in activating thymic and peripheral *i*NKT cells. Mice deficient in the peroxisomal enzyme glyceronephosphate *O*-acyltransferase (GNPAT), essential for the synthesis of ether lipids, had significant alteration of the thymic maturation of *i*NKT cells and fewer *i*NKT cells in both thymus and peripheral organs, which confirmed the role of ether-bonded lipids as *i*NKT cell antigens. Thus, peroxisome-derived lipids are nonredundant self antigens required for the generation of a full *i*NKT cell repertoire.

Natural killer T cells that express a semi-invariant T cell antigen receptor (TCR; *i*NKT cells) are a homogenous and polyclonal population of T cells with important immunoregulatory functions[1–3]. These T cells are selected in the thymus in a manner dependent on the antigen-presenting molecule CD1d and survive in peripheral organs via homeostatic mechanisms that, in contrast, are CD1d independent[4,5]. When activated by antigens, *i*NKT cells exert a variety of effector functions, including the secretion of cytokines, killing of target cells, B cell help and maturation of dendritic cells (DCs)[6–9]. Various foreign antigens involved in this activation have been identified, including microbial lipids[10–14], which supports the idea of an important role for *i*NKT cells during infection[15]. Stimulatory lipids have been extracted from house dust[16], and although their nature remains unknown, such lipids might be important in the pathogenesis of asthma. The stimulation of *i*NKT cells by self lipid antigens in infection, autoimmune disease, allergic responses and immunity to cancer has been suggested[2,17,18]. Self antigens also drive the selection and maturation of *i*NKT cells in the thymus and their population expansion in the periphery[2,19,20]. Several self lipids, including glycosphingolipids and phospholipids, have been suggested as potential *i*NKT cell self antigens; however, their nature and relevance in thymic selection are unknown. Among glycosphingolipids, isogloboside-3 stimulates *i*NKT cells[21], but its presence in the thymus is still under debate[22]. Indirect evidence suggests that unknown stimulatory glycosphingolipids are generated after activation of DCs induced by Toll-like receptor 9 (ref. 23) and during bacterial infection in an α-galactosidase-dependent manner[24]. By a series of indirect approaches, both of those studies[23,24] focused the attention on the possible involvement of glycosphingolipids. However, those studies neither identified which type of self glycosphingolipid is stimulatory in the tested experimental models nor provided biochemical evidence of glycosphingolipid antigenicity. Another published study has identified glucosylceramide as a self antigen that stimulates *i*NKT cells[25]. The synthesis of glucosylceramide is upregulated during infection, and antigenicity is dependent on the presence of unique alkyl chains. The involvement of glucosylceramide in thymic development remains to be investigated.

Other studies have suggested that additional types of lipids, with a non-glycosphingolipid nature, may also stimulate *i*NKT cells. Among the lipids in this group, lysophosphatidylcholine and lysosphingomyelin activate human *i*NKT cells at very high concentrations[26]. By indirect approaches, a subsequent study has also provided additional evidence, without identification of the stimulatory lipid, that non-glycosphingolipid molecules may stimulate autoreactive *i*NKT hybridoma cells[27]. Finally, antigen-independent activation of *i*NKT cells in the periphery has been shown as well[16,25]. Thus, despite the large amount of published data, the antigens responsible for the thymic maturation of *i*NKT cells remain elusive[22]. It is probable that several endogenous antigens are involved that resemble self peptides that select and expand major histocompatibility complex–restricted T cells. That possibility is in agreement with the variety of lipids associated with CD1d molecules[25,28–30] and with structural studies showing how the semi-invariant TCR of *i*NKT cells recognizes different

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Received 1 December 2011; accepted 23 January 2012; published online 18 March 2012; doi:10.1038/ni.2245

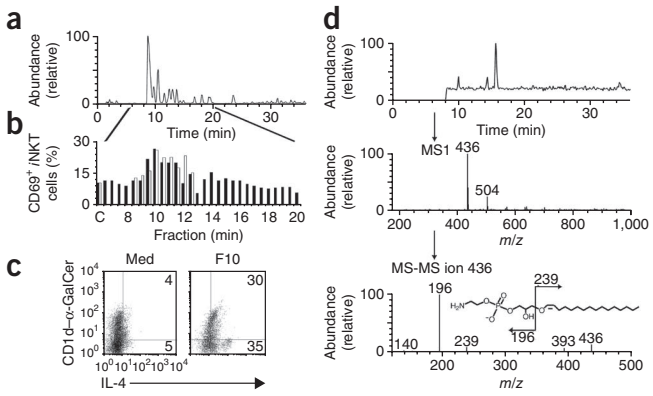

Figure 1 Identification of *i*NKT cell–stimulatory lipids extracted from mouse thymocytes. (**a**) HPLC separation of fraction G. (**b**) Frequency of *i*NKT thymocytes that upregulated CD69 after stimulation with untreated lipids (filled bars) or alkaline-treated lipids (open bars), collected every 0.5 min. (**c**) Intracellular IL-4 in wild-type *i*NKT thymocytes 36 h after stimulation with immobilized CD1d in the presence of medium (Med) or the subfraction that eluted at 10 min (F10). Numbers in quadrants indicate percent cells in each throughout. (**d**) Rechromatography of the subfraction that eluted at 10 min (top); nonfragmented mass spectrometry (MS1) of the main peak above, showing only one ion in negative ionization (mass/charge (*m/z*), 436; middle); and tandem mass spectrometry (MS-MS) of ion 436 (bottom); results are presented relative to the signal of the ion with the greatest intensity, set as 100. Inset, structure of pLPE, predicted by diagnostic ion fragments. Data are representative of four independent experiments with similar results.

antigens[31–33]. Only through the identification of the natural lipid antigens that stimulate *i*NKT cells will it be possible to understand their role during thymic selection and maturation, whether they are functionally redundant, and whether lipid antigens involved in the development of *i*NKT cells in the thymus are also important in their nonhomeostatic peripheral stimulation.

RESULTS

Identification of *i*NKT cell–stimulatory lipids in thymocytes

To identify *i*NKT cell–stimulatory ligands, we extracted lipids from mouse thymocytes, fractionated the lipids according to their polarity and used them to stimulate freshly isolated *i*NKT thymocytes in activation assays based on immobilized mouse CD1d molecules. Three fractions induced substantial upregulation of the activation marker CD69 (**Supplementary Fig. 1**), and one fraction (G) was very active. When we purified the lipids of fraction G by HPLC (**Fig. 1a**), those that eluted between 9.5 min and 11.5 min induced upregulation of CD69 (**Fig. 1b**) and secretion of interleukin 4 (IL-4) by *i*NKT thymocytes (**Fig. 1c**). Alkaline treatment of lipids that eluted between 8.5 min and 12.5 min did not affect the *i*NKT cell response (**Fig. 1b**), which indicated that the stimulatory lipids did not contain ester-bonded acyl chains. We reassessed the most active fraction (which eluted at 10 min) by HPLC; this yielded a single dominant peak (**Fig. 1d**, top). Analysis by mass spectrometry identified an ion with a mass/charge value of 436 (**Fig. 1d**, middle). Tandem mass spectrometry in the negative-ionization mode identified the 'diagnostic' ions (products whose formation provides information of their precursor) 196 and 140, indicative of a phosphoethanolamine, and an ion with a mass/charge value of 239, which suggested the presence of an alkyl chain with a vinyl ether bond (**Fig. 1d**, bottom). The spectrum was compatible with the structure of 1-*O*-1′-(Z)-hexadecenyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (C16-alkenyl-LPE; mass/charge, 436)[34]. Both the HPLC retention time and mass spectra matched those of a synthetic analog (**Supplementary Fig. 2**). We observed similar spectral features in the active fractions that eluted at 11–11.5 min (**Fig. 1a**), which demonstrated the presence of 1-O-1′,9′-(Z,Z)-octadecadienyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine, with a mass/charge value of 462 (**Supplementary Fig. 3**). Both masses were also present in the active alkaline-treated fractions with the same HPLC retention times (data not shown). Thus, we identified and isolated at least two (or a family of) *i*NKT cell–stimulatory compounds from mouse thymocytes.

Synthetic ether-bonded lipids stimulate *i*NKT cells

To confirm the stimulatory activity of the family of compounds noted above, we used synthetic ether-bonded plasmalogen C16-lysophosphatidylethanolamine (pLPE; vinyl ether at sn1, hydroxyl at sn2 and ethanolamine as headgroup at sn3), and C16-alkanyl-lysophosphatidic acid (eLPA; ether bond at sn1, hydroxyl at sn2 and phosphoric acid headgroup at sn3) to stimulate *i*NKT thymocytes and compared their results with those of synthetic ester-bonded C16-lysophosphatidylethanolamine (LPE) and C16-lysophosphatidic acid (LPA). We found that pLPE induced substantial upregulation of CD69 and secretion of IL-4 similar to that induced by α-galactosylceramide (α-GalCer), whereas LPE was inactive (**Fig. 2a**,**b** and **Supplementary Fig. 4**). Moreover, eLPA was also active, although at higher doses, whereas LPA was inactive (**Fig. 2a**,**b**). The thymocyte response to pLPE was inhibited by monoclonal antibody (mAb) to CD1d (**Fig. 2a**), which confirmed the CD1d restriction of this recognition. The proposal of the physiological relevance of pLPE was supported by the high potency of its effect on *i*NKT thymocytes (median effective dose, ~10 nM; **Fig. 2a** and **Supplementary Fig. 5**). At low nanomolar concentrations, pLPE also induced upregulation of CD69 and secretion of IL-4 by *i*NKT cells freshly isolated from the liver (**Fig. 3a**) and activated two *i*NKT cell hybridomas (**Fig. 3b** and data not shown). We found that pLPE and eLPA activated a fraction of liver *i*NKT cells, whereas LPE and LPA were inactive (**Fig. 3c**); this demonstrated the relevance of the ether bond and also indicated that these antigens stimulated both thymic and peripheral *i*NKT cells.

We also investigated the ability of pLPE to stimulate human *i*NKT cells; pLPE activated human *i*NKT cells in a dose- and CD1d-dependent

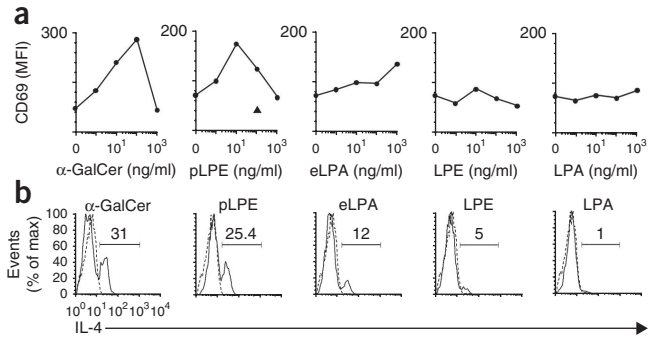

Figure 2 Synthetic mono-alkyl glycerophosphates stimulate thymic *i*NKT cells. Expression of CD69 (**a**) and intracellular IL-4 (**b**) in wild-type *i*NKT thymocytes (identified as cells with dull fluorescence of TCRβ and positive or dull fluorescence of the CD1d–α-GalCer dimer; **Supplementary Fig. 12**) stimulated with immobilized CD1d and various doses of α-GalCer and synthetic lipids (**a**; horizontal axes) or 5 ng/ml of α-GalCer and 10 ng/ml of the synthetic lipids (**b**). Triangle (**a**, second from left) shows blockade of CD69 upregulation after the addition of mAb to CD1d. Numbers above bracketed lines (**b**) indicate percent IL-4$^+$ *i*NKT thymocytes; solid lines, staining in the presence of antigen; dashed lines, staining in the absence of antigen. MFI, median fluorescence intensity. Data are representative of four independent experiments.

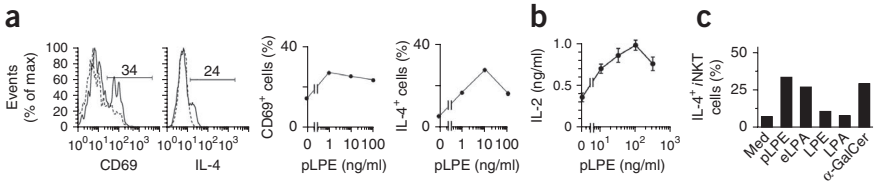

Figure 3 Synthetic mono-alkyl glycerophosphates stimulate peripheral *i*NKT cells. (**a**) Expression of CD69 and intracellular IL-4 (left) in freshly isolated liver *i*NKT cells stimulated for 36 h with immobilized CD1d and pLPE (10 ng/ml), and frequency of CD69$^+$ cells and IL-4$^+$ (intracellular IL-4) cells (right) among freshly isolated liver *i*NKT cells stimulated with various doses of pLPE. Numbers above bracketed lines (left) indicate percent CD69$^+$ or IL-4$^+$ *i*NKT thymocytes. (**b**) Activation of autoreactive *i*NKT hybridoma cells with various doses of pLPE (horizontal axis), assessed by enzyme-linked immunoassay of IL-2 at 24 h after stimulation. (**c**) Frequency of IL-4$^+$ *i*NKT cells (intracellular IL-4) freshly isolated from liver and stimulated for 36 h with immobilized CD1d and medium, lipid antigens (10 ng/ml) or α-GalCer (5 ng/ml). Data are representative of five experiments (**a**,**c**) or two experiments (**b**; error bars, s.d.).

manner to induce the release of IL-4 and granulocyte-macrophage colony-stimulating factor (**Supplementary Fig. 6a**,**b**). Six human *i*NKT cell clones that differed in their TCR β-chain complementarity-determining region 3 released these cytokines after stimulation with pLPE and DCs (**Supplementary Fig. 6d**), which suggested that several TCR β-chains may complement the invariant α-chain in the recognition of pLPE.

Fewer *i*NKT cells in mice that lack ether lipids

The synthesis of both pLPE and eLPA, but not that of LPE or LPA, is initiated in peroxisomes, and the enzyme GNPAT (glyceronephosphate *O*-acyltransferase; also known as dihydroxyacetonephosphate acyltransferase) is key to their synthesis[35] (**Supplementary Fig. 7**). *Gnpat* was expressed by mouse thymocytes (**Supplementary Fig. 8**), which indicated that these cells also produce ether-bonded lipids. To directly assess whether these lipids were necessary for the maturation and population expansion of *i*NKT cells, we studied *Gnpat*^{tm1Just} mice, which do not synthesize ether-bonded lipids and lack all types of plasmalogens[36]. These mice (called '*Gnpat*$^{-/-}$' here) have multiple abnormalities, such as male infertility, defects in eye development, cataracts and optic-nerve hypoplasia, and thus resemble human rhizomelic chondrodysplasia punctata type 2 syndrome, a peroxisomal disorder that usually results in death in early childhood[37]. Furthermore, the mice that survive develop hypomorphism, which partially resolves after 12 weeks of age.

Both the frequency and absolute number of *i*NKT cells were significantly lower in the thymus, liver and spleen of *Gnpat*$^{-/-}$ mice than in those of wild-type mice (**Fig. 4a**,**b**). To exclude the possibility of abnormalities associated with growth retardation, we also investigated mice at week 12 and found that *Gnpat*$^{-/-}$ mice continued to have substantially fewer *i*NKT cells than wild-type mice had (**Supplementary Table 1**). When we investigated *i*NKT thymocytes at each maturation stage, we found no fewer cells in *Gnpat*$^{-/-}$ mice than in wild-type mice at the very immature stage 0 (**Fig. 4c**,**d**), which excluded the possibility of intrinsically altered commitment to the *i*NKT lineage. Instead, there were fewer total *i*NKT cells in *Gnpat*$^{-/-}$ mice than in wild-type mice at other maturation stages (stages 1–3; **Fig. 4c**–**e**), which confirmed the proposal that the *Gnpat*$^{-/-}$ thymus has a smaller population of *i*NKT cells. Cells at stages 1 and 2 outnumbered those at stage 3 (the most mature stage) in *Gnpat*$^{-/-}$ mice (**Fig. 4c**–**e**), which suggested a maturation block at the later checkpoint[38] in these mice. That result was also supported by the finding of a much lower total number and frequency of *i*NKT cells that expressed CD69 in *Gnpat*$^{-/-}$ mice than in wild-type mice (**Fig. 4f**,**g**). In contrast to *i*NKT cells, other thymic and peripheral cell populations were normal in *Gnpat*$^{-/-}$ mice (**Supplementary Fig. 9**).

In *Gnpat*$^{-/-}$ *i*NKT thymocytes, the expression of CD132 (common γ-chain) and CD122 (receptor chains IL-2Rβ and IL-15Rβ), all molecules involved in the transition to stage 3 (ref. 4), was normal (**Fig. 5a**), as was the number of apoptotic cells (**Fig. 5b**). Furthermore, the abundance of proliferating *Gnpat*$^{-/-}$ *i*NKT thymocytes at stages 1, 2 and 3 was <50% the abundance of the equivalent cells in wild-type mice (**Fig. 5c**). Other thymic and peripheral cell populations were

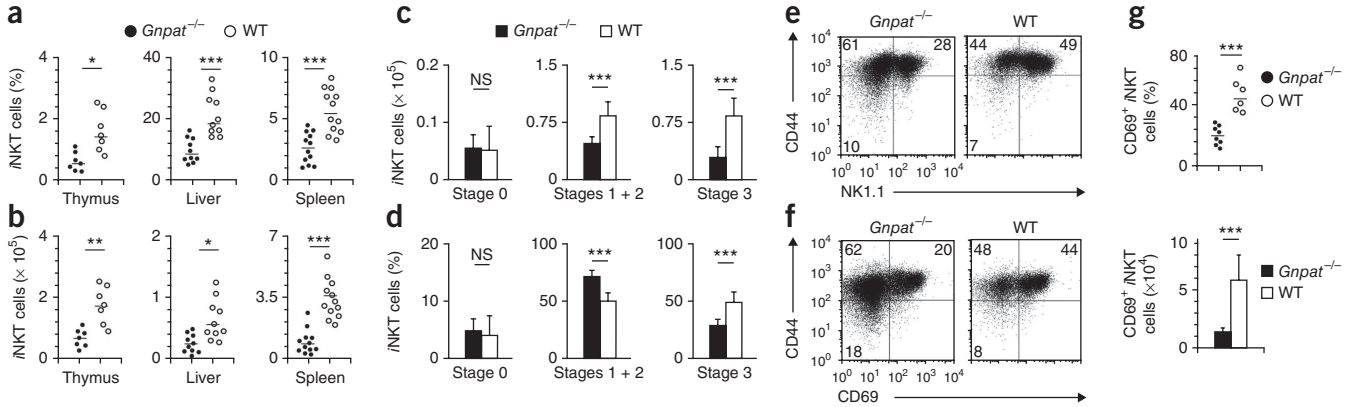

Figure 4 Fewer *i*NKT cells in *Gnpat*$^{-/-}$ mice. (**a**,**b**) Frequency (**a**) and absolute number (**b**) of *i*NKT cells in the thymus, liver and spleen of *Gnpat*$^{-/-}$ mice and their wild-type (WT) littermates. Each symbol represents an individual mouse; small horizontal bars indicate the median. (**c**,**d**) Total number (**c**) and frequency (**d**) of *i*NKT thymocytes (identified as TCRβ$^+$CD1d–α-GalCer$^+$) at stages 0–3 (according to expression of the markers CD24, CD44 and NK1.1) in *Gnpat*$^{-/-}$ mice and their wild-type littermates. (**e**) Expression of CD44 and NK1.1 on thymocytes from *Gnpat*$^{-/-}$ mice and their wild-type littermates. (**f**) Expression of CD44 and CD69 on *i*NKT thymocytes from *Gnpat*$^{-/-}$ mice and their wild-type littermates. (**g**) Frequency (top) and absolute number (bottom) of CD69$^+$ *i*NKT thymocytes. Each symbol represents an individual mouse; small horizontal bars indicate the median. NS, not significant. $^*P \le 0.05$, $^{**}P \le 0.01$ and $^{***}P \le 0.001$ (Mann-Whitney–Wilcoxon test). Data are representative of four independent experiments (**a**–**e**) or three experiments (**f**,**g**; errors bars (**c**,**d**,**g**), s.d.).

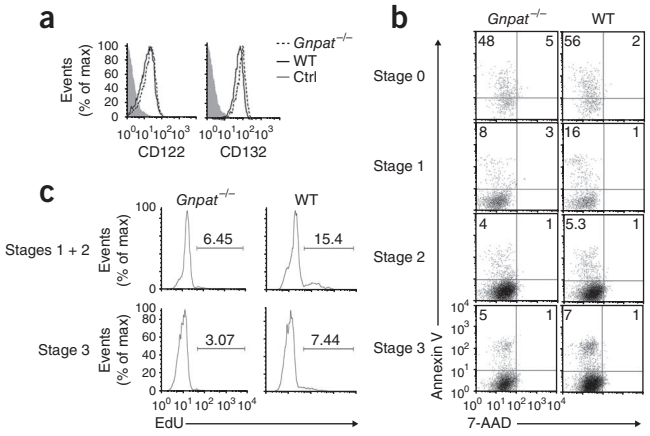

Figure 5 Analysis of *Gnpat*$^{-/-}$ *i*NKT thymocytes. (**a**) Expression of CD122 and CD132 by *Gnpat*$^{-/-}$ and wild-type *i*NKT thymocytes (identified as TCRβ^{+}CD1d–α-GalCer^{+}) stained with mAb to CD122 or mAb to CD132. Ctrl (filled histograms), staining with irrelevant isotype-matched mAb. Results are for stage 2–gated cells. Median fluorescence intensity: CD122, 15 ± 2 (*Gnpat*$^{-/-}$) or 19 ± 4 (wild-type); CD132, 56 ± 7 (*Gnpat*$^{-/-}$) or 78 ± 4 (wild-type). (**b**) Apoptosis of *i*NKT thymocytes at stages 0–3 (identified by enrichment by magnetic-activated cell sorting with CD1d–α-GalCer and by gating on selected subpopulations according to expression of CD24, CD44 and NK1.1), assessed by staining with annexin V and the membrane-impermeable DNA-intercalating dye 7-AAD. (**c**) Proliferation of *Gnpat*$^{-/-}$ and wild-type *i*NKT thymocytes (TCRβ^{+}CD1d–α-GalCer^{+}) at stages 1 and 2 (top) or 3 (bottom), assessed as incorporation of the thymidine analog EdU. Numbers above bracketed lines indicate percent proliferating cells. Data are representative of three independent experiments with similar results.

also normal and proliferated as wild-type cells did (**Supplementary Fig. 10**). These findings showed that in *Gnpat*$^{-/-}$ mice, *i*NKT thymocytes and not other thymic populations were less able to proliferate and mature. This alteration might have been due to an intrinsic low responsiveness to antigens or, more probably, to a lack of a set of endogenous ether lipid antigens.

Antigen-presenting cells and *i*NKT cells in *Gnpat*$^{-/-}$ mice

The residual *Gnpat*$^{-/-}$ peripheral *i*NKT cells functioned normally, as assessed by upregulation of CD69 after stimulation with α-GalCer and isogloboside-3 (**Fig. 6a**), which excluded the possibility of an intrinsic alteration in responsiveness to antigen stimulation. Peripheral *Gnpat*$^{-/-}$ *i*NKT cells also responded normally to bacterial antigens such as GSL-1 from *Sphingomonas paucimobilis* and to DCs treated with heat-inactivated *S. paucimobilis* (**Fig. 6a**). The surface expression of CD1d was also normal on *Gnpat*$^{-/-}$ thymocytes and DCs (**Supplementary Fig. 11**), which excluded the possibility of low CD1d expression as a possible mechanism of the lower abundance of *i*NKT cells. We investigated the presentation ability of *Gnpat*$^{-/-}$ antigen-presenting cells and found less stimulation of autoreactive *i*NKT hybridoma cells when we used both thymocytes and DCs, whereas α-GalCer was presented normally (**Fig. 6b**). Overall these findings excluded the possibility that *Gnpat*$^{-/-}$ cells had impaired antigen responsiveness, as well as less antigen-presentation capacity, and indicated a possible alteration in the repertoire of stimulatory self lipids.

Gnpat$^{-/-}$ thymocytes fail to select *i*NKT cells

Two main defects may explain the altered maturation of *i*NKT cells in GNPAT-deficient mice. The first is that *Gnpat*$^{-/-}$ thymocytes may have intrinsic defects that prevent the full maturation of *i*NKT cells. This defect could be ascribed to altered membrane lipid composition and a consequent defect in TCR signaling during thymic development. Although other thymocytes develop normally in *Gnpat*$^{-/-}$ mice, this defect might be more pronounced in developing *i*NKT cells. A second defect is that *Gnpat*$^{-/-}$ thymocytes may be less able to promote the thymic population expansion of *i*NKT cells because they lack relevant stimulatory antigens.

To determine which of those alterations applied to *Gnpat*$^{-/-}$ mice, we used two experimental approaches. In the first, we sorted *i*NKT thymocytes at stages 1 and 2 from wild-type C57BL/6 (CD45.2^{+}) mice without triggering their TCRs and injected those cells into *Gnpat*$^{-/-}$ or *Gnpat*$^{+/+}$ thymus (both expressing the CD45.1 marker). After 5 d, we killed the recipient mice and evaluated the *i*NKT cell maturation stage and CD69 expression of the injected wild-type cells (**Fig. 7a**,**b**).

We observed the following significant difference: in *Gnpat*$^{+/+}$ thymus, 96% of the donor cells had matured to stage 3 (**Fig. 7a**) and 91% of wild-type *i*NKT cells were CD69^{+} (**Fig. 7b**), whereas in *Gnpat*$^{-/-}$ thymi, the frequency of those donor cells was significantly lower (72% had matured to stage 3 and 73% were CD69^{+}; **Fig. 7a**,**b**). These findings showed that *Gnpat*$^{-/-}$ thymocytes had an impaired ability to activate and promote the full maturation of wild-type *i*NKT thymocytes.

In a second set of experiments, we investigated two groups of chimeric mice. The first group consisted of C57BL/6 mice reconstituted with a mixture of bone marrow cells from *Gnpat*$^{-/-}$ and C57BL/6 mice or a mixture from their GNPAT-sufficient (*Gnpat*$^{+/-}$ or *Gnpat*$^{+/+}$) littermates and C57BL/6 mice, as a control (**Fig. 7c**). In these groups, C57BL/6 wild-type thymocytes drove the proper positive selection of other cells. *Gnpat*$^{-/-}$ precursors matured normally, like GNPAT-sufficient cells, to stage 3 and reached a frequency of 80% of *i*NKT thymocytes (**Fig. 7c**), which showed that GNPAT deficiency did not intrinsically preclude the full population expansion and maturation of *i*NKT cells. The second group of chimeras was *Cd1d*$^{-/-}$ mice reconstituted with a mixture of bone marrow cells from *Cd1d*$^{-/-}$ mice and *Gnpat*$^{-/-}$ mice. In this experimental setting, CD1d^{+} *Gnpat*$^{-/-}$ thymocytes were the antigen-presenting cells that selected developing *i*NKT cells from *Cd1d*$^{-/-}$ precursors. The control groups were *Cd1d*$^{-/-}$ mice reconstituted with bone marrow cells from *Cd1d*$^{-/-}$ mice and GNPAT-sufficient littermates. We found considerably fewer *i*NKT cells at

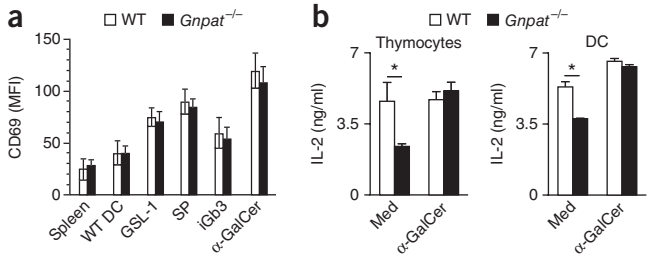

Figure 6 Normal antigen responsiveness of residual *Gnpat*$^{-/-}$ *i*NKT cells and normal antigen presentation by *Gnpat*$^{-/-}$ antigen-presenting cells. (**a**) Expression of CD69 on splenic *Gnpat*$^{-/-}$ or wild-type *i*NKT cells (gated on TCRβ^{+} CD1d–α-GalCer^{+} cells) after stimulation without exogenous antigens (Spleen) or with wild-type DCs (WT DC) or in the presence of GSL-1 (10 μg/ml), heat-inactivated *S. paucimobilis* (SP; 200:1, bacteria/DCs), isogloboside-3 (iGb3; 10 μg/ml) or α-GalCer (20 ng/ml). (**b**) Response of autoreactive *i*NKT hybridoma cells to *Gnpat*$^{-/-}$ or wild-type thymocytes (left) and to DCs (right) in the absence of exogenous antigens (Med) or in the presence of α-GalCer, assessed by enzyme-linked immunoassay of IL-2 at 24 h after stimulation. *$P \leq 0.001$ (Student's *t*-test). Data are representative of at least two experiments (**a**) or four experiments (**b**; error bars, s.d.).

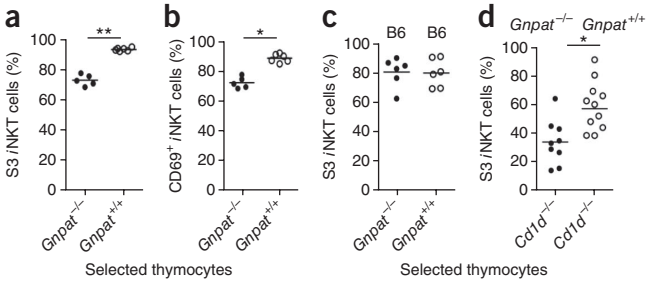

Figure 7 *Gnpat*$^{-/-}$ thymocytes inefficiently select *i*NKT cells but develop normally when selected by GNPAT-sufficient thymocytes. (**a**,**b**) Frequency of *i*NKT cells at stage 3 (S3; **a**) and of CD69^{+} GNPAT-sufficient *i*NKT cells (**b**) 5 d after injection into *Gnpat*$^{-/-}$ or *Gnpat*$^{+/+}$ thymus. (**c**) Frequency of *i*NKT cells at stage 3 in chimeric recipients of *Gnpat*$^{-/-}$ or *Gnpat*$^{+/+}$ bone marrow (identified by CD45.1 expression) plus C57BL/6 bone marrow; top, selecting thymocytes (B6, C57BL/6). (**d**) Frequency of *i*NKT cells at stage 3 in chimeric recipients of *Cd1d*$^{-/-}$ bone marrow plus *Gnpat*$^{-/-}$ or *Gnpat*$^{+/+}$ bone marrow; top, selecting thymocytes. Each symbol represents an individual mouse; small horizontal bars indicate the median. $*P \leq 0.01$ and $**P \leq 0.001$ (Mann-Whitney–Wilcoxon test). Data are representative of two independent experiments.

stage 3 among *Cd1d*$^{-/-}$ *i*NKT thymocytes selected on *Gnpat*$^{-/-}$ cells than among those selected on *Gnpat*$^{+/+}$ cells (**Fig. 7d**). Together, the data obtained by the intrathymic injection of immature *i*NKT thymocytes and with chimeric mice supported the conclusion that *Gnpat*$^{-/-}$ thymocytes did not have an intrinsic defect that prevented the full maturation of *i*NKT cells in the thymus but instead were poor *i*NKT cell–selecting cells.

DISCUSSION

The development of *i*NKT cells in the thymus occurs via a series of maturation events marked by the surface appearance of activation, adhesion and interleukin-receptor molecules[1,2,38]. Full maturation occurs after a first phase of selection, when T cells expressing the semi-invariant TCR of the NKT cell become committed to develop as *i*NKT cells. In a second phase, TCR engagement induces the population expansion and further maturation of immature *i*NKT thymocytes before they exit the thymus. Self lipids are very probably involved in the selection and maturation of thymic *i*NKT cells, as suggested by indirect evidence. The first such evidence is that CD1d is necessary for the development of *i*NKT cells[39,40]. As CD1d is associated with a variety of endogenous lipids[25,28–30], it is probable that CD1d–lipid antigen complexes, and not empty CD1d molecules, drive the selection of *i*NKT cells. Defective maturation of *i*NKT cells also results from altered presentation of lipid antigens in lipidoses characterized by lysosomal accumulation of lipids[21,41,42]. In mice with such deficiencies, the residual *i*NKT cells have an altered TCR repertoire and CD4 expression, which suggests that selecting CD1d-antigen complexes contain various types of lipid antigens[41]. Other indirect evidence indicating intralysosomal loading of CD1d with lipid molecules is that the presence of lipid-binding proteins in lysosomes and trafficking of CD1d through lysosomal compartments are necessary for the proper maturation of *i*NKT cells[15]. Finally, the accumulation of stimulatory endogenous self lipids is probably the cause of CD1d-dependent *i*NKT cell autoreactivity and recognition of neutrophils activated by serum amyloid A 1 (ref. 43), of suppressor monocytes[44], of antigen-presenting cells stimulated with type I interferon[23] and of CD1d^{+} cells infected with bacteria[11,25].

Several studies have addressed the nature of the self lipids that are able to activate peripheral *i*NKT cells[21,23–25,27,45]. These investigations suggest the existence of many self antigens. Whether these are also involved in the thymic maturation of *i*NKT cells remains to be investigated.

We set up a method to fractionate lipids according to their biophysical characteristics and evaluated their immunogenicity *in vitro*. This approach allows detection of antigenic lipids and facilitates the identification of those present in detectable quantities and with moderate to high potency. We have identified mono-alkyl glycerophosphates as an important lipid family that contributes to the maturation of *i*NKT cells. Of this family, we identified ether-bonded pLPE as active agonist. We also carefully evaluated fractions containing glycosphingolipids, but they never stimulated *i*NKT hybridomas, freshly isolated *i*NKT thymocytes or splenocytes under stringent conditions (CD1d plate-bound assays without IL-12). Our results are in agreement with a published study showing that self glycosphingolipids are not able to stimulate autoreactive *i*NKT hybridoma cells[27]. These negative findings do not formally exclude the possibility that glycosphingolipids could be important in the development of *i*NKT cells. Their stimulatory activity could have remained undetected because of low abundance, insufficient presentation or lack of IL-12. Whatever the reason, it remains unproven that they are involved in the maturation of *i*NKT cells.

The activity of pLPE was very potent, as low nanomolar concentrations induced cytokine release from freshly isolated *i*NKT cells and *i*NKT hybridoma cells. Notably, the potency of pLPE was in the same range as that of the strong agonist α-GalCer, which suggested that a few CD1d-pLPE complexes may have efficiently stimulated the mouse *i*NKT cells. We found that pLPE also stimulated human *i*NKT cells, albeit with lower potency. That weaker stimulation might be ascribed to the reported differences between human and mouse CD1d as well as those between human and mouse *i*NKT TCRs[46].

The ether bond and type of polar head present in pLPE were necessary for efficient stimulation of *i*NKT cells. Indeed, LPE, which lacks ether bonded alkyl chain, and eLPA, which has a terminal phosphate instead of phosphoethanolamine, were inactive and much less potent than pLPE, respectively. They are also structurally different from the previously identified lysophosphatidylcholine and lysosphingomyelin[26] (two peroxisome-independent lipids with one alkyl chain or a single base, respectively) that stimulate autoreactive human *i*NKT cells, and these differences are probably responsible for the very different potencies of these molecules. These structural differences might also explain why lysophosphatidylcholine does not activate mouse *i*NKT cells[25,27].

The proposal of the physiological relevance of ether-bonded lipids in the maturation of *i*NKT cells was supported by the finding of considerably fewer (>60%) *i*NKT cells in *Gnpat*$^{-/-}$ mice. GNPAT is the enzyme that initiates the synthesis of plasmalogen precursors. This enzyme is located in peroxisomes and its activity is not redundant. Hence, *Gnpat*$^{-/-}$ mice do not have ether-bonded lipids and lack plasmalogens[36]. These mice had fewer total *i*NKT thymocytes, and among the remaining cells, there was a greater frequency of immature *i*NKT cells at stages 1 and 2. *Gnpat*$^{-/-}$ *i*NKT thymocytes also underwent much less proliferation, but other thymic populations did not. Both defects indicate less population expansion of *i*NKT cells[1,2] and a block in their final maturation[5,38]. In contrast, *Gnpat*$^{-/-}$ mice had normal numbers of immature *i*NKT thymocytes at stage 0, as well as normal numbers and proliferation of other thymic cell populations.

The defect observed in *Gnpat*$^{-/-}$ mice was not dependent on altered TCR signaling, as supported by much evidence, such as the normal responsiveness of residual *Gnpat*$^{-/-}$ *i*NKT cells to lipid agonists and the ability of *Gnpat*$^{-/-}$ *i*NKT thymocytes to reach stage 3 when selected by wild-type cells, as observed in chimeric mice. The defect

cannot be ascribed to alterations in the β-chains and common γ-chains of the IL-2 receptor or of SLAM costimulatory molecules, which seemed to have normal expression (data not shown). Instead, the defect was probably a consequence of impaired stimulation by ether lipids that are the self lipids missing from *Gnpat*$^{-/-}$ mice. Both the lower expression of CD69 and inefficient stimulation of autoreactive *i*NKT hybridoma cells are in agreement with that interpretation. Two types of reconstitution experiments also supported that possibility. In the first, *Gnpat*$^{-/-}$ thymocytes seemed less efficient than *Gnpat*-sufficient thymocytes in activating and inducing the maturation of intrathymically injected wild-type immature *i*NKT thymocytes. In a second, with chimeric mice, *Gnpat*$^{-/-}$ thymocytes did not efficiently select CD1d-deficient *i*NKT cells, whereas *Gnpat*$^{-/-}$ *i*NKT cells matured normally in wild-type thymus. Thus, the maturation of *Gnpat*$^{-/-}$ *i*NKT cells was altered only in the *Gnpat*$^{-/-}$ environment and was affected by insufficient intrathymic selection.

The lower CD69 expression and diminished proliferation, total number and transition to stage 3 of *Gnpat*$^{-/-}$ *i*NKT thymocytes suggested that ether lipids are important in the population-expansion phase[1,2] and in the final maturation in the thymus[5,38]. Ether lipids may be redundant for the first *i*NKT cell checkpoint, as *i*NKT cells at stage 0 were normal in *Gnpat*$^{-/-}$ mice, and other lipids might drive positive selection and commitment to *i*NKT cell lineage, facilitated by the degenerated mode of recognition of the *i*NKT cell TCR[31–33]. We anticipate that although several weak agonists (not detected by our screening method) may positively select *i*NKT cells at the first checkpoint[38], only more potent agonists such as pLPE are able to induce the population expansion and final maturation of *i*NKT cells.

Our findings also showed that a residual population of *i*NKT cells (<40%) was able to mature in the absence of ether lipids in *Gnpat*$^{-/-}$ mice. This set of GNPAT-independent antigens were not able to promote normal development of *i*NKT cells in the thymus or their recovery in peripheral lymphoid organs, which proves the essential and nonredundant contribution of ether lipids. The identification of mono-alkyl glycerophosphates as endogenous ligands of *i*NKT cells provides new tools with which to investigate the immunological function of this T cell lineage, the rules of *i*NKT cell selection in thymus, and the mechanisms for inducing *i*NKT cell autoreactivity in the periphery.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank W. Just (University of Heidelberg) for *Gnpat*$^{-/-}$ mice; J. Mattner (Cincinnati Children's Hospital) for *S. paucimobilis*; P. Seeberger (Max Plank Institute of Colloids and Interfaces, Potsdam) for GSL-1; E. Palmer, K. Karjalainen and P. Dellabona for discussions; P. Cullen for proofreading the manuscript; and L. Angman for technical assistance. Supported by the Swiss National Foundation (3100AO-122464/1 and Sinergia CRS133-124819 to G.D.L., and Oncosuisse to L.M. and G.D.L.) and the Yunnan High-End Technology Professionals Introduction Program (2010CI117 to C.X.).

AUTHOR CONTRIBUTIONS

F.F. designed and did most of the experiments and participated in manuscript preparation; G.S.R. obtained and interpreted MS data; M.L. did experiments and participated in discussions; S.S. prepared and purified lipid extracts; M.C. and M.K. did experiments; S.F.-P. and J.B. provided genotyped *Gnpat*$^{-/-}$ mice; G.N. and C.X. provided synthetic compounds; A.C. and A.S. did experiments; L.M. supervised the work, contributed to discussions and prepared the manuscript; and G.D.L. conceived of the experiments, supervised the work and prepared the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Gnpat*^{tm1Just} (*Gnpat*$^{-/-}$) mice (provided by W. Just) were maintained at the Medical University of Vienna. *Gnpat*$^{-/-}$ mice and their wild-type littermates obtained from heterozygous matings were genotyped according to a published method[36]. Mice were analyzed between 3 and 6 weeks of age. In two experiments 12-week-old mice were investigated and deficiencies in *i*NKT cells similar to those of younger mice were observed. C57BL/6 and *Cd1d*$^{-/-}$ mice[40] were bred and kept at the animal facility of the University Hospital Basel. This study was approved by the Kantonales Veterinäramt Basel-Stadt of Basel, Switzerland.

Bone marrow chimeras. Bone marrow chimeras were set up as described[47]. Grafted bone marrow cells originated from C57BL/6, *Gnpat*$^{+/+}$, *Gnpat*$^{-/-}$ or *Cd1d*$^{-/-}$ mice. In one set of experiments, CD45.2$^+$ C57BL/6 recipients were reconstituted with CD45.2$^+$ C57BL/6 bone marrow cells in combination with CD45.1$^+$ *Gnpat*$^{+/+}$ or *Gnpat*$^{-/-}$ bone marrow cells. In a second set of studies, CD45.2$^+$ *Cd1d*$^{-/-}$ mice were reconstituted with CD45.2$^+$ *Cd1d*$^{-/-}$ bone marrow cells in combination with CD45.1$^+$ *Gnpat*$^{+/+}$ or *Gnpat*$^{-/-}$ bone marrow cells. Bone marrow was depleted of T cells by negative selection with CD90.2 MicroBeads (N. 130-049-101; Miltenyi Biotech), and 3×10^6 bone marrow cells from each donor were injected into lethally irradiated recipients (950 rads) pretreated with anti–asialo GM1 rabbit antiserum (20 µl per mouse; Wako BioProducts) intraperitoneally 2 d before transfer. Lymphoid organs of chimeric mice were analyzed for long-term reconstitution 6 weeks after transfer.

Intrathymic injection. Thymocyte samples from 10-day-old C57BL/6 mice were depleted of CD24$^+$ and CD8$^+$ thymocytes by treatment with mAb to CD24 (B2A2) and mAb to CD8 (3.168.8.1; both produced in-house) and rabbit complement (HD Supplies)[41]. CD44^{hi}NK1.1$^-$DX5$^-$CD69$^-$ cells sorted with mAb to CD44 (IM7; Biolegend), mAb to NK1.1 (PK136; Biolegend), mAb to CD49b (DX5; Biolegend) and mAb toCD69 (H1.2F3; BD Bioscience) were injected intrathymically (1×10^5 to 3×10^5 cells) into *Gnpat*$^{+/+}$ or *Gnpat*$^{-/-}$ mice. Before injection, the phenotype of the sorted cells was analyzed by flow cytometry to confirm purity, with mAb to CD44, mAb to NK1.1, mAb to TCRβ (H57/597; Biolegend) and the tetramer CD1d–α-GalCer (US National Institutes of Health Tetramer Facility). Then, 5 d after that injection, mice were killed and *i*NKT cells were analyzed by staining with CD1d–α-GalCer tetramer, mAb to TCRβ, mAb to CD44, mAb to NK1.1, mAb to CD69 and mAb to CD45.2 (104; Biolegend).

Cells. Thymocytes were prepared for analysis of *i*NKT cells according to one of two protocols. In the first, thymocyte populations were depleted of CD24$^+$ cells by treatment with mAb B2A2 (purified in-house) plus rabbit complement (HD Supplies) and a lympholyte-M gradient (Cedarlane Laboratories) as described[48]. In the second, *i*NKT thymocytes were positively sorted by autoMACS (Miltenyi Biotech) after being labeled with R-phycoerythrin–conjugated CD1d–α-GalCer dimers bound to magnetic beads coated with mAb to R-phycoerythrin (130-048-801; Miltenyi Biotech). Liver mononuclear cells were prepared as described[49]. Splenocytes and bone marrow cells were obtained by standard methods. DCs were prepared by culture of bone marrow progenitors for 8 d with mouse granulocyte-macrophage colony-stimulating factor. Mouse *i*NKT hybridomas FF13 and FF5 were derived and maintained as described[41]. Human *i*NKT cell clones BGA1, BGA84, BGA89, JS7, JS63 and VM-D5 were derived from peripheral blood of healthy donors and were characterized for their response to α-GalCer and expression of the TCR α-chain variable region 24 (V_α24) and β-chain variable region 11 (V_β11)[50].

Flow cytometry. The *i*NKT cells were identified by staining with the mouse CD1d–α-GalCer dimer as described[48]. Cells were stained with combinations of the following antibodies: mAb to TCRβ (H57/597), mAb to CD4 (RM4-5 and GK1.5), mAb to CD44 (IM7), mAb to NK1.1 (PK136), mAb to CD49b (DX5), mAb to CD24 (M1/69), mAb to CD45.1 (A20), mAb to CD45.2 (104) and mAb to CD154 (MR1; all from Biolegend); mAb to CD8α (53-6.7), mAb to CD1d (1B1), mAb to CD69 (H1.2F3), mAb to CD62L (MEL-14), mAb to CD132 (4G3), mAb to CD45R (RA3-6B2), mAb to CD11b (M1/70) and mAb to CD11c (HL3; all from BD Pharmingen); and mAb to CD122 (TM-Beta1), mAb to CD137 (17B5) and mAb to CD134 (OX86; all from eBioscience). Directly labeled mAbs were used with the following fluorochromes: fluorescein isothiocyanate, Alexa Fluor 488, R-phycoerythrin, phycoerythrin-indodicarbocyanine, phycoerythrin-indotricarbocyanine, Pacific blue, allophycocyanin or Alexa Fluor 647. Biotinylated mAbs were detected with streptavidin-allophycocyanin or streptavidin–allophycocyanin–Alexa Fluor 750 tandem (Invitrogen). Samples were analyzed by a CyAn ADP flow cytometer (DakoCytomation), with gating to exclude doublets and nonviable cells on the basis of pulse width and incorporation of propidium iodide (gating strategy, **Supplementary Fig. 13**).

The death of thymic *i*NKT cells sorted from total thymocytes by magnetic-activated cell sorting with CD1d–α-GalCer dimers and antibodies to cell surface markers (as described above) was assessed by staining with annexin V–fluorescein isothiocyanate and 7-AAD (7-amino-actinomycin D) according to the manufacturer's instructions (BD Pharmingen).

All flow cytometry data were analyzed with FlowJo software (TreeStar).

***In vivo* thymocyte proliferation.** Mice were injected intravenously with 1 mg EdU (5-ethynyl-2′-deoxyuridine) 3 h before analysis according to the manufacturer's protocol (EdU Click-iT staining kit; Invitrogen). Mice were killed and *i*NKT thymocytes were sorted with mouse CD1d–α-GalCer dimers and magnetic-activated cell sorting (as described above). After staining of additional surface markers, cells were fixed with 4% paraformaldehyde and made permeable with saponin. For analysis of DNA content, cells were incubated with RNase A and 7-AAD for 30 min before analysis with a CyAn ADP flow cytometer.

Antigen-presentation assay. For assessment of endogenous antigen, increasing numbers of DCs were incubated with the FF5 or FF13 mouse *i*NKT hybridoma cells (1×10^5 cells per well) without the addition of exogenous antigens in the presence or absence of mAb to CD1d (20 µg/ml; 1B1; eBioscience) or of irrelevant isotype-matched mAb (R35-38; BD Pharmingen) at the same concentration. Mouse IL-2 released into the supernatants was assessed by enzyme-linked immunoassay with antibody pairs from BD Pharmingen.

For exogenous antigen assessments mouse thymocytes (0.5×10^6 cells per well) or DCs (5×10^4 cells per well) were preincubated for 2 h at 37 °C with serial dilutions of sonicated antigens before the addition of mouse *i*NKT hybridoma FF5 or FF13 cells (1×10^5 cells per well). Cytokines released in the supernatants were assessed by enzyme-linked immunoassay. Recombinant CD1d loaded with antigen or not was immobilized onto plates and used to stimulate mouse *i*NKT cells. The activation of *i*NKT cells was evaluated by surface expression of CD69 (H1.2F3; BD Pharmingen) and staining of intracellular IL-4 (11B11; BD Pharmingen).

Lipids and lipid analysis. Total lipids were fractionated as described[51] with minor modifications. Lipids were separated into ten fractions on aminopropyl cartridges (Waters Corporation) with the following eluting solvents: fraction A was eluted with ethyl acetate and hexane (15:85, vol/vol); fraction B was eluted with chloroform and methanol (23:1, vol/vol); fraction C was eluted with diisopropyl ether and acetic acid (98:5, vol/vol); fraction D was eluted with acetone and methanol (9:1.35, vol/vol); fraction E was eluted with chloroform and methanol (2:1, vol/vol); fraction F was eluted with methanol; fraction G was eluted with isopropanol and 3 N HCl in methanol (4:1, vol/vol); fraction H was eluted with methanol and 3 N HCl in methanol (9:1, vol/vol); fraction I was eluted with chloroform and methanol (2:1, vol/vol); and fraction J was eluted with chloroform, methanol and 3.6 M aqueous ammonium acetate (30:60:8, vol/vol/vol). The active fractions were further analyzed by reverse-phase HPLC electrospray-ionization mass spectrometry with a Nucleodur C18 polar endcapped column (particle diameter, 3 µm; internal diameter, 3 mm; length, 125 mm; Macherey-Nagel). Mobile phase A was methanol, water and formic acid (74:25:1, vol/vol/vol) and mobile phase B was methanol and formic acid (99:1, vol/vol), both adjusted to a pH of 4.0 with ammonium formate and formic acid. Mobile phase A was 80% from time 0 to min 1 and decreased to 50% in 1 min, was maintained at 50% for 2 min and then decreased to 0% in 30 min and was maintained 20 min at 0%. Then, mobile phase A was increased to 80% and maintained for 5 min. A flow splitter was installed before the mass-spectrometer inlet for the collection

of 75% of separated lipids into individual fractions every 30 s. Data from mass spectrometry (MS_n, where the subscripted 'n' indicates the number of tandem mass spectrometry experiments) were acquired with an LXQ ion-trap mass spectrometer equipped with a heated electrospray-ionization source (Thermo). Synthetic standards of pLPE (1-O-1-(Z)-hexadecenyl-2-hydroxy-sn-glycero-3-phospho-ethanolamine), eLPA (1-O-hexadecyl-2-hydroxy-sn-glycero-3-phosphate) and LPE (1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine) were from Avanti Polar Lipid; LPA (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphate) was synthesized in-house. Alkaline hydrolysis was done with 1 M NaOH for 1 h at 20 °C, then the solution was neutralized with HCl and lipids were extracted with chloroform. Cleavage of ester bonds in phospholipids was confirmed by mass spectrometry.

Isogloboside-3 and α-GalCer were from Alexis. GSL-1 was provided by P. Seeberger.

Statistics. For cytokine assays, data were analyzed with a two-tailed unpaired Student's *t*-test with Welch's correction. For staining assays, data were analyzed with Mann-Whitney-Wilcoxon multiple-comparison test.

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doi:10.1038/ni.2245