Role for lysosomal phospholipase A2 in iNKT cell-mediated CD1d recognition

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Invariant natural killer T (iNKT) cells recognize self lipid antigens presented by CD1d molecules. The nature of the self-antigens involved in the development and maturation of iNKT cells is poorly defined. Lysophospholipids are self-antigens presented by CD1d that are generated through the action of phospholipases A1 and A2. Lysosomal phospholipase A2 (LPLA2, group XV phospholipase A2) resides in the endocytic system, the main site where CD1d antigen acquisition occurs, suggesting that it could be particularly important in CD1d function. We find that *Lpla2*$^{-/-}$ mice show a decrease in iNKT cell numbers that is neither the result of a general effect on the development of lymphocyte populations nor of effects on CD1d expression. However, endogenous lipid antigen presentation by CD1d is reduced in the absence of LPLA2. Our data suggest that LPLA2 plays a role in the generation of CD1d complexes with thymic lipids required for the normal selection and maturation of iNKT cells.

antigen processing | lipid storage disease

CD1d molecules are β_2-microglobulin (β_2m)-associated MHC class I homologs that bind lipid antigens and recycle between the plasma membrane and endocytic compartments. Endogenous and exogenous lipid binding can occur during transit through the secretory pathway and in the endocytic system, which is the main site of antigen loading (1). CD1d-lipid complexes are recognized by natural killer T (NKT) cells, a subset of T cells that shares characteristics of both the innate and adaptive arms of the immune system. NKT cells rapidly respond to stimulation by antigen-presenting cells (APCs) and release cytokines that can alter the strength and character of immune responses. They do so by transactivating, for example, NK cells, $CD4^+$ and $CD8^+$ T cells, and B cells, and by shifting cytokine responses to or from T helper (Th)1, Th2, and Th17 profiles. NKT cells have been shown to participate in inflammation, autoimmunity, cancer, and infectious diseases (2–4), and are characterized by the expression of both T-cell receptor (TCR) and NK cell markers. There are two main types: type I or invariant (iNKT), which express a semi-invariant TCR (Vα14-Jα18 in mice and Vα24-Jα18 in humans, paired with a restricted set of TCR-β chains, Vβ8.2, -7, or -2 in mice and Vβ11 in humans), and type II or noninvariant NKT cells that express more diverse TCRs (5–13).

iNKT cells arise from double-positive (DP; $CD4^+CD8^+$) thymocytes and are selected by DP thymocytes that present ill-defined endogenous lipids associated with CD1d (14–26). Isoglobotrihexosylceramide was shown to bind to CD1d and stimulate iNKT cells and was proposed to play a major role in their thymic selection (27); however, subsequent studies have challenged this hypothesis (28, 29). A recent study has identified plasmalogens as natural lipid antigens required for iNKT cell thymic maturation (30). Presentation of the appropriate lipid repertoire is essential for the proper iNKT cell development and function.

CD1d predominantly acquires lipids during recycling through the endocytic pathway, probably by the exchange of an existing lipid in the CD1d binding groove for a newly available one. These lipids (endogenous or exogenous) must be extracted from the membranes they reside in to become available for CD1d binding. Lysosomal lipid-binding proteins, such as saposins, Niemann-Pick type C2, the GM2 activator protein, and CD1e, have been shown to facilitate lipid binding by CD1d (31–35). Microsomal triglyceride transfer protein has been proposed to facilitate CD1d loading with lipids during the time spent in the endoplasmic reticulum (ER) and to have an ill-understood distal effect on CD1d recycling and antigen acquisition in the endocytic system (36–39). However, whether other molecules that facilitate lipid loading exist and precisely how any of them work remains poorly understood.

Lysophospholipids are natural detergents that are generated from their respective phospholipids by the action of phospholipases A1 and A2. These enzymes cleave the acyl-ester bonds at sn-1 and sn-2 positions, respectively, leaving only a single fatty acid side chain. Recent studies have identified lysophosphatidyl choline (LPC) and lysophosphosphatidyl ethanolamine in association with CD1d molecules (40, 41) and lysophospholipids can be recognized by NKT cells, as shown by the identification of human type II NKT cells from myeloma patients that specifically recognize LPC presented by CD1d (42) and of human type I NKT cell clones recognizing LPC-pulsed CD1d-positive cells (43). Moreover, lysophosphatidyl ethanolamine generated in hepatocytes as a result of hepatitis B infection can stimulate liver type II NKT cells (44). Therefore, lysophospholipids may have a dual role in CD1d biology: they could either serve as genuine CD1d antigens presented to T cells or as accessory detergent-like molecules that regulate lipid acquisition by CD1d-β_2m complexes.

Lysosomal phospholipase A2 (LPLA2, group XV PLA2) cleaves the acyl chain of various phospholipids (preferentially phosphatidyl ethanolamine and phosphatidyl choline) at the sn-2 position with optimal activity at acidic pH (45–47). The enzyme can also act as a phospholipase A1 depending on the physicochemical characteristics of the acyl groups located at the sn-1 and sn-2 positions and on the lipid bilayer structure (48). LPLA2 also shows transacylase activity; the acyl chain cleaved from the phospholipid substrate through the phospholipase activity can be transferred to an acceptor, such as *N*-acetyl-sphingosine (49). LPLA2 is ubiquitously expressed, but the highest enzymatic activity is in alveolar macrophages. Because LPLA2 resides in endocytic compartments, the main site for CD1d antigen acquisition, we examined its role in CD1d antigen presentation. We show that in the absence of the enzyme the iNKT cell population is negatively affected as a result of altered CD1d endogenous antigen presentation and defective thymic selection.

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Results

iNKT Cell Development Is Impaired in the Absence of LPLA2. Thymus, spleen, and liver of 6- to 8-wk-old WT and *Lpla2*$^{-/-}$ mice (50) were examined for the presence of iNKT cells by staining with anti-CD3ε antibody and α-GalCer (PBS57)-mouse CD1d tetramers (CD1d-Tet) within the thymic CD8^{low} or spleen and liver B220^{low} populations (Fig. 1*A*). *Lpla2*$^{-/-}$ mice showed a significant reduction in iNKT cells, both as percentage of total live cells (Fig. 1*B*) and in cell numbers (Fig. 1*C*), in all of the organs tested. No reductions in the CD4^{high}CD3^{high} (Fig. S1*A*) and CD8^{high}CD3^{high} (Fig. S1*B*) T-cell populations were detected in the *Lpla2*$^{-/-}$ mice, suggesting that iNKT lymphocytes were specifically affected. Furthermore, no defect in the size of DP thymic compartment, which is responsible for NKT cell selection, was observed in the *Lpla2*$^{-/-}$ mice (Fig. S1*C*).

Mouse iNKT cells express Vα14Jα18 TcR subunits paired with Vβ2, Vβ7, and Vβ8.2 subunits (5). No significant modification in the frequency of Vβ7 and Vβ8.2 plus Vβ8.1 use was observed for thymic iNKT cells in *Lpla2*$^{-/-}$ mice compared with WT controls (Fig. S2*A*). To examine if the defect in iNKT cells specifically affects either CD4^{+} or CD4^{-}CD8^{-} iNKT cells (5), iNKT cells from the CD8^{low} population in the thymus, spleen, and liver were assessed for CD4 expression by flow cytometry. No significant difference in the percentage of CD4^{+} iNKT cells among all iNKT cells in the thymus, spleen, or liver of WT and *Lpla2*$^{-/-}$ mice was detected (Fig. S2*B*). Thus, the absence of LPLA2 negatively affects the development of iNKT cells but does not induce preferential selection and/or expansion of specific subsets.

iNKT cells arise in the thymus from DP thymocytes and pass through well-characterized developmental stages. The earliest detectable cells, best visualized in the thymus of young mice, are CD24^{hi}CD44^{-}NK1.1^{-} (stage 0). They progress to stage 1 (CD24^{low}CD44^{low}NK1.1^{-}), stage 2 (CD44^{hi}NK1.1^{-}), and finally to the mature stage 3 (CD44^{hi}NK1.1^{hi}) (14, 17, 51). Thymocytes from 3- to 4-wk-old mice were depleted of CD8α^{+} cells and the CD3ε^{int}CD1d-Tet^{+} iNKT population was analyzed for surface expression of CD24, CD44, and NK1.1. As in 6- to 8-wk-old *Lpla2*$^{-/-}$ mice, the younger mice had fewer thymic iNKT cells (Fig. S2*C*). In fact, the reduction was greater in young mice compared with adult mice (70% versus 50%), indicating that the phenotype attenuates with age. The apparent slight increase in stage 0 (CD24^{hi}) iNKT cells in the *Lpla2*$^{-/-}$ mice (Fig. 2*A*) was not statistically significant (Fig. 2*B*). *Lpla2*$^{-/-}$ mice showed a statistically significant decrease in percentage of iNKT cells in the immature stages 0+1 and 2 compared with WT and implicitly an increase in mature stage 3 populations (Fig. 2 *C* and *D*). Immature NK1.1^{-} iNKT cells can exit the thymus and undergo CD1d-dependent maturation in the periphery (20, 52). Because *Lpla2*$^{-/-}$ mice showed a reduction in iNKT cells both in the thymus and periphery (Fig. 1), peripheral maturation was analyzed by NK1.1 expression. A statistically significant increase in the NK1.1^{+} iNKT cells population was observed in the spleen and liver of *Lpla2*$^{-/-}$ mice compared with WT controls (Fig. 2*E*). Thus, thymic and peripheral maturation of iNKT cells are not impeded when LPLA2 is absent.

***Lpla2*$^{-/-}$ iNKT Cells Respond Normally to Exogenous α-GalCer in Vivo.** iNKT cells acquire a memory phenotype and the ability to rapidly respond to antigens. To determine if the iNKT cells that do develop in *Lpla2*$^{-/-}$ mice can recognize and respond to the prototypical antigen α-GalCer, 6- to 8-wk-old *Lpla2*$^{-/-}$ or WT mice were injected i.p. with α-GalCer and livers and spleens were analyzed 2 h postinjection for iNKT cell activation. No significant difference was observed in the level of cytokine production or CD69 expression by iNKT cells in *Lpla2*$^{-/-}$ and WT mice receiving the vehicle control (Fig. S3). When α-GalCer–stimulated iNKT cells were compared, similar levels of IFN-γ (Fig. 3, *Left Upper* and *Lower*), IL-4 (Fig. 3, *Center Upper* and *Lower*), and CD69 (Fig. 3, *Right Upper* and *Lower*) were detected in both organs. These data suggest that the iNKT cells that develop in *Lpla2*$^{-/-}$ mice are not intrinsically defective.

CD1d Presentation of Endogenous Antigens Is Altered in *Lpla2*$^{-/-}$ Thymus. LPLA2 is expressed in endolysosomal compartments where CD1d acquires most of its endogenous antigens and could directly affect CD1d expression or function. Expression was examined by flow cytometry in total cells isolated from thymus, spleen, liver, or enriched splenic dendritic cells (DCs) of 6- to 8-wk-old *Lpla2*$^{-/-}$ and WT mice. There were no dramatic differences in the expression levels of CD1d, with the exception of a ∼10% and ∼20% decrease, respectively, in the thymus and splenic DCs from *Lpla2*$^{-/-}$ mice (Fig. S4*A*). To assess if endogenous antigenic presentation is modified as a result of this decrease and/or LPLA2 deficiency, *Lpla2*$^{-/-}$ or WT thymocytes from 6- to 8-wk-old mice were cocultured with three autoreactive NKT hybridomas (N37-1H5a, N38-2C12, and N57-2C12) (53, 54). N37-1H5a responded equally to *Lpla2*$^{-/-}$ and WT thymocytes, whereas N38-2C12 and N57-2C12 showed an average reduced response to the *Lpla2*$^{-/-}$ thymocytes of 30% and 50%, respectively (Fig. 4*A*). These differences were independent of target:effector ratio (Fig. S4*B*). Because the N37-1H5a response was comparable, the slight difference in CD1d expression between WT and *Lpla2*$^{-/-}$ thymus is unlikely to explain the reduced responses of N38-2C12 and N57-2C12. These are more likely to be caused by differences in the CD1d-associated lipid repertoire. A similar assay was performed using total splenocytes or enriched splenic DC (40–60% CD11c^{+} cells); all three

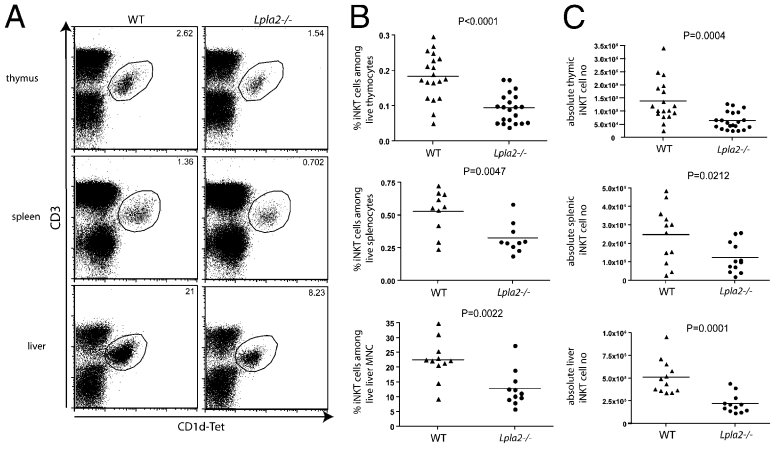

Fig. 1. Diminished iNKT population in *Lpla2*$^{-/-}$ mice. (*A*) Total cells obtained from the individual organs (thymus, spleen, liver) of WT and *Lpla2*$^{-/-}$ mice were stained with CD1d-Tet, anti–CD8α-FITC (thymus), anti–B220-FITC (spleen, liver), and anti–CD3ε-PerCP-Cy5.5 Abs and examined by flow cytometry. Numbers within dot plots indicate percent of CD3^{int}CD1d-Tet^{high} within CD8^{low} (thymus) or B220^{low} (spleen, liver) populations. Data representative of at least three experiments with three mice per group. (*B* and *C*) Statistical analysis of the percentage (*B*) and absolute numbers (*C*) of iNKT cells among live thymocytes, splenocytes, and liver MNCs of WT and *Lpla2*$^{-/-}$ mice. Each symbol represents one mouse. Horizontal bar represents arithmetic mean of samples in each column. Data are pooled from of at least three experiments with three mice per group.

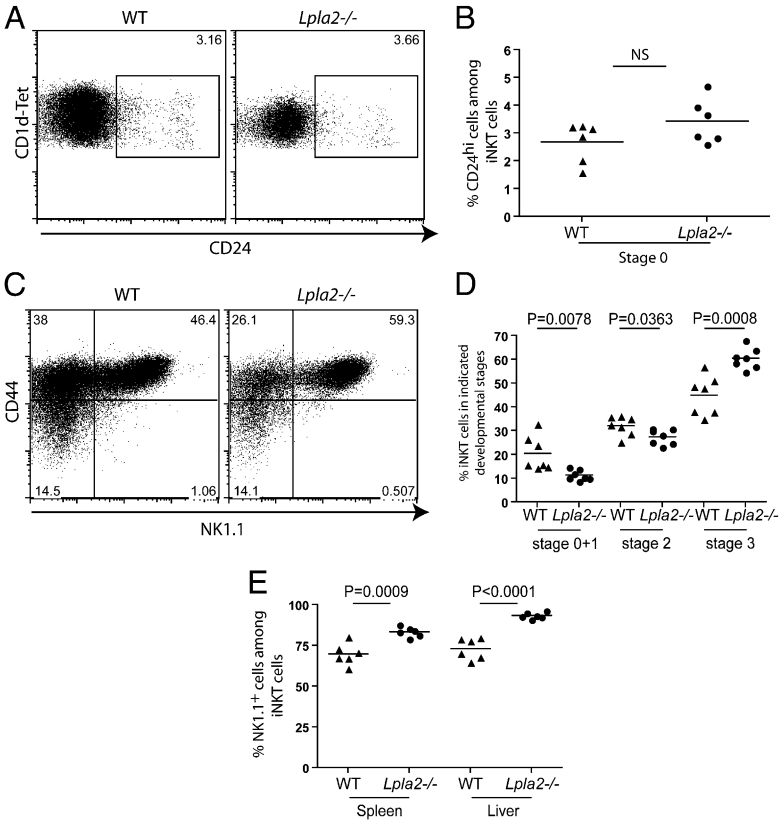

Fig. 2. iNKT subpopulations are equally affected in the absence of LPLA2. (*A*) Dot plots showing CD24 expression by iNKT cells from WT or *Lpla2*$^{-/-}$ 3- to 4-wk-old mice thymi. CD8α^{+}-depleted cells were stained with anti–CD24-PE, anti–CD3ε-PerCP-Cy5.5, and CD1d-Tet. Numbers indicate percentage among total iNKT cells. Data show one representative result from two experiments with three mice per group. (*B*) Statistical analysis of the percentage of CD24^{+} iNKT cells in WT and *Lpla2*$^{-/-}$ 3- to 4-wk-old mice thymi. Data pooled from two experiments with three mice per group. (*C*) Dot plots showing CD44 and NK1.1 expression by iNKT cells from WT or *Lpla2*$^{-/-}$ 3- to 4-wk-old mice thymi. CD8α^{+}-depleted cells were stained with anti–CD44-FITC, anti–NK1.1-PE, anti–CD3ε-PerCP-Cy5.5, and CD1d-Tet. Data show one representative result from two experiments with three to four mice per group. Numbers indicate percentage of each iNKT cells subpopulation. (*D*) Statistical analysis of the percentage of iNKT cells at different developmental stages (based on CD44 and NK1.1 expression) in the thymus of 3- to 4-wk-old WT and *Lpla2*$^{-/-}$ mice. Data pooled from two experiments with three to four mice per group. (*E*) Statistical analysis of the percentage of NK1.1^{high} iNKT cells in spleen and liver of 6- to 8-wk-old WT and *Lpla2*$^{-/-}$ mice. Data pooled from two experiments with three mice per group. (*B*, *D*, and *E*) Each symbol represents one mouse. Horizontal bar represents arithmetic mean of samples in each column. NS, not significant.

hybridomas gave the same response to *Lpla2*$^{-/-}$ and WT APCs (Fig. 4 *B* and *C*). The difference between the stimulatory capacity of thymocytes but not splenic APCs for N38-2C12 and N57-2C12 suggests a thymus-specific defect in endogenous lipid processing and presentation in *Lpla2*$^{-/-}$ mice.

CD1d Presentation of Exogenous Antigens Is Altered in Peripheral *Lpla2*$^{-/-}$ APCs. To investigate the effects of LPLA2 on endocytic processing of an exogenous lipid, we used galactosyl-α1–2-galactosyl ceramide (GalGalCer), which requires enzymatic cleavage in the endocytic system to release antigenic α-GalCer (55). DN32.D3 hybridoma cells were used because they are highly responsive to α-GalCer presentation. Thymocytes, splenocytes, and enriched splenic DCs (Fig. 5 *A* and *B*) from 6- to 8-wk-old mice were pulsed with GalGalCer and cocultured with DN32.D3 cells. Interestingly, *Lpla2*$^{-/-}$ and WT thymocytes showed similar stimulatory capacity (Fig. 5*A*), whereas splenocytes and enriched splenic DCs from *Lpla2*$^{-/-}$ mice showed a significant reduction in exogenous antigen presentation (Fig. 5 *A* and *B*). Thus, exogenous antigen processing and presentation is affected in the spleen of *Lpla2*$^{-/-}$ mice but not in the thymus, the opposite result observed for endogenous antigen presentation (Fig. 4). The defect in exogenous antigen presentation in enriched splenic *Lpla2*$^{-/-}$ DCs is unlikely to result from the marginal decrease in CD1d expression (Fig. S4*A*) because these cells stimulated the three autoreactive hybridomas similarly (Fig. 4*C*). Lysosomal lipid storage, in this case phospholipidosis, was previously reported in *Lpla2*$^{-/-}$ alveolar macrophages (50), and similar storage diseases can affect the stimulation of CD1d-reactive iNKT cells. To determine the extent of lysosomal storage in *Lpla2*$^{-/-}$ cells, we stained cells with LysoTracker Red DND-99, in which staining correlates with numbers and/or size of lysosomes (56). *Lpla2*$^{-/-}$ thymocytes, splenocytes, and splenic DCs showed increased staining compared with the WT control cells (Fig. 5*C*), consistent with lysosomal storage, which could explain the reduced GalGalCer processing and presentation by *Lpla2*$^{-/-}$ APCs.

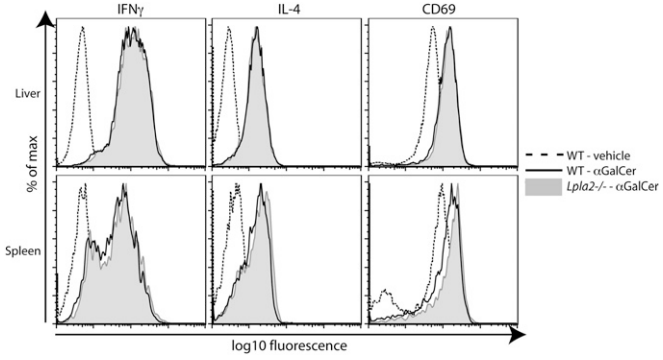

Fig. 3. Normal response of *Lpla2*$^{-/-}$ iNKT cells to α-GalCer in vivo stimulation. Six- to 8-wk-old WT and *Lpla2*$^{-/-}$ mice were injected with vehicle control or 2 μg α-GalCer and iNKT cells were analyzed 2 h later. Total cells were stained with anti–B220-FITC, anti–CD69/IFNγ/IL-4-PE, anti–CD3ε-PerCP-Cy5.5, and CD1d-Tet-APC as described in *Materials and Methods*. Histograms show production of IFN-γ (*Left*), IL-4 (*Center*), and expression of CD69 (*Right*) by iNKT cells from liver (*Upper*) and spleen (*Lower*). Data show one experiment representative of two using three mice per genotype (two mice injected with α-GalCer, one mouse vehicle-treated).

Discussion

The identity of endogenous lipid antigens presented to iNKT cells by CD1d molecules, generally acquired in the endocytic pathway, remains unclear. Here we have examined the role of LPLA2, a lysosomal phospholipase with broad substrate specificity (57), in CD1d antigen presentation. Our data show that LPLA2 deficiency

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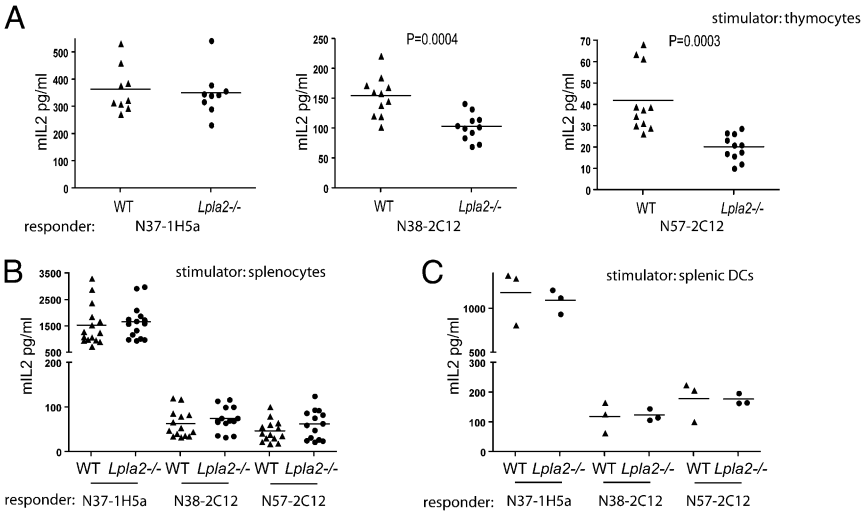

Fig. 4. Defective endogenous antigen presentation of *Lpla2*$^{-/-}$ thymocytes. WT or *Lpla2*$^{-/-}$ thymocytes (*A*), splenocytes (*B*), or enriched splenic DCs (*C*) were cocultured for 24 h with CD1d-restricted hybridoma cell lines. Graphs show statistical analysis of mIL-2 secretion by N37-1H5a, N38-2C12, and N57-2C12. (*A* and *B*) Data cumulated from at least three experiments with three mice per group. (*C*) One representative experiment (three mice per group) of five. Each symbol represents one mouse. Horizontal bar represents arithmetic mean of samples in each column. mIL-2, mouse interleukin-2.

results in impaired iNKT cell development that appears to be the result of an alteration in the lipids presented by CD1d molecules.

iNKT cells arise from BM precursors that undergo differentiation in the thymus, a process that involves selection, proliferation, and maturation (24). Assessment of CD1d endogenous antigen presentation by *Lpla2*$^{-/-}$ thymocytes using three iNKT mouse autoreactive hybridomas that differ in Vβ use and/or CDR3β sequence showed defective stimulation for two of them (Fig. 4*A*), suggesting that in the absence of LPLA2 the endogenous lipid repertoire presented by CD1d is modified. Modifications to the lipid repertoire in the absence of LPLA2 could affect iNKT cells at multiple levels. Positive and negative selections are the earliest developmental steps that require CD1d antigen presentation (14, 16, 19, 20, 58). *Lpla2*$^{-/-}$ thymic iNKT cells showed no signs of stage-specific developmental blockade when CD24, CD44, and NK1.1 expression was examined (Fig. 2), suggesting maturation is not affected once the cells enter stage 0. However, the endogenous antigen presentation defect observed in thymocytes (Fig. 4) and the reduced numbers of iNKT but not conventional CD4 or CD8 T cells in *Lpla2*$^{-/-}$ mice (Fig. S1) suggests that iNKT selection is modified in the absence of LPLA2. iNKT cells require a second round of CD1d-antigen recognition for final maturation and acquisition of NK-like function, including NK1.1 expression, and this can happen in the thymus and in peripheral organs (59). However, acquisition of the NK1.1 maturation marker in the thymus and periphery is not impeded in the absence of LPLA2 (Fig. 2).

Lpla2$^{-/-}$ thymocytes were defective in stimulating autoreactive hybridomas but splenocytes were not (Fig. 4*D*), even though processing and presentation of the model lysosomal exogenous antigen, GalGalCer, was severely impaired (Fig. 5*A*). This suggests that the thymic and peripheral CD1d lipid repertoires differ and/or that they are differentially affected by LPLA2 deficiency, and that intact CD1d function and lipid repertoire at both sites are required for iNKT cell development and maturation. Splenocytes from the *Lpla2*$^{-/-}$ mice showed evidence of increased lysosomal storage based on LysoTracker Red DND-99 staining (Fig. 5*C*). Defects in iNKT cell populations have been reported in mouse models of lysosomal storage diseases (28, 56, 60), and it has been suggested that lysosomal storage per se may induce defects in mouse CD1d antigen presentation through spatial restrictions in the endocytic system and not necessarily because antigens are absent. However, this does not seem to be the case for human CD1d because iNKT cells levels are normal in Niemann-Pick disease type C and Fabry patients, which might be the

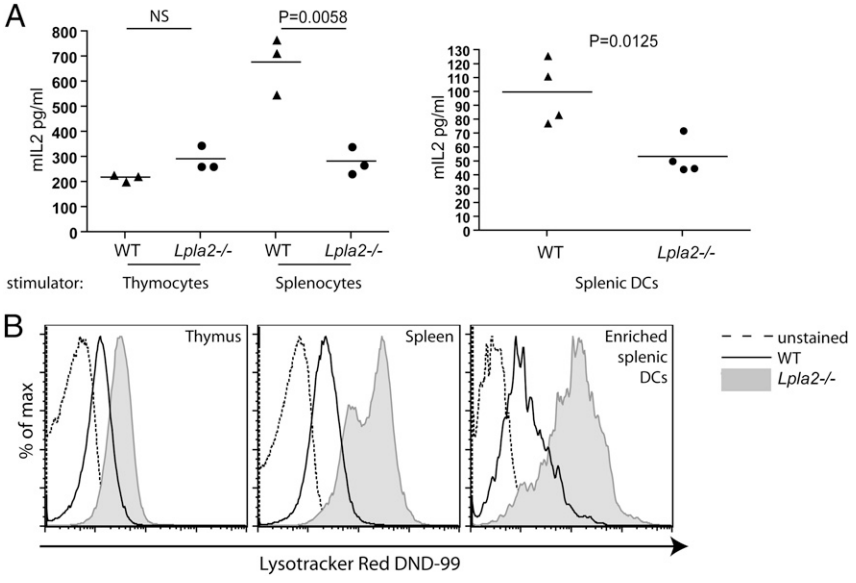

Fig. 5. *Lpla2*$^{-/-}$ splenocytes show deficiency in GalGalCer processing and α-GalCer presentation. (*A*) WT and *Lpla2*$^{-/-}$ thymocytes, splenocytes, or enriched splenic DCs were pulsed with 100 ng/mL GalGalCer overnight, for 4 h, or 2 h, respectively, and cocultured with DN32.D3 cell line. Graphs show one out of three individual experiments with three mice per group. Each symbol represents one mouse. Horizontal bar represents arithmetic mean of samples in each column. (*B*) Histograms showing LysoTracker DND-99 uptake by WT or *Lpla2*$^{-/-}$ thymocytes (*Left*), splenocytes (*Center*), and enriched splenic DCs (*Right*). Data are representative of two experiments with three mice per group.

result of the differences in intracellular trafficking of mouse and human CD1d molecules (61, 62). LPLA2 is a lysosomal enzyme, and its absence induces phospholipidosis in alveolar and peritoneal macrophages (50); thus, reduced stimulation by *Lpla2*$^{-/-}$ APCs could potentially result from modified lysosomal structure and function. However, the capacity of *Lpla2*$^{-/-}$ thymocytes to process and present α-GalCer generated lysosomally from its precursor, GalGalCer, makes phospholipid storage an unlikely explanation for defective thymic iNKT cell development.

The exact role of LPLA2 in CD1d antigen generation and loading is unknown. Lysolipids, the reaction products of LPLA2, are emerging as a category of antigens presented by CD1d molecules. They were identified among lipids eluted from CD1d-β_2m complexes (40–42) and they can stimulate type I and type II human NKT cells as well as mouse type II NKT cells (42–44). Because the mouse NKT cells recognizing lysophospholipids were type II and not type I (44, 63), the impact of LPLA2 absence on iNKT cells may not be directly related to lysophospholipids as CD1d antigens. Lysolipids have a single hydrocarbon chain and resemble detergents, which can induce lipid exchange by CD1d molecules in vitro (33). Lysolipids can also modify the curvature and the fluidity of membrane bilayers (64). Lipid transfer proteins have been shown to facilitate antigen loading onto CD1d (31–34), and lysolipids could indirectly participate in the process by altering physicochemical properties of the cellular membranes. Lysophospholipids can bind to CD1d (43) but the stability of the complex is low (65), particularly at an acidic pH characteristic of the lysosome, suggesting that they might act as temporary occupants of the CD1d lipid-binding groove that selectively allow access of new lipids in low pH endocytic compartments. In the human CD1d-LPC structure, the F′ channel is occupied by LPC and the A′ by two spacer lipids. An extreme shift of the α helices results in disruption of the A′ "roof" (65), making CD1d loaded with LPC much like an empty CD1d structure (66). An intriguing possibility is that lysolipids might help eliminate or load the spacer molecules found in several reported CD1d-ligand structures (67–70). LPLA2-generated lysolipids could therefore serve as antigens or as accessory molecules.

The self-antigen(s) presented by CD1d are poorly defined. It is already known that absence of LPLA2 modifies the total cellular lipid content and the phospholipid composition in alveolar macrophages (50). Similar changes may be found in other cell types, although the magnitude of the change is likely to be less in cell types with lower LPLA2 levels. LPLA2 mRNA expression level is higher in the spleen than in thymus (71) and the total phospholipid content was 30% higher in *Lpla2*$^{-/-}$ spleen than in WT, whereas the difference in phospholipid content was not significant in the thymus (57). However, changes in other lipid classes might exist. Future analysis of the lipid antigens presented by CD1d in the absence of LPLA2 may provide more insights into the nature and identity of the antigens required for iNKT cell development and maturation.

LPLA2 control of iNKT cell biology could have physiological and pathophysiological relevance because lysophospholipid levels increase during inflammation, viral infections, and cancer. Interestingly, aged *Lpla2*$^{-/-}$ mice exhibit a phenotype resembling systemic lupus erythematosus (57), an autoimmune disease in which reduced frequency of iNKT cells is inversely correlated with IgG levels (72). Finally, many cationic amphiphilic drugs, such as antiarrhythmics, antipsychotics, and antibiotics, are linked with toxic phospholipidosis (73), with impaired LPLA2 activity (74) and/or liver toxicity; these are serious side effects that could involve hepatic iNKT cells, which represent a ∼30% of liver B220^{low} cells.

In summary, LPLA2 appears to play a role in the generation of thymic lipid-CD1d complexes required for effective iNKT selection. In its absence, fewer iNKT cells pass the positive/negative selection points, resulting in reduced population in the thymus and periphery. However, the remaining iNKT cells undergo normal development and retain the capacity to respond to stimuli.

Materials and Methods

Mice. Three- to 9-wk-old mice on C57BL/6 background were used. C57BL/6 mice were purchased from The Jackson Laboratory. *Lpla2*$^{-/-}$ mice were described previously (50). All mice were bred and maintained in compliance with Yale University's institutional animal care and use committee regulations.

Reagents and Antibodies. Antibodies were purchased from BD Biosciences, eBioscience, Invitrogen, and Santa Cruz Biotechnology. APC-labeled CD1d: PBS-57 (α-GalCer) tetramers (CD1d-Tet) and unloaded controls were provided by the National Institute of Health Tetramer Core Facility at Emory University. Cells were maintained as previously described (53, 54).

Flow Cytometry. Thymocytes, splenocytes, and liver cells were obtained by mechanical disruption of the organs. Gradient separation was used for enrichment of liver mononuclear cells. Cells were stained for four-color flow cytometry analysis with the appropriate antibodies after Fc (crystallizable fragment of an antibody) receptor blocking with Fc Block (CD16/CD32, 2.4G2). The following antibodies (and their appropriate isotype controls) were used: anti–CD8α-FITC (53-6.7), anti–B220-FITC (RA3-6B2), anti–CD44-FITC (IM7), anti–CD24-PE (M1/69), anti–NK1.1-PE (PK136), anti–CD69-PE (H1.2F3), anti–CD4-PE (GK1.5), anti–Vβ7-PE (TR310), anti–Vβ8.1&8.2-PE (MR5-2), anti–IFNγ-PE (XMG1.2), anti–IL4-PE (BVD4-1D11), and anti–CD3ε-PerCp-Cy5.5 (145-2C11). CD1d was detected using biotin–anti-CD1d (1B1) in combination with streptavidin-PerCP. All samples were analyzed on BD FACScalibur with CellQuest and FlowJo software.

In Vivo α-GalCer Stimulation. Six- to 8-wk-old WT and *Lpla2*$^{-/-}$ mice were injected i.p. with 2 μg α-GalCer in 0.05% (vol/vol) Tween20/PBS vehicle solution and euthanized after 2 h. Throughout processing, splenocytes and liver cells were maintained in brefeldin A solution to block protein secretion (GolgiPlug, BD Pharmingen). Cells were stained for four-color flow cytometry using anti–B220-FITC, anti–IFNγ-PE, anti–IL4-PE, anti–CD69-PE, anti–CD3ε-PerCP-Cy5.5, and CD1d-Tet.

In Vitro Antigen Presentation. A total of 10^6 thymocytes and splenocytes from 6- to 8-wk-old WT and *Lpla2*$^{-/-}$ mice were cocultured with CD1d-restricted hybridoma cell lines at 10:1 target:effector ratio for 18–24 h. For exogenous antigen presentation, cells were pulsed overnight or for 4 h, respectively, with 100 ng/mL GalGalCer, extensively washed, and cocultured with DN32.D3 hybridoma cells at 10:1 target:effector ratio for 18–24 h. Splenic DCs were enriched from single-cell suspension using CD11c microbeads (Miltenyi Biotec) and cocultured at 1:5 target:effector ratio with hybridoma cells for 18–24 h. For exogenous antigen presentation, enriched splenic DCs were pulsed for 2 h with 100 ng/mL GalGalCer, extensively washed, and cocultured with DN32.D3 cells at 1:5 target:effector ratio for 18–24 h. Mouse IL-2 production in the coculture supernatant was detected using mouse IL-2 ELISA kit (BD Pharmingen).

LysoTracker Staining. Thymocytes, splenocytes, and enriched splenic DCs from 6- to 8-wk-old WT and *Lpla2*$^{-/-}$ mice were incubated with 0.5 μM LysoTracker DND-99 (Invitrogen) in RPMI-1640 for 15 min at 37 °C and analyzed by flow cytometry.

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