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This information is current as of April 15, 2017.

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*J Immunol* 2012; 189:5129-5138; Prepublished online 24 October 2012;
doi: 10.4049/jimmunol.1201570
http://www.jimmunol.org/content/189/11/5129

**Supplementary Material**  
http://www.jimmunol.org/content_supp/2012/10/25/jimmunol.120157

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ATP-Binding Cassette Transporter G1 Intrinsically Regulates Invariant NKT Cell Development

Duygu Sag,* Gerhard Wingender,† Heba Nowyhed,* Runpei Wu,* Abraham K. Gebre,‡ John S. Parks,† Mitchell Kronenberg,‡ and Catherine C. Hedrick*

ATP-binding cassette transporter G1 (ABCG1) plays a role in the intracellular transport of cholesterol. Invariant NKT (iNKT) cells are a subpopulation of T lymphocytes that recognize glycolipid Ags. In this study, we demonstrate that ABCG1 regulates iNKT cell development and functions in a cell-intrinsic manner. Abcg1−/− mice displayed reduced frequencies of iNKT cells in thymus and periphery. Thymic iNKT cells deficient in ABCG1 had reduced membrane lipid raft content, and showed impaired proliferation and defective maturation during the early stages of development. Moreover, we found that Abcg1−/− mice possess a higher frequency of Vβ7+ iNKT cells, suggesting alterations in iNKT cell thymic selection. Furthermore, in response to CD3e/CD28 stimulation, Abcg1−/− thymic iNKT cells showed reduced production of IL-4 but increased production of IFN-γ. Our results demonstrate that changes in intracellular cholesterol homeostasis by ABCG1 profoundly impact iNKT cell development and function. The Journal of Immunology, 2012, 189: 5129–5138.

Natural killer T cells are a unique subset of T lymphocytes that share characteristics with both NK cells and conventional T cells. There are two main classes of NKT cells, type I and type II. Type I NKT cells, also referred to as invariant NKT (iNKT) cells, express an invariant TCR (iTCR) α-chain, utilizing Vo14Jo18 in mice and Vo24Jo18 in humans. This iTCR α-chain is paired with a limited TCR β-chain, predominantly Vβ8, Vβ7, or Vβ2 in mice, and Vβ11 in humans (1). Unlike conventional MHC class I- and class II-reactive T cells, which recognize peptide Ags, iNKT cells recognize glycolipid Ags presented by CD1d, a MHC class I-like molecule (1). At least two classes of glycolipids have been reported to bear antigenic activity for iNKT cells, as follows: glycosphingolipids, as exemplified by Ags found in *Sphingomonas* bacteria (2, 3), and diacylglycerols, found, for example, in *Borrelia* bacteria (4). There are reports that other types of Ags, including cholesterol-containing molecules from *Helicobacter pylori*, are recognized by iNKT cells (5). The best-studied Ag for iNKT cells is the glycosphingolipid, α-galactosylceramide (α-GalCer) (6). Upon activation with α-GalCer, iNKT cells are able to produce large quantities of both Th1 cytokines (IFN-γ) and Th2 cytokines (IL-4, IL-10, and IL-13) (7, 8). Thus, iNKT cells can modulate immunity in a broad range of diseases and conditions, including atherosclerosis, autoimmunity, cancer, diabetes, allergy, and infection (7, 8).

ATP-binding cassette (ABC) transporters are transmembrane proteins that facilitate the transport of specific substrates across the membrane in an ATP-dependent manner (9). ABC transporter G1 (ABCG1) is a member of the ABC transporter family that regulates cholesterol homeostasis in the cell (10). Cholesterol homeostasis is crucial for the growth and survival of cells, because cholesterol is a key component of cell membranes and lipid rafts (11). ABCG1 is expressed in many tissues, including spleen, brain, lung, and kidney, and in many cell types, including lymphocytes, myeloid cells, and endothelial cells (12). Whereas ABCG1 can localize to the plasma membrane, it resides mostly intracellularly. Although initial studies suggested that the main function of ABCG1, similar to the other cholesterol transporter ABC transporter A1 (ABCA1), is to promote cholesterol efflux from cells (12, 13), recent reports by us and others show that ABCG1 is also important for the intracellular transport of cholesterol (14, 15). We previously reported that ABCG1 regulates the transfer of cholesterol from outer to inner membrane leaflets in secretory granules, including insulin granules in the pancreas (14). A recent study by Tarling and Edwards (15) has demonstrated that ABCG1 localizes to endocytic vesicles to facilitate the redistribution of intracellular cholesterol away from the endoplasmic reticulum. Thus, a key function of ABCG1 may be to regulate membrane cholesterol content to facilitate proper membrane fluidity and cholesterol homeostasis.

ABCG1 expression in immune cells, such as macrophages and lymphocytes, impacts their function. ABCG1-deficient macrophages display increased inflammatory activity (16, 17) in response to LPS (18), and when loaded with cholesterol (19). We (20) and others (21) have reported that changes in cholesterol and lipid raft content in the absence of ABCG1 increase TCR signaling and proliferation of CD4+ T cells. Like other lymphocytes, iNKT cells express ABCG1; however, the role of ABCG1 and cholesterol homeostasis in iNKT cell biology is not known. In this study, we demonstrate that changes in intracellular cholesterol homeostasis by ABCG1 profoundly impact thymic iNKT cell development and function.
Materials and Methods

Mice

C57BL/6j mice (000664) and B6.129S7-Rag1tm1Mom/j (00216) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Abcg1−/− R26Z knock-in mice were purchased from Deltagen (San Mateo, CA) and are congenic on a C57BL/6j background. B6.SJL-Ptprca/Boy/1TAc mice (CD45.1 congenic, 004007) were purchased from Taconic Farms (Germantown, NY). Mice were fed a standard rodent chow diet and were housed in microisolator cages in a pathogen-free animal facility of the La Jolla Institute for Allergy and Immunology. All experiments followed guidelines of the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, and approval for use of rodents was obtained from the La Jolla Institute for Allergy and Immunology according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Mice were euthanized by CO2 inhalation.

Reagents

Flow cytometry Abs, including anti-mouse CD45.2 (104), CD4 (RM4-5), TCRβ (H57-597), IL-4 (BV2-24G2), CD44 (IM7), NK.1.1 (PK136), and CD1d (1B1), were purchased from eBioscience (San Diego, CA); CD45.1 (A20), IFN-γ (XMG1.2), BV7 (TR310), and BV8.1/2 (MR5-2) were purchased from BD Biosciences (San Jose, CA); CD19 (L243) and CD16/CD32 (2.4G2) Abs were purchased from BioLegend. For activation of primary NKT cells, RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA), and PBS was purchased from Thermo Scientific (Waltham, MA).

Primary cell preparation

Single-cell suspensions were prepared from the thymus, spleen, and liver. Spleens and thymi were meshed through a 40-μm filter (Fisher Scientific, Pittsburgh, PA). RBCs in spleen were lysed in RBC lysis buffer, according to the manufacturer’s protocol. Prior to extraction, the liver was perfused with PBS via the portal vein until opaque and meshed through a 100-μm strainer and washed. Total liver cells were then resuspended in a 37.5% isotonic Percoll solution (Amersham Biosciences, Piscataway, NJ) and centrifuged for 30 min at 850 
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Flow cytometry

Cells were resuspended in 100 μl flow cytometry staining buffer (1% BSA plus 0.1% sodium azide in PBS). FcγRs were blocked with CD16/32 blocking Ab for 10 min, and surface Abs on cells were stained for 30 min at 4°C. Cells were labeled with CD1d tetramer together with the other surface Abs in staining buffer. LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) was used for analysis of viability, and forward- and side-scatter parameters were used for exclusion of doublets from analysis. Staining protocols are listed above.

For measurement of membrane lipid rafts, cells were stained for an additional 10 min at 4°C in PBS with 10 ng Alexa Fluor 488-labeled cholera toxin B (CT-B) from Vybrant lipid-raft labeling kits (Invitrogen).

For intracellular staining, cells were fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) after the cell surface staining. Cells were stained for 30 min at 4°C with directly conjugated fluorescent of IL-4 and IFN-γ Abs.

Calculations of percentages were based on live cells as determined by forward- and side-scatter and viability analysis. Cell fluorescence was assessed using LSR-II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

In vitro stimulation assays

For activation of iNKT cells, 30 μM 1,2-dioctanoylglycerol (1,2-DAG) with 2 μg/ml LPS was incubated with the indicated TCR hybridoma at 0°C. The cells were washed twice with PBS, and thymi were plated at 2 × 10^7 cells/well in RPMI 1640 medium supplemented with 5% FBS and 1% penicillin/streptomycin. Soluble αCD28 Ab (2 μg/ml) and GolgiPlug (BD Biosciences) were added, and the cells were incubated at 37°C for 4 h. Thymocytes were stimulated with PMA (1 μg/ml) and ionomycin (200 ng/ml) for 4 h in the presence of GolgiPlug at 37°C. IL-4 and IFN-γ production by iNKT cells was assessed by flow cytometry.

In vivo BrdU proliferation assay and detection of apoptosis

C57BL/6 (B6) and Abcg1−/− mice were injected i.p. with 0.3 mg BrdU (in 100 μl PBS) three times every 4 h. Thymini were harvested the next day, and single-cell suspensions were stained with fluorophore-conjugated Abs and CD1d tetramer. After cell surface staining, cells were analyzed for BrdU incorporation using FITC or allophycocyanin BrdU flow kit (BD Biosciences), according to the manufacturer’s instructions. Apoptosis of thymin iNKT cells was measured by flow cytometry using a PE Annexin V Apoptosis Detection Kit 1 (BD Biosciences), according to the manufacturer’s instructions.

Generation of bone marrow chimeras

Recipient mice (Rag1−/−) were irradiated in two doses of 450 rad each (for a total of 900 rad) 4 h apart. Bone marrow cells from both femurs and tibias of donor mice (B6.SJL and Abcg1−/−) were collected under sterile conditions. Bones were centrifuged for the collection of marrow, and cells were washed, mixed at a 1:1 ratio, and resuspended in PBS for injection. Approximately 5 × 10^6 bone marrow cells from B6.SJL and Abcg1−/− mice (total 10^7 cells) in 200 μl PBS were delivered retro-orbitally into each recipient mouse. Recipient mice were housed in a barrier facility under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, mice were provided autoclaved acidified water with antibiotics (trimethoprim-sulfamethoxazole) and were fed autoclaved food. Mice were analyzed 12 wk after bone marrow reconstitution.

Cholesterol content in iNKT cells

Surface Ags on thymocytes from B6 and Abcg1−/− mice were stained, as described above, followed by iNKT cell (CD8a−, TCRβ+, CD1d tetramer+) sorting with a FACSAria cytometer (BD Biosciences). Approximately 2 × 10^5 events were collected for gas chromatography analysis. Sorted thymic iNKT cells were pelleted by low-spin centrifugation. After several washes with PBS, the cell pellet was extracted with chloroform:methanol (2:1) containing 5-cholestane as internal standard. Total and free cholesterol content was determined by gas–liquid chromatography and normalized to cellular protein, as previously described. 20S Ergosterol ester was calculated as (total cholesterol − free cholesterol) × 1.67. Multiplying by 1.67 corrects for the average fatty acid mass that is lost during saponification.

Quantitative real-time PCR

iNKT cells were FACs sorted from thymus, and total cellular RNA was collected with an RNAeasy Plus Micro Kit, according to the manufacturer’s protocol (Qiagen, Valencia, CA). RNA purity and quantity were measured with a nanodrop spectrophotometer. Approximately 500 ng RNA was used for synthesis of cDNA with an Iscript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Total cDNA was diluted 1:20 in H2O, and a volume of 9 μl was used for each real-time condition with a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and TaqMan Gene Expression Mastermix and ABCG1 and ABCA1 TaqMan primers (Invitrogen). Data were analyzed and presented on the basis of the relative expression method (24). The formula for this calculation is as follows: relative expression = 2^(-ΔΔCt) where ΔCt is the difference in the threshold cycle between the gene of interest and the housekeeping gene (18S). S is the Abcg1−/− mouse, and C is the B6 control mouse.

Statistical analysis

Data for all experiments were analyzed with Prism software (GraphPad, San Diego, CA). Unpaired Student t test was used for comparison of experimental groups. The p values <0.05 were considered statistically significant.

Results

Abcg1−/− mice display impaired iNKT cell development

To investigate the impact of ABCG1 deficiency on iNKT cell development, iNKT cell frequencies in thymus, liver, and spleen of four- to six-week-old Abcg1−/− and control B6 mice were assessed by flow cytometry (Fig. 1A, 1B). Abcg1−/− mice had...
FIGURE 1. *Abcg1*^-/-^ mice display reduced iNKT cell frequencies in thymus and liver. (A) Thymocytes, liver mononuclear cells, and splenocytes from four- to six-week-old wild-type B6 and *Abcg1*^-/-^ mice (n = 9) were stained with fluorophore-conjugated Abs and CD1d tetramer. Representative contour plots show CD19^−^CD8α^−^TCRβ^+^CD1d-tetramer^+^ iNKT cells. Bar graphs show (B) frequency and (C) total cell number of iNKT cells in thymus, liver, and spleen (iNKT cells [%]: % of live cells). Data are pooled from two to three independent experiments (three to five mice per group for each experiment) with similar results. (D) Graph shows the frequency of NRP-1^+^CD69^+^iNKT cells in spleen. Representative data of two independent experiments with four-week-old mice (five to six mice per group) are shown. (E) Thymocytes, liver mononuclear cells, and splenocytes from B6 (n = 5) and *Abcg1*^-/-^ mice (n = 5) were stained with fluorophore-conjugated CD19, CD8α, TCRβ, VB7, VB8.1/2, and VB2 Abs, and CD1d tetramer, and analyzed by flow cytometry. Graphs show frequencies of VB7^+^ (top), VB8.1/2^+^ (middle), and VB2^+^ (bottom) B6 and *Abcg1*^-/-^ iNKT cells in thymus, liver, and spleen. (F) Graphs show absolute cell numbers of VB7^+^ (top), VB8.1/2^+^ (middle), and VB2^+^ (bottom) iNKT cells in thymus, liver, and spleen. Data are representative of two independent experiments. Error bars represent means ± SEM. Asterisks denote the significance of differences between groups (*p < 0.05, **p < 0.01, ***p < 0.001, two-tailed Student *t* test).
significantly lower frequencies of iNKT cells in both thymus and liver compared with control mice (Fig. 1A, 1B). Absolute cell numbers of iNKT cells in thymus and liver were also lower in Abcg1−/− mice compared with control mice (Fig. 1C). A similar tendency was observed in the spleen; however, this did not reach statistical significance in all experiments (Fig. 1A–C). Recently, Milpied et al. (25) reported that recent thymic emigrant NKT cells can be divided into CD4+ and CD4− subsets, and NKT cells with diverse CDR3 regions (1). There are data suggesting that Abcg1−/− mice have a higher affinity for the endogenous ligand(s) presented during positive selection of NKT cells in the thymus (27). Thus, the frequency of Vβ7+ iNKT cells has been used as a surrogate marker for the overall avidity for the iTCR toward its endogenous selecting ligand(s) in the thymus. To determine whether ABCG1 deficiency could impact the selection of NKT cells in the thymus, we first analyzed Vβ7+ iNKT cells in Abcg1−/− mice. As shown in Fig. 1E, the percentages of Vβ7+ iNKT cells in thymus, liver, and spleen of Abcg1−/− mice were significantly higher than in the control B6 mice. Next, we analyzed Vβ8.1/2+ and Vβ2+ iNKT cells in these organs and found that in thymus and spleen of Abcg1−/− mice, the percentages of Vβ8.1/2+ and Vβ2+ iNKT cells were significantly lower than in the B6 mice, which is in line with the increased frequency of Vβ7+ iNKT cells in the Abcg1−/− mice (Fig. 1E). In liver, the percentage of Vβ8.1/2+ iNKT cells was significantly lower in Abcg1−/− mice compared with B6 mice; however, the percentages of Vβ2+ iNKT cells in Abcg1−/− mice and B6 mice were comparable (Fig. 1E). Next, we determined the absolute cell numbers of Vβ7+ iNKT cells in Abcg1−/− mice. In line with the reduced total iNKT cell frequency in thymus of Abcg1−/− mice compared with control B6 mice (Fig. 1A, 1B), the absolute cell number of Vβ7+ iNKT cells in thymus was also significantly lower in Abcg1−/− mice (Fig. 1F). In liver, the absolute cell number of Vβ7+ iNKT cells tended to be lower in Abcg1−/− mice, but the

FIGURE 2. ABCG1 deficiency affects iNKT cell development via a cell-intrinsic mechanism. (A) Thymocytes from B6 mice (n = 5) and Abcg1−/− mice (n = 4) were stained with CD4, CD8a, and CD1d Abs and analyzed by flow cytometry. Bar graph shows mean fluorescence intensity (MFI) of CD1d on CD4+ CD8+ thymocytes from B6 and Abcg1−/− mice. Data are representative of two independent experiments with similar results. (B) NKT cell hybridoma DN3A4-1.2 cells were cocultured with thymocytes from either B6 (n = 4) or Abcg1−/− (n = 4) mice in the presence of titrated amounts of α-GalCer in vitro. After 18 h, IL-2 production was detected by ELISA. Data are representative of two independent experiments with similar results. (C) and (D) Bone marrow chimeras were generated by reconstituting irradiated Rag1−/− mice (n = 11) with 1:1 mixed bone marrow cells from CD45.1+ B6.SJL and CD45.2+ Abcg1−/− donor mice. Single-cell suspension from thymus was analyzed by flow cytometry 12 wk following reconstitution. (C) Representative contour plots show CD19−, CD8α−, TCRβ+, CD1d-tetramer+ iNKT cells, which are gated on CD45.1+ and CD45.2+ to identify Abcg1+/+ B6.SJL and Abcg1−/− iNKT cells, respectively. (D) Bar graph shows percentages of Abcg1+/+ B6.SJL and Abcg1−/− iNKT cells in thymus. Data are pooled from three independent experiments (three to four mice per group for each experiment) with similar results. (E) Graph shows frequency of Vβ7+ CD45.1+ B6 and CD45.2+ Abcg1−/− iNKT cells in thymus. Data are pooled from two independent experiments (three mice per group for each experiment) with similar results (***p < 0.001, ****p < 0.0001).
difference was not statistically significant (p = 0.067) (Fig. 1F). As
the frequencies of total iNKT cells in spleen of Abcg1−/− mice and B6 mice were comparable (Fig. 1A, 1B) and Abcg1−/− mice had a higher percentage of Vβ7+/iNKT cells in spleen (Fig. 1E), the absolute cell numbers of Vβ7+/iNKT cells in spleen were significantly higher in Abcg1−/− mice (Fig. 1F). These data demonstrate that thymic selection of Vβ7+/iNKT cells is favored in the absence of ABCG1.

**ABCG1 deficiency affects iNKT cell development via a cell-intrinsic mechanism**

A critical factor driving iNKT cell development in the thymus is the generation of a CD1d-restricted iTCR by CD45.2+/CD44high NK1.1+ DP thymocytes, we examined whether DP thymocytes expressing an ABCG1 deficient thymocyte that presents endogenous selecting ligand(s), the iTCR-expressing DP thymocyte is positively selected to enter the thymic precursor pool (30). Subsequently, these iNKT cell precursors undergo a series of proliferation and differentiation/maturation stages (31). Because iNKT cells are positively selected by DP thymocytes, we examined whether DP thymocytes from Abcg1−/− mice had lower CD1d expression. If so, this would be a likely explanation for the observed decrease in iNKT cell frequency. As shown in Fig. 2A, DP thymocytes from Abcg1−/− and B6 mice had similar levels of surface CD1d expression. Next, to investigate whether ABCG1 deficiency affects CD1d-mediated lipid Ag presentation, we incubated the iNKT cell hybridoma DN3A4-1.2 with thymocytes isolated from either Abcg1−/− or B6 mice in the presence of titrated amounts of α-GalCer in vitro. We found that Abcg1−/− thymocytes were as efficient as B6 thymocytes at stimulating IL-2 production by iNKT cell hybridomas (Fig. 2B). Overall, these results show that the impairment in iNKT cell development observed in Abcg1−/− mice was not due to changes in CD1d surface expression on DP thymocytes or their CD1d-mediated Ag presentation.

To determine whether the impact of ABCG1 deficiency on iNKT cell development is mediated through cell-intrinsic factor(s), we used a mixed bone marrow chimera approach. Irradiated Rag1−/− mice were reconstituted with both CD45.1+ B6.SJL and CD45.2+ Abcg1−/− bone marrow mixed at a 1:1 ratio and analyzed 12 wk after reconstitution. We did not observe any difference in the frequency of Abcg1−/− and B6 DP thymocytes (Supplemental Fig. 2A) or in their surface CD1d expression in the chimeric mice (Supplemental Fig. 2B). However, when analyzing iNKT cells in the thymus, we found that ~3-fold fewer iNKT cells developed from the Abcg1−/− bone marrow than from the B6 bone marrow (Fig. 2C, 2D). These results demonstrate that the impact of ABCG1 deficiency on iNKT cell development in the thymus is cell intrinsic.

To determine whether the increased frequency of Vβ7+/iNKT cells in Abcg1−/− mice (Fig. 1E) was also caused by a cell-intrinsic mechanism, we evaluated the percentage of Vβ7+/iNKT cells in the 1:1 mixed bone marrow chimeric mice. Abcg1−/−-derived iNKT cells in the thymus of the chimeric mice displayed a higher frequency of Vβ7+ population compared with B6-derived iNKT cells (Fig. 2E). These data demonstrate that thymic selection of Vβ7+/iNKT cells is favored in the absence of ABCG1 via an iNKT cell-intrinsic mechanism.

**ABCG1 deficiency affects the maturation of iNKT cells in the thymus**

After positive selection in thymus, iNKT cells proliferate and differentiate/mature along stages that are defined by the surface expression of CD44 and NK1.1 (31). Cells with a stage 1 phe-

![FIGURE 3. ABCG1 deficiency affects the maturation of iNKT cells in thymus. Single-cell suspensions from thymi of B6/Abcg1−/− 1:1 mixed chimeric mice (n = 10) were stained with fluorophore-conjugated CD45.1, CD45.2, TCRβ, CD44, NK1.1 Ab, and CD1d tetramer and analyzed by flow cytometry. TCRβ+ and CD1d-tetramer+ cells were further gated to distinguish stage 1 (stg1, CD44low NK1.1−), stage 2 (stg2, CD44high NK1.1−), and stage 3 (stg3, CD44high NK1.1+) iNKT cells. (A) Representative contour plots. Bar graphs show (B) percentages and (C) absolute cell numbers of CD44.1+ B6 and CD45.2+ Abcg1−/− iNKT cells of indicated maturation stages. Results are representative of two independent experiments with similar results. (D) Graphs show frequencies of Vβ7+ thymic iNKT cells at stage 1–3 in B6 (n = 8) and Abcg1−/− (n = 7) mice. Data are pooled from two independent experiments (three to four mice per group for each experiment) with similar results (*p < 0.05, **p < 0.01, ***p < 0.001).](http://www.jimmunol.org/DownloadedFrom)
notype (CD44low NK1.1−) are followed by stage 2 cells, which have increased CD44 expression (CD44high NK1.1−). Stage 1 and stage 2 nNKT cells are highly proliferative (32, 33). The upregulation of NK1.1 expression by nNKT cells marks stage 3 (CD44high NK1.1+) cells, which are mature but less proliferative (32, 34). Whereas most nNKT cells exit the thymus at stage 2 and complete their maturation in the periphery, some enter stage 3 in the thymus and remain as long-term thymus-resident nNKT cells (31). To determine where during nNKT cell development ABCG1 plays a role, we evaluated the developmental stages of nNKT cells in the 1:1 mixed bone marrow chimeric mice. The percentage of Abcg1−/− nNKT cells in stage 1 (CD44low NK1.1−) was ~2-fold larger compared with B6 nNKT cells, whereas the percentage of nNKT cells in stage 2 (CD44high NK1.1−) was reduced by 33%. There were no differences in the percentages of stage 3 (CD44high NK1.1+) nNKT cells between both genotypes (Fig. 3A, 3B). We calculated the absolute cell numbers of Abcg1−/− and B6 nNKT cells at these three maturation stages, and, in line with the reduced relative frequency of Abcg1−/− nNKT cells in the thymus of the chimeric mice (Fig. 2C, 2D), the total number of Abcg1−/− nNKT cells was dramatically reduced at stages 2 and 3 compared with B6 nNKT cells (Fig. 3C). Subsequently, we analyzed Vβ7+nNKT cells in stage 1–3 of thymic development in Abcg1−/− mice. Similar to the observed increase in the frequency of total thymic Vβ7+nNKT cells in Abcg1−/− mice (Fig. 1E), the frequencies of Vβ7+nNKT cells in all three maturation stages were significantly higher in Abcg1−/− mice than in B6 mice (Fig. 3D). These data indicate that ABCG1 deficiency impairs the proper maturation of nNKT cells at early stages of nNKT cell development.

**Abcg1−/− nNKT cells have reduced proliferation**

To determine whether the reduced frequency of nNKT cells in the absence of ABCG1 is due to a decrease in proliferation, we measured proliferation of thymic nNKT cells at different maturation stages in vivo in Abcg1−/− and B6 control mice by BrdU incorporation. In Abcg1−/− mice, the frequency of BrdU+ nNKT cells at stages 1 and 2 was significantly reduced compared with nNKT cells from B6 mice (Fig. 4A, 4B). In stage 3, the frequency of BrdU+ nNKT cells also tended to be lower in Abcg1−/− mice, but the difference was not statistically significant (p = 0.051) (Fig. 4A, 4B). Next, we measured BrdU incorporation of Vβ7+nNKT cells in Abcg1−/− and B6 mice. In line with the reduced proliferation of total nNKT cells in Abcg1−/− mice (Fig. 4A, 4B), the frequency of BrdU+ Vβ7+nNKT cells was significantly lower in Abcg1−/− mice compared with B6 mice (Supplemental Fig. 4).

To investigate whether increased cell death also contributes to the reduced nNKT cell numbers in the absence of ABCG1, we examined apoptosis of Abcg1−/− and B6 nNKT cells at different stages of thymic development by annexin V staining. We found that the percentages of apoptotic (annexin V+) nNKT cells at stage 1–3 in Abcg1−/− and B6 mice were comparable (Fig. 4C, 4D). These results indicate that the reduced frequency of nNKT cells

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**FIGURE 4.** Abcg1−/− nNKT cells display reduced proliferation in early stages of development. B6 (n = 7) and Abcg1−/− (n = 8) mice were injected with BrdU three times every 4 h. The next day, thymi were harvested and single-cell suspensions were stained with fluorophore-conjugated CD8α, TCRβ, CD44, NK1.1, BrdU Ab, and CD1d tetramer and analyzed by flow cytometry. TCRβ+ and CD1d-tetramer+ cells were further gated to distinguish stage 1 (stg1, CD44low NK1.1−), stage 2 (stg2, CD44high NK1.1−), and stage 3 (stg3, CD44high NK1.1+) nNKT cells. (A) Representative contour plots and (B) bar graphs show BrdU incorporation by nNKT cells at each stage. Data are pooled from two independent experiments (three to four mice per group for each experiment) with similar results. (C and D) Thymocytes from B6 (n = 3) and Abcg1−/− (n = 3) mice were cultured overnight and the next day stained with fluorophore-conjugated CD8α, TCRβ, CD44, NK1.1, annexin V Ab, CD1d tetramer, and a live/dead marker and analyzed by flow cytometry. (C) Representative contour plots and (D) bar graphs show percentages of apoptotic (annexin V+ live) nNKT cells at stage 1–3. Data are representative of two independent experiments with similar results (**p < 0.01, ***p < 0.001).
in thymus in the absence of ABCG1 is not due to increased cell death but reduced proliferation, particularly during the early stages of development.

**ABCG1 deficiency affects lipid raft content in iNKT cells**

We next measured the cholesterol content in iNKT cells isolated from thymus of B6 and Abcg1<sup>2/2</sup> mice using gas chromatography. We found no significant changes in the cholesteryl ester, free cholesterol, or total cholesterol content in Abcg1<sup>2/2</sup>iNKT cells (Fig. 5A). However, as ABCG1 is important in intracellular cholesterol transport (14, 15) and cholesterol is an important component of membrane lipid rafts (11), we measured lipid raft content in thymic iNKT cells. Lipid rafts are specialized regions of the cell membrane that are rich in cholesterol and gangliosides.

**FIGURE 5.** Abcg1<sup>2/2</sup>iNKT cells display no change in cholesterol content, but have lower lipid raft content. (A) iNKT cells were FACS sorted from thymus of B6 and Abcg1<sup>2/2</sup> mice (<em>n</em> = 9; 27 mice, 3 mice were pooled for each sample per group), and free cholesterol (FC), cholesteryl ester (CE), and total cholesterol (TC) were measured by gas chromatography. Data are pooled from three independent experiments (3 samples per group for each experiment) with similar results. (B and C) Thymocytes from B6 (<em>n</em> = 4) and Abcg1<sup>2/2</sup> (<em>n</em> = 4) mice were stained with fluorophore-conjugated Abs, CD1d tetramer, and CT-B, and analyzed by flow cytometry. (B) Representative plot shows lipid raft staining (CT-B) of iNKT cells in thymus. (C) Graph shows mean fluorescence intensity (MFI) of CT-B of iNKT cells in thymus. Data are representative of two independent experiments with similar results (**<em>p</em> < 0.01).
and act as platforms to colocalize proteins involved in intracellular signaling pathways (35, 36). In contrast to the comparable cholesterol levels, the lipid raft content of Abcg1+/− NKT cells in thymus was 43% lower than in B6 NKT cells (Fig. 5B, 5C). These results suggest that ABCG1 plays a role in regulation of membrane lipid raft content in thymic NKT cells, which is most likely important for their proper development.

**ABCG1 deficiency affects cytokine production of iNKT cells**

Because Abcg1−/− NKT cells have reduced lipid raft content (Fig. 5B, 5C) and the TCR is associated with lipid rafts (37), we next investigated whether deficiency of ABCG1 in iNKT cells would affect their TCR-driven activation. We stimulated thymocytes from B6:Abcg1−/−:12.1 mixed bone marrow chimeric mice with plate-bound αCD3ε and soluble costimulatory αCD28 Ab in vitro and measured IL-4 and IFN-γ production of thymic NKT cells by intracellular staining. We found that CD45.2+ Abcg1−/− iNKT cells had reduced IL-4 production, but enhanced IFN-γ production compared with CD45.1+ B6 iNKT cells (Fig. 6A, 6B). Previous studies have shown that stage 1 and stage 2 NKT cells produce abundant IL-4, but less IFN-γ, whereas stage 3 NKT cells make more IFN-γ, but less IL-4 (32, 34, 38). Our results demonstrate that the frequency and the total cell number of stage 2 NKT cells are reduced in the absence of ABCG1 (Fig. 3A–C). Therefore, the diminished production of IL-4 by Abcg1−/− iNKT cells might be due to the lower percentages of the potent IL-4 producer stage 2 NKT cells in the absence of ABCG1. To address this possibility, we stimulated thymocytes from Abcg1−/− and B6 mice with αCD3ε and αCD28 Abs in vitro and measured IL-4 and IFN-γ production of thymic NKT at different stages by intracellular staining. Stage 2 and stage 3 Abcg1−/− iNKT cells produced significantly less IL-4 and more IFN-γ compared with B6 control (Fig. 6C), demonstrating that the altered cytokine production of Abcg1−/− NKT cells is not due to the reduced percentages of stage 2 NKT cells in the absence of ABCG1. Furthermore, to elucidate that the changes in the cytokine production by iNKT cells in the absence of ABCG1 are related to TCR triggering, we stimulated thymocytes from Abcg1−/− and B6 mice in vitro with PMA/ionomycin, which bypasses TCR triggering to induce activation. We then measured IL-4 and IFN-γ production of thymic NKT cells by intracellular staining. As seen in Fig. 6D, PMA/ionomycin stimulation induced IL-4 and IFN-γ production by Abcg1−/− iNKT cells to a similar degree as in the B6 NKT cells, indicating that Th1-biased cytokine production we observed in Abcg1−/− iNKT cells is indeed related to TCR triggering. Overall, these results demonstrate that deficiency of ABCG1 in iNKT cells skews their cytokine production, leading to a Th1 bias following TCR-driven activation.

**Discussion**

In this study, we demonstrate that changes in cholesterol homeostasis caused by the absence of ABCG1 impair iNKT cell development. ABCG1-deficient thymic iNKT cells displayed reduced proliferation in vivo and defective maturation during the early stages of development. Considering the number of thymic iNKT cells, the developmental block was particularly evident at stage 2, although the reduced proliferation of stage 1 cells most likely contributes to the deficit. Moreover, in the absence of ABCG1, thymic iNKT cells had reduced membrane lipid raft content, which was accompanied by a Th1-biased cytokine production in response to TCR stimulation. The defects were cell intrinsic, meaning that they occur in the iNKT cell precursor rather than in the DP thymocyte responsible for iNKT cell-positive selection. Therefore, although it is theoretically possible that a cholesterol-containing compound is an important self-ligand mediating positive selection, our results rule out differences in self-ligand presentation as the responsible factor. In addition, thymic selection of Vβ7+ iNKT cells was favored in the absence of ABCG1 via an iNKT cell-intrinsic mechanism. Importantly, to our knowledge, our work illustrates for the first time an iNKT cell-intrinsic modulation of the TCR Vβ repertoire during thymic selection. Furthermore, to our knowledge, our work is the first to demonstrate that changes in intracellular cholesterol homeostasis profoundly impact iNKT cell development and function.

We (20) and Bensinger et al. (21) have previously reported that alterations in intracellular cholesterol homeostasis in the absence of ABCG1 lead to a hyperproliferative phenotype of conventional T cells. In our previous study, we showed that ABCG1-deficient CD4+ T cells displayed enhanced TCR signaling and proliferation as a result of increased cholesterol and lipid raft content (20). In contrast, this study reveals an opposite role of ABCG1 in iNKT cells, as ABCG1-deficient iNKT cells displayed decreased proliferation and lower membrane lipid raft content. iNKT cells differ from naïve CD4+ T cells in that they have an activated/memory phenotype and they produce large amounts of both Th1 and Th2 cytokines following activation with glycolipid Ags (8). Therefore, it is plausible that iNKT cells have differences in cholesterol metabolism and, as such, different requirements for cholesterol homeostasis than do CD4+ T cells. Excess cholesterol is exported out of the cell by the cholesterol transporters ABCA1 and ABCG1 (39, 40). ABCA1 efluxes cholesterol to lipid-poor apolipoprotein A1 (41), whereas ABCG1 promotes cholesterol eflux to mature high-density lipoprotein particles (13). iNKT cells express both ABCG1 and ABCA1 (Supplemental Fig. 3A, 3B). ABCA1 expression is enhanced in Abcg1−/− iNKT cells compared with B6 iNKT cells (Supplemental Fig. 3B). This is not surprising, because many previous studies have shown that genetic deletion of one cholesterol transporter, either ABCA1 or ABCG1, is compensated for by an upregulation of the other transporter (20, 42, 43). Nonetheless, neither ABCA1 nor ABCG1 can fully compensate for the loss of the other (10). However, we cannot rule out the possibility that changes in ABCA1 expression contributed to our observed findings of altered iNKT cell development and function in Abcg1−/− mice. Whereas, in the absence of ABCG1, the total cholesteryl ester content of CD4+ T cells increases (20), the total cholesteryl ester of iNKT cells is unchanged (Fig. 5A). These results suggest that, in terms of regulating cholesteryl ester content, ABCA1 can substitute for the lack of ABCG1 in iNKT cells, but not in T cells. Future studies of the role of ABCA1 in iNKT cells using floxed mice may be useful to delineate the different roles of these two transporters in iNKT cell development.

Apart from cholesterol eflux, ABCG1 is also important for intracellular cholesterol transport (14, 15). Cholesterol is an essential component of membrane lipid rafts (11). Because iNKT cells have a reduction in the lipid raft content (Fig. 5B, 5C) without any changes in the overall cholesterol content (Fig. 5A) in the absence of ABCG1, the critical role of ABCG1 in iNKT cells seems to be the regulation of the intracellular transport of cholesterol. Miguel et al. (44) have recently shown that the amount of membrane lipid rafts present in CD4+ T cells correlates closely with immunological synapse formation and CD4+ T cell proliferation and activation. Therefore, based on our data, we hypothesize that ABCG1 deficiency causes a decrease in the transport of cholesterol to the cell membrane, affecting lipid rafts, which impacts iNKT cell development and function.

Our results demonstrate that, in the absence of ABCG1, thymic selection of Vβ7+ iNKT cells is favored in a cell-intrinsic manner. Previous studies have shown that Vβ7+ iNKT cells have a lower
affinity toward α-GalCer (45), whereas, based on the analysis of CD1d“I+” heterozygous mice, they may have a higher affinity for the endogenous ligand(s) for the positive selection of iNKT cells in thymus (27). Therefore, the frequency of thymic Vβ7“I+” iNKT cells may be a surrogate marker for the overall avidity of the iTCR toward its endogenous selecting ligand(s) in the thymus. In previous studies, which showed that the thymic selection of the Vβ7“I+” iNKT cells was favored, the reduced avidity of the iTCR toward the selecting ligand(s) was due to reduced expression of CD1d on DP thymocytes (27). However, in our study, the surface expression levels of CD1d on DP thymocytes in Abcg1“I−” mice were unchanged (Fig. 2A). Furthermore, the expression levels of the TCR/CD3 complex were similar in the Abcg1“I−” iNKT cells compared with control data (not shown). As the effects we observed were iNKT cell intrinsic (Fig. 2E), this altogether suggests that the differences in the avidity of the iTCR–CD1d interaction in the Abcg1“I−” mice are due to changes in the iTCR itself. Based on our data, we hypothesize that the alterations in the lipid raft content in the absence of ABCG1 modify the distribution of the TCR in iNKT cells so that it is less colocalized in lipid rafts. These changes may make the iTCR less sensitive to the endogenous ligand(s) presented by CD1d for the positive selection of iNKT cells in thymus. Such a reduction may favor the selection of relatively high-avidity Vβ7“I+” iNKT cells in the thymus and lead to reduced export of iNKT cells to the periphery. Changes in the composition/organization of the iTCR also alter cytokine production of iNKT cells. More work is needed, however, to examine how ABCG1 deficiency leads to a Th1-biased cytokine production.

In summary, we demonstrate a novel role for ABCG1-mediated cholesterol homeostasis in iNKT cell development. iNKT cells have been implicated in the development of atherosclerosis, rheumatoid arthritis, several forms of allergy, as well as autoimmunity (7, 8). All of these diseases have known or proposed lipid constituents that increase risk of disease development. For example, ABCG1-deficient (43, 46), as well as iNKT cell-deficient mice are protected from atherosclerosis development (47–51). Therefore, linking the role of lipid transporters and glycolipid-sensitive iNKT cells could lead to the development of entirely new therapeutic approaches for diseases that have a hyperlipidemic component, such as atherosclerosis.

Acknowledgments

We thank Amy Blatchley, Archana Khurana, the Department of Laboratory Animal Care, and the flow cytometry facility at the La Jolla Institute for Allergy and Immunology for excellent technical assistance. We also thank Dr. Isaac Engel and Dr. Meng Zhao for valuable scientific contributions.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figures

Fig 1

Thymus

Spleen

NK1.1-CD4-  NK1.1+CD4-  NK1.1+CD4+  NK1.1-CD4+  B6  Abcg1-/-

INKT cells (%)

Fig 2

A

CD4+ CD8+ cells (%)

B

CD4+ CD8+ thymocytes

CD1d

MFI

NK1.1-CD4-  NK1.1+CD4-  NK1.1+CD4+  NK1.1-CD4+  B6  Abcg1-/-
Fig 3

A. ABCG1 mRNA relative expression in Thymus iNKT cells

B. ABCA1 mRNA relative expression in Thymus iNKT cells

Fig 4

BrdU+Vβ7+ iNKT cells (%)
Supplemental Figure Legends

FIGURE 1. Abcg1−/− mice display no change in phenotypic iNKT cell subsets. Thymocytes and splenocytes from B6 mice (n = 8) and Abcg1−/− mice (n = 8) were stained with fluorophore-conjugated antibodies and CD1d-tetramer and analyzed by flow cytometry. TCRβ+ and CD1d-tetramer+ cells were further gated to distinguish NK1.1− CD4−, NK1.1− CD4+, NK1.1+ CD4− and NK1.1+ CD4+ iNKT cell subsets. Bar graphs show frequency of iNKT cells in thymus (left) and spleen (right). Data are pooled from 2 independent experiments (4 mice per group for each experiment) with similar results.

FIGURE 2. B6:Abcg1−/− 1:1 mixed chimeric mice do not display differences in the frequency of Abcg1−/− and B6 DP thymocytes and their surface CD1d expression. Single-cell suspensions from thymi of B6:Abcg1−/− 1:1 mixed chimeric mice (n = 10) were stained with fluorophore-conjugated CD45.1, CD45.2, CD4, CD8α and CD1d antibody and analyzed by flow cytometry. Bar graphs show A, frequency of CD45.1+ B6 and CD45.2+ Abcg1−/− CD4+ CD8α DP thymocytes and B, mean fluorescence intensity (MFI) of CD1d on DP thymocytes. Data are representative of 2 independent experiments with similar results.

FIGURE 3. iNKT cells express ABCG1 and ABCA1. iNKT cells were sorted from thymus of B6 and Abcg1−/− mice (n = 6 per group) and expression of A, ABCG1 and B, ABCA1 was measured by quantitative real-time PCR. Data are pooled from 2 independent experiments (3 mice per group for each experiment) with similar results. Error bars represent means ± SEM. Asterisks denote the significance of differences between groups. (***p < 0.001, two-tailed Student’s t test)
FIGURE 4. *Abcg1*−/− Vβ7+ iNKT cells display reduced proliferation. B6 (n = 4) and *Abcg1*−/− (n = 3) mice were injected with BrdU 3 times every four hours. The next day, thymi were harvested and single-cell suspensions were stained with fluorophore-conjugated antibodies including BrdU antibody and CD1d-tetramer and analyzed by flow cytometry. Graph shows BrdU incorporation by Vβ7+ iNKT cells. Data is representative of 2 independent experiments with similar results. (*p < 0.05)*