Glucosylceramide Synthase Is Involved in Development of Invariant Natural Killer T Cells

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Invariant natural killer T (iNKT) cells represent a unique population of CD1d-restricted T lymphocytes expressing an invariant T cell receptor encoded by Vα14-Jα18 and Vα24-Jα18 gene segments in mice and humans, respectively. Recognition of CD1d-loaded endogenous lipid antigen(s) on CD4/CD8-double positive (DP) thymocytes is essential for the development of iNKT cells. The lipid repertoire of DP thymocytes and the identity of the decisive endogenous lipid ligands have not yet been fully elucidated. Glycosphingolipids (GSL) were implicated to serve as endogenous ligands. However, further in vivo investigations were hampered by early embryonal lethality of mice deficient for the key GSL-synthesizing enzyme glucosylceramide (GlcCer) synthase [GlcCer synthase (GCS), EC 2.4.1.80]. We have now analyzed the GSL composition of DP thymocytes and shown that GlcCer represented the sole neutral GSL and the acidic fraction was composed of gangliosides. Furthermore, we report on a mouse model that by combination of Vav-promoter-driven iCre and floxed GCS alleles (VavCreGCSf/f) enabled an efficient depletion of GCS-derived GSL very early in the T cell development, reaching a reduction by 99.6% in DP thymocytes. Although the general T cell population remained unaffected by this depletion, iNKT cells were reduced by approximately 50% in thymus, spleen, and liver and showed a reduced proliferation and an increased apoptosis rate. The Vβ-chains repertoire and development of iNKT cells remained unaltered. The GSL-depletion neither interfered with expression of CD1d, SLAM, and Ly108 molecules nor impeded the antigen presentation on DP thymocytes. These results indicate that GlcCer-derived GSL, in particular GlcCer, contribute to the homeostatic development of iNKT cells.

Keywords: CD1, glycosphingolipid, glucosylceramide, glucosylceramide synthase, natural killer T cell, thymus

INTRODUCTION

Natural killer T (NKT) cells represent a unique T cell population co-expressing NK cell markers such as NK1.1 (CD161) (1, 2). Initially, a subset of NKT cells bearing an invariant T cell receptor (TCR) α-chain (Vα14-Jα18 in mouse and Vα24-Jα18 in human) paired with a limited repertoire of β-chains (Vβ2, Vβ7, Vβ8.2 in mouse and Vβ11 in human) could be identified, hence the designation...
as invariant NKT cells [invariant natural killer T (iNKT) or type I NKT] (3–6). iNKT cells are important mediators of tumor surveillance, peripheral tolerance and antimicrobial defense (7–15).

In contrast to conventional T cells, iNKT cells recognize lipid antigens presented by non-polymorphic MHC class I-like CD1 molecules (16, 17). Human genome encodes for five CD1 molecules that—based on the amino acid sequence—can be assigned to either group I (CD1a, -b, -c, and -e) or group II (CD1d) (18). Mice lack group I CD1 molecules and have two group II Cd1 genes termed Cd1d1 and Cd1d2, from which only Cd1d1 seems to encode for a functional protein (19). Whereas presentation of peptide antigens on HMC molecules of thymic cortical epithelial cells is a prerequisite for the development of conventional T cells, positive selection of iNKT cells requires presentation of lipid antigens by CD1 molecules of double positive (CD4+CD8+) thymocytes (20–22). In addition, lysosomal proteases and sphingolipid activator proteins, also known as saposins, are indispensable for normal thymic iNKT cell development suggesting that loading of lipid antigens onto CD1 molecules plays a crucial role in this process (23–26).

Several microbial, i.e., exogenous, lipid antigens recognized by iNKT cells have been identified (27, 28). α-Galactosylceramide (αGalCer, also referred to as KRN7000), which is derived from the marine sponge *Agelas mauritania*, is the most potent member of this group (29, 30). Other α-anomeric microbial lipids with striking structural similarities to αGalCer and stimulatory effects toward iNKT cells have been found in *Sphingomonas* spp. (31, 32), *Borrelia burgdorferi* (33), and *Streptococcus pneumoniae* (34).

By contrast, lipid antigens mediating positive selection and peripheral homeostasis of iNKT cells are obviously of endogenous and not of microbial origin as implicated by the fact that germ-free mice show an unaltered iNKT cell population (35). A variety of endogenous lipids (mostly phospholipids and sphingolipids) have been shown to be captured by CD1d during endosomal–lysosomal recycling or on the secretory pathway (36–39). However, most iNKT cells do not respond to these lipids and the reactivity toward them is restricted to singular iNKT cell clones (40).

Despite an extensive research, the identity of the endogenous lipid antigen(s) responsible for the thymic selection of iNKT cells remains partially unresolved (41, 42). It has been demonstrated that mice deficient for glyceronephosphate O-acyltransferase (GNPAT) show an altered iNKT cell population (43). Based on the observation that cells deficient in glucosylceramide (GlCer)-based glycosphingolipids (GSL) (*Figure 1*) were unable to stimulate iNKT cell hybridomas, it was suggested that the endogenous selecting ligand might be GlCer or a GlCer-derived GSL (44). Subsequent studies pinpointed to GlCer as an endogenous lipid antigen mediating activation of iNKT cells in response to microbial danger signals (45). However, later, the same group reported that a minor—hitherto unidentified—lipid co-purifying with GlCer might function as the actual self-lipid antigen (46). Until now, in vivo experiments addressing the putative role of GlCer-derived GSL during thymic iNKT cell development were hampered by an early embryonic lethality of mice deficient for GlCer synthase (GCS) (47).

In the present study, we have analyzed the GSL composition of double-positive (DP) thymocytes and shown that besides GlCer, these cells expressed GlcCer-derived acidic GSL from the ganglioside series such as GM1a, GM1b, GD1a, and GD1c. Furthermore, we have circumvented the lethality of GCS-deficient embryos by investigating mice with tissue-specific deletion of the GCS gene (Vavε+/GCSf/f) and demonstrated that depletion of GlCer-derived GSL in DP thymocytes resulted in a significant reduction of the iNKT cell population. Thus, GlCer-derived GSL represent relevant endogenous lipids contributing to the development of iNKT cells.

**MATERIALS AND METHODS**

**Experimental Mice**

Mice with floxed GCS (Ugcg, EC 2.4.1.80) alleles were described previously (48). TCRα14-Jta281 transgenic mice were kindly provided by Agnes Leheuen (49). CD1d-deficient (50) and Vavε+/transgenic (51) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All strains were backcrossed for more than 10 generations to the C57BL/6 genetic background (Charles River Wiga, Sulzfeld, Germany) and housed under specific pathogen-free conditions. Vavε-negative littermates were used as wild-type (WT) controls. Animal experiments were performed in compliance with the German guidelines on animal protection.

**Organ Preparation, Flow Cytometry, and Cell Sorting**

Single cell preparations from organs were prepared as described previously (52). Flow cytometry was performed as described in Ref. (53). The following monoclonal antibodies were used: anti-CD1d (clone: 1B1); anti-CD3ε (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD11c (HL3), anti-CD19 (MB19-1), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD150/SLAM (9D1), and anti-NK1.1 (PK136), provided by NIH Tetramer Core Facility at Emory University (Atlanta, GA, USA). BrdU and Annexin V experiments were performed according to the manufacturer's protocol (both BD). Analysis of flow cytometry data was performed using Flow Cytometry Analysis Software (BD) and FlowJo (Tree Star, Flow Cytometry Analysis Software) by gating on lymphocytes in the forward and side scatter. Double-positive thymocytes were sorted using FACSARia™ (BD) by gating stringently on CD4+/CD8+ DP lymphocytes and excluding 7AAD-positive dead cells.

**RNA Isolation and Quantitative PCR**

RNA was extracted from cell pellets using the phenol/chloroform extraction method (54) followed by digestion by RNase-free DNasel (turbo DNA free, Ambion, Huntingdon, UK). A
total of 3 µg of total RNA were reverse transcribed in 20-µl total volume using SuperscriptII (Invitrogen, Karlsruhe, Germany) according to the manufacturers’ instructions. RT-PCR was performed with 1 µl cDNA and GCS primers: forward 5′—gat cta aga ggg tga agg cgc a—3′ and reverse 5′—ctg cct tgc aat cct gtc tgt c—3′.

Isolation and Analysis of GSL

Glycosphingolipids were extracted from lyophilized cell pellets as described in detail in Ref. (55, 56). For thin layer chromatography (TLC) analysis, an amount corresponding to 0.2 mg protein was loaded on a TLC plate (Merck, Darmstadt, Germany). Running solvent was CHCl₃/CH₃OH/H₂O (62.5:30:6, v/v/v) for neutral GSL and CHCl₃/CH₃OH/0.2% CaCl₂ in H₂O (60:35:8, v/v/v) for acidic GSL, respectively. Sialidase treatment was performed as described in Ref. (57). 0.05 U Vibrio cholerae sialidase in 0.2 M Na-acetate buffer, 2 mM CaCl₂, pH 5.2, was used to digest acidic GSL on a polyisobutylmethacrylate-fixed TLC plate at room temperature for 8 h.

Mass Spectrometric Analyses

Sphingolipids from DP thymocytes were extracted as previously described with slight modifications (58). Briefly, sorted thymocytes (~5 × 10⁶) were dried with 1-propanol and extracted twice at 37°C for 15 min with a chloroform/methanol/water mixture of 10/10/1 (v/v/v) and once with 30/60/8. The residual cell pellets were used for protein determination according to the Lowry method. The combined lipid extracts were dried under air flow and subsequently subjected to mild alkaline hydrolysis with 0.1 M potassium hydroxide in methanol for 2 h at 37°C. Saponified extracts were finally desalted by reverse-phase (C18) column chromatography. Aliquots corresponding to 30 µg of protein were dissolved in 1 ml 95% methanol containing the following internal standard mixture: Cer (d18:1;14:0), Cer (d18:1;19:0), Cer (d18:1;25:0), Cer (d18:1;31:0) each 4 pmol; GlcCer (d18:1;14:0), GlcCer (d18:1;19:0), GlcCer (d18:1;25:0), and GlcCer (d18:1;31:0) each 2 pmol.

For quantification of lipid extracts, UPLC–ESI–MS/MS analyses were performed as described in Ref. (59) with following
In Vitro and In Vivo Experiments with iNKT and T Cells

Double-positive thymocytes were isolated from WT, Vav\textsuperscript{CreGCS\textsuperscript{f/f}}, and CD1d\textsuperscript{−/−} thymus after depleting cells reactive with PBS57-loaded CD1d tetramers. 0.5 × 10\textsuperscript{5} DP thymocytes per well were placed in 96-well-plate and incubated with αGalCer (Avanti Polar Lipids, Alabaster, AL, USA) at indicated concentrations. iNKT cells were enriched by antibodies against CD1d and CD90.2 (Miltenyi Biotec) and applied at 50,000/well. Activation of T cells was performed as described in Ref. (60). Briefly, splenic T-cells were enriched by anti-CD90.2 micro beads (Miltenyi Biotec) and applied at 50,000/well. Activation of T cells in vitro was performed as described in Ref. (60). Briefly, splenic T-cells were enriched by anti-CD90.2 micro beads (Miltenyi Biotec) and incubated with 0.5 mg/ml calcium ionophore A23187 and 10 ng/mL phorbol 12-myristate 13-acetate (PMA, both Sigma). Supernatants were collected after 18 h and analyzed for IFN\textgamma. and incubated with 0.5 mg/ml calcium ionophore A23187 and 10 ng/mL phorbol 12-myristate 13-acetate (PMA, both Sigma). Supernatants were collected after 18 h and analyzed for IFN\textgamma. and IL4 concentrations by cytometric bead array technique (BD). For the in vivo testing of iNKT cells function, mice were injected i.p. with 0.2 or 3 µg αGalCer and sacrificed 8 h later.

Super-Resolution Microscopy

Thymocytes of WT and Vav\textsuperscript{CreGCS\textsuperscript{f/f}} mice were enriched by magnetic separation using CD5 beads (Miltenyi), spun down using cytosin system (4 × 10\textsuperscript{5} cells/slide) and fixed in 1% paraformaldehyde in PBS for 15 min at room temperature. Cells were then incubated with antibodies against CD1d-FITC (BD), early endosome antigen 1 (EEA1) (Cell Signaling), Rab7 (Santa Cruz Biotechnology), and lysosome-associated membrane protein 1 (LAMP1) (eBioscience). After washing, corresponding Alexa-Fluor 546-conjugated secondary antibodies were added and the slides were incubated for 1 h at room temperature in the dark. DAPI was used for nuclear visualization. Negative controls contained DAPI staining and Alexa-Fluor 546-conjugated secondary antibodies (for EEA1, Rab7, and LAMP1), or DAPI staining only (for CD1d). Images were acquired using the Olympus IX81 motorized microscope equipped with the MT20 illumination system; the C3, GFP, and DAPI HC-Filter sets; and Hamamatsu Orca-ER CCD camera. Two hundred images from each channel were acquired for each region using the 100x/1.4 PlanApo objective, and five regions were analyzed from each sample. Images were post-processed with ImageJ (http://rsbweb.nih.gov/ij) to obtain super-resolution optical fluctuation images—www.ncbi.nlm.nih.gov/pubmed/20018714. On average, 20 cells were analyzed for co-localization between red and green using the ImageJ's co-localization plugin, and the ratio of co-localized and total green area was plotted and statistically analyzed. The images of lysosomes were further analyzed automatically with the same parameters using ImageJ macro developed at DKFZ Light Microscopy Core Facility (Heidelberg, Germany). Shortly, images of lysosomes were thresholded and segmented using the Find Maxima tool with the Segmented Particles above lower threshold option activated. The segmented particles above the minimum area limit of 10 pixels (0.022 µm\textsuperscript{2}) were further counted for each cell using ImageJ's Analyze Particles tool.

Statistical Analysis

Unpaired two-tailed Student's t-test was performed to compare data sets. Differences were considered significant if \( p < 0.05 \). Numbers of independent observations per group are indicated for each result.

RESULTS

GSL Composition of DP Thymocytes

Because the development of INKT cells depends on presentation of lipid antigens on DP thymocytes, we analyzed the latter cell population for its GSL composition in WT mice. In the neutral GSL fraction, hexosylceramides represented the major fraction (Figure 2A). In the acidic fraction, two compounds running at the height of GM1 and GD1, respectively, emerged (Figure 2A). In order to further characterize these substances, the acidic fraction was digested by neuraminidase and the products were subsequently separated into acidic and neutral fractions (Figure 2B). The hereby obtained acidic compound ran at the height of the GM1 standard suggesting that this band probably consisted of a mixture of non-digestible GM1 or was derived from GD1b after the release of the terminal sialic acid. By contrast, the neutral compound was not represented in the standards; however, due to its running properties, it likely corresponded to neutral ganglioside GA1 (Figure 2B). To identify this product, the original acidic fraction was on-plate digested with neuraminidase and subsequently immunostained using anti-GA1 antibodies (Figure 2C). Both the upper and the lower compounds had a neutral backbone of GA1 (Figure 2C, left panel). Based on the running properties and the comparison with the standards, these compounds likely correspond to GM1a, GM1b, GD1b, and GD1c.

Characterization of Vav\textsuperscript{CreGCS\textsuperscript{f/f}} Mice

Although several lines of evidence have implicated that GlcCer-based GSL might belong to the lipid antigens relevant for the INKT cell development, a direct proof of this hypothesis was precluded by the early embryonal lethality of GCS-deficient mice (47). To overcome this problem, we implemented a tissue-specific characterization of Vav\textsuperscript{CreGCS\textsuperscript{f/f}} mice under the control of the Vav-promoter (VavCre) that activates the recombinase activity very early in T-cell development with virtually 100% of DN1 (CD25\textsuperscript{−}/CD44\textsuperscript{+}) thymocytes being already positive (51). In terms of TLC analysis, no GSL could be detected in DP thymocytes from Vav\textsuperscript{CreGCS\textsuperscript{f/f}} mice (Figure 3A). This was in
line with extensive and significant reduction of GCS mRNA in these cells (Figure 3B). Mass spectrometry performed on FACS-sorted DP thymocytes revealed a 99.6% reduction of the GlcCer content in Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} mice as compared to WT (Figure 3C). This reduction occurred independently of the analyzed acyl moiety (Figure 3D). By contrast, the ceramide content of Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} DP thymocytes was indistinguishable from WT (Figures 3E,F).

Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} mice reproduced normally and progeny were born at expected Mendelian ratios (data not shown). Newborn and adult Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} mice did not exhibit any overt growth, developmental or behavioral defects. Body weight, the weight, and cellularity of thymus and spleen were indistinguishable from Vav\textsuperscript{Cre}-negative littermates (Figure 4A). Similarly, no aberration in the maturation of the conventional thymocytes could be revealed by flow cytometry in Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} mice (Figure 4B). In spleens, the amount of CD3- and CD19-positive T- and B-lymphocytes, respectively, was unaffected by the deletion of the GCS gene (Figure 4C). The expression levels of CD1d on Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} DP thymocytes and splenic CD11c\textsuperscript{+}/MHCII\textsuperscript{+} dendritic cells were indistinguishable from WT mice (Figures 4D,E). The expression of SLAM (CD150) and Ly108 molecules, which provide important signals on DP thymocytes during the thymic iNKT cell development (61), did not significantly differ between Vav\textsuperscript{Cre}GCS\textsuperscript{f/f} and WT mice (Figures 4F,G).

Furthermore, CD1d trafficking was analyzed using super-resolution microscopy and EEA1, Rab7, and LAMP1 as markers of early endosomes, late endosomes and lysosomes, respectively (Figures 5A–C). The quantification of signal co-localization
Figure 3 | Glycosphingolipid (GSL) depletion in Vav\textsuperscript{Cre}GCS\textsuperscript{ff} double-positive (DP) thymocytes. (A) GSL were extracted from enriched DP thymocytes from 8-week-old wild-type (WT) and Vav\textsuperscript{Cre}GCS\textsuperscript{ff} mice, separated into neutral and acidic fractions and analyzed by thin layer chromatography. In DP thymocytes from Vav\textsuperscript{Cre}GCS\textsuperscript{ff} mice, no residual GSL could be detected. The substances in the neutral fraction marked by asterisks do not show the typical orcinol color for GSL and therefore very unlikely represent those compounds. Shown are results from four different experimental animals per group. (B) FACS-sorted DP thymocytes were analyzed for the expression of GlcCer synthase (GCS) by quantitative PCR. The expression was normalized to 18S rRNA. Shown are means ± SEM, N = 7 per group. (C–F) GSL were extracted from FACS-sorted DP thymocytes. The content of GlcCer (C,D) and ceramide (E,F) was quantified by mass spectrometry and normalized for the protein amount in the sample. Panels (C,E) show the total amount of GlcCer and ceramide, respectively. In panels (D,F), the composition of acyl moieties of GlcCer and ceramide, respectively, are displayed. Some of the bars for Vav\textsuperscript{Cre}GCS\textsuperscript{ff} are barely visible due to very low levels. Shown are means ± SEM, N = 6 and 7 per group, respectively. Statistically significant differences between WT and Vav\textsuperscript{Cre}GCS\textsuperscript{ff} mice are indicated: ** p < 0.01; *** p < 0.001.
revealed a statistically significant shift of CD1d from late to early endosomes in Vav<sup>Cre</sup>GCS<sup>ff</sup> mice. In contrast, the CD1d amount in lysosomes remained unaffected (Figure 5D). Although a tendency toward less but larger lysosomes could be seen in DP thymocytes of Vav<sup>Cre</sup>GCS<sup>ff</sup> mice, the difference was not statistically significant (Figure 5E).
### Significant Reduction of the iNKT Cell Population in VavCreGCSff Mice

In newborn and adult mice, the iNKT cell populations were characterized by flow cytometry using PB57-loaded CD1d tetramers. Adult VavCreGCSff mice showed a significant reduction of the iNKT population in terms of absolute numbers and percentages in thymus, spleen, and liver as compared to WT littermates (Figure 6A). In all three organs, a reduction of the iNKT cell population by approximately 50% could be observed. In VavCreGCSff mice, the remaining iNKT cells could be clearly identified and discerned from any unspecific staining as visualized by comparison with CD1d-deficient mice that do not produce iNKT cells (Figure 6A). Newborn mice showed identical results (data not shown). To test for possible unspecific effects of the VavCre transgene, iNKT cell frequencies and absolute numbers were compared between VavCre-positive and VavCre-negative GCS+/- mice showing no statistically significant differences (Table S1 in Supplementary Material).

In course of thymic maturation, iNKT cells upregulate expression of NK1.1 and CD44 allowing the identification of three developmental stages: immature, CD44+/NK1.1-; semi-mature, CD44+/NK1.1+; and mature, CD44+/NK1.1+ (62). In terms of absolute numbers, iNKT cells were significantly reduced in all three developmental stages in VavCreGCSff mice. However, no significant difference was observed in the percentual distribution among these three stages (Figure 6B).

In iNKT cells, the invariant Vα14-chain pairs almost exclusively with Vβ2, 7, or 8.2 (63). We tested whether the depletion of GCS-derived GSL would lead to a shift of the Vβ-chain repertoire in VavCreGCSff mice. However, no statistically significant difference in the percentage distribution of the Vβ-chains could be found between VavCreGCSff mice and WT littermates. In terms of absolute numbers, a decrease corresponding to the diminished iNKT cell population could be observed (Figure 6C).

Measurements of proliferation and apoptosis rate by BrdU incorporation and Annexin V staining, respectively, revealed that in VavCreGCSff mice, thymic iNKT cells showed a significantly reduced proliferation and an increased apoptosis as compared to WT mice. By contrast, conventional thymocytes were unaffected (Figures 6D,E).

### Deletion of GCS Did Not Affect the Processes of Antigen Presentation and Recognition

Depletion of GCS-derived GSL in DP thymocytes might not only alter the repertoire of lipid antigens but also impact the processes of their presentation. Thus, in order to test the antigen presenting capacity of VavCreGCSff DP thymocytes, these cells were exposed to increasing concentrations of the exogenous antigen αGalCer and co-incubated with WT responder iNKT cells enriched from livers of TCRVα14-αβ28 transgenic mice. As measured by secretion of IFNγ and IL4, no statistically significant difference could be observed between the antigen presentation on DP thymocytes from VavCreGCSff and WT mice (Figures 7A,B).

Furthermore, we have subjected VavCreGCSff iNKT cells to functional tests in vivo and in vitro. Upon injection of αGalCer, upregulation of CD69 on iNKT cells was unaltered in VavCreGCSff mice (Figure 7C). IFNγ levels were significantly lower in VavCreGCSff mice injected with 3 µg αGalCer. Similarly, IL4 levels tended to be lower in the VavCreGCSff mice although a statistical significance was not reached (Figure 7C). Therefore, we tested the reactivity of VavCreGCSff iNKT cells in vitro with equalized cell numbers. To this end, WT DP thymocytes
FIGURE 5 | Continued
The paradox that iNKT cell development remains unaltered after depletion of singular GlcCer-derived GSL groups offers three explanations: (a) lipids other than GSL, (b) other—yet unaddressed—GlcCer-derived GSL, or (c) the GlcCer itself mediate the positive selection of iNKT cells. Several lines of evidence have shown that also lipids other than GSL might be important for the iNKT cell activation and development (38, 43). The first publication has demonstrated that ether-bonded mono-alkyl glycerophosphates stimulated iNKT cells and that deficiency for GNPAT led to an approximately 50% reduction of the iNKT cell population in vivo (43). However, GNPAT-deficient mice have multiple severe abnormalities and those that survive develop hypomorphism (66), altogether making the exclusion of any unspecific effects on the iNKT cell population very challenging. By contrast, Brennan et al. have pinpointed to βGlcCer as the self-antigen responsible for activation of iNKT cells by dendritic cells upon recognition of microbial danger signals (45). However, their later findings implicated that not βGlcCer but a rare, yet unknown, component of the GlcCer fraction should be responsible for the stimulation of iNKT cells (46). Recently, Kain et al. could identify trace amounts of α-anomeric GSL in mammalian immune cells and demonstrate their stimulatory capacity toward iNKT cells (67).

DISCUSSION

Although substantial progress in understanding the function of iNKT cells has been achieved since their discovery two decades ago, the identity of the endogenous lipid antigen(s) mediating their thymic positive selection and peripheral activation remains largely elusive. Originally, it has been shown that cells deficient in GlcCer-based GSL were unable to stimulate iNKT cell hybridomas, thus implicating that the endogenous ligand might be GlcCer or a GlcCer-derived GSL (Figure 1) (44). However, mice deficient for singular series of GlcCer-derived GSL such as gangliosides, globosides, isoglobosides, and sulfatides were shown to have normal iNKT cell numbers (46). Recently, Brennan et al. could identify trace amounts of α-anomeric GSL in mammalian immune cells and demonstrate their stimulatory capacity toward iNKT cells (67).

Independently of these ambiguous results on GlcCer with regard to its stimulatory role for iNKT cells in the periphery, it remained

were loaded with αGalCer and co-incubated with iNKT cells enriched from spleens and livers of VavCreGCSff or WT mice. No functional deficiency could be observed between iNKT cells from VavCreGCSff and WT mice as measured by IFNγ secretion (Figure 7D).

In line with the latter result, the general T cell population of VavCreGCSff mice was unaffected and showed an unaltered production of IFNγ in response to TCR-independent (PMA/calcium ionophore) or TCR-dependent (CD3/CD28) stimulation (Figure 7E).

FIGURE 5 | CD1d trafficking through early endosomes, late endosomes, and lysosomes. (A–C) In double-positive (DP) thymocytes, super-resolution microscopy was used to determine intracellular localization of CD1d molecules in early endosomes, late endosomes, and lysosomes visualized in the red channel by early endosome antigen 1 (EEA1), Rab7, and lysosome-associated membrane protein 1 (LAMP1), respectively. Co-localization areas were presented in white (right panels). DAPI was applied to visualize the nucleus (bar = 5 µm). (D) DP thymocytes were analyzed for co-localization between green and red signals using the ImageJ’s co-localization plugin, and the ratio of co-localized and total green area was plotted and statistically analyzed using the unpaired t-test. Although a significant shift from late to toward early endosomes could be observed in VavCreGCSff DP thymocytes, the amount of CD1d in lysosomes was equal. Shown are means ± SEM, N = 20 cells per group. (E) A tendency toward less but larger LAMP1-positive lysosomes could be seen in DP thymocytes of VavCreGCSff mice, however, the difference was not statistically significant. The bars show means ± SEM, N = 7 cells per group.
unknown whether GlcCer-derived GSL (be it α- or β-anomers) might represent also the endogenous lipid antigen in the process of thymic iNKT cell selection.

We describe here the first functional *in vivo* model that has allowed for a depletion of GlcCer and GlcCer-derived GSL in DP thymocytes and that has shown a significant reduction
in iNKT cells. Due to the very early activation of the VαVβ promoter in hematopoietic progenitors (51), it was possible to achieve not only a deletion of the GCS mRNA but also a highly efficient depletion of its product GlcCer that averaged at 99.6% in DP thymocytes. The finding of the residual 0.4% GlcCer in VavCreGCSf/f DP thymocytes might have several explanations: (a) it represents residual, not yet catabolized, traces of intrinsic GlcCer in VavCreGCSf/f DP thymocytes. (b) Thymocytes could potentially utilize blood-derived GSL in vivo as it has been shown also for other cell types (68). Of note, we have omitted any exposure of the thymocytes to fetal calf serum or albumin during their ex vivo preparation and sorting. (c) Contamination by epithelial cells or cell fragments before or during the sorting might have artificially contributed to the measured residual GlcCer levels. For conventional T cells, it has been demonstrated that even a single antigen–MHC complex can elicit their activation (69, 70). Therefore, it cannot be excluded that also such trace amounts of GlcCer still found on DP thymocytes would enable sufficient activation of iNKT cells defined as CD3+/PBS57-CD1d+ thymocytes. Shown are relative and absolute numbers (right and left panels, respectively) of iNKT cells expressing the corresponding TCRVβ-chain. No statistically significant differences could be observed between WT and VavCreGCSf/f mice in terms of relative numbers (i.e., distribution among the three stages). The significant reduction in absolute numbers reflected the overall diminished iNKT cell population in VavCreGCSf/f mice. N = 16/group in the left panel and 10/group in the right panel, respectively. (C) Usage of TCRVβ-chains by splenic iNKT cells was investigated in 8-week-old mice. Analyses were gated on CD19+/PBS57-CD1d+/CD44+ splenocytes. Shown are relative and absolute numbers (left and right panels, respectively) of iNKT cells expressing the corresponding TCRVβ-chain. No statistically significant differences could be observed between WT and VavCreGCSf/f mice in terms of relative numbers (i.e., distribution among the three TCRVβ-chains). The reduction in the absolute numbers reflected the diminished iNKT cell population in VavCreGCSf/f mice. N = 9/group in the left panel and 6/group in the right panel, respectively. (D, E) Proliferation and apoptosis of thymic iNKT cells were measured in 8-week-old mice using BrdU incorporation and Annexin V staining, respectively. In VavCreGCSf/f mice, iNKT cells (CD3+/PBS57-CD1d+ thymocytes) showed a significantly reduced proliferation and an increased apoptosis as compared to WT controls. By contrast, conventional thymocytes were unaffected. N = 5/group. Bars represent means ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ns, non-significant.
FIGURE 7 | Antigen presentation and recognition in Vav<sup>CreGCS<sup>ff</sup> mice. (A,B) Double-positive (DP) thymocytes were tested for their antigen presentation capacity toward invariant natural killer T (iNKT) cells in vitro. To this end, iNKT-depleted wild-type (WT), Vav<sup>CreGCS<sup>ff</sup> mice, and CD1<sup>d−/−</sup> DP thymocytes were exposed to increasing concentrations of αGalCer and co-incubated with responder WT iNKT cells enriched from livers of TCR<sup>α14-Jα281</sup> transgenic mice. The activation measured as secretion of IFN<sub>γ</sub> (A) and IL4 (B) did not differ between WT and Vav<sup>CreGCS<sup>ff</sup> DP thymocytes. CD1<sup>d−/−</sup> DP thymocytes served as negative controls, and the corresponding bars cannot be discriminated from the zero line in all but one concentration. Shown are means ± SEM, N = 6–9 per group. (C) Activation of iNKT cells was tested in vivo. WT and Vav<sup>CreGCS<sup>ff</sup> mice were i.p. injected with either 0.2 or 3 µg αGalCer. Eight hours later, splenic iNKT cells were analyzed for surface CD69 expression by flow cytometry by gating on CD19<sup>−</sup>/PBS57-CD1d<sup>+</sup>/CD44<sup>+</sup> lymphocytes. Expression of CD69 did not differ between WT and Vav<sup>CreGCS<sup>ff</sup> DP thymocytes. In parallel, serum was analyzed for IFN<sub>γ</sub> and IL4 levels. In Vav<sup>CreGCS<sup>ff</sup> mice injected with 3 µg αGalCer, IFN<sub>γ</sub> levels were significantly lower than in the WT controls. All other measurements did not show a statistically significant difference. Shown are means ± SEM, N = 3 per group. (D) Activation of iNKT cells was tested in vitro. iNKT cells from livers and spleens of WT and Vav<sup>CreGCS<sup>ff</sup> mice were exposed to αGalCer-loaded WT DP thymocytes. The activation measured as IFN<sub>γ</sub> secretion did not differ between WT and Vav<sup>CreGCS<sup>ff</sup> iNKT cells. Shown are means ± SEM, n = 3–6 per group. (E) Splenic conventional T cells were tested for their T cell receptor (TCR)-independent and TCR-dependent activation in vitro. WT and Vav<sup>CreGCS<sup>ff</sup> splenic T cells were activated by PMA/calcium ionophore A23187 or by plate-bound anti-CD3/anti-CD28 antibodies. Vehicle (media)-treated cells served as controls. No statistically significant differences could be found in the IFN<sub>γ</sub> secretion between WT and Vav<sup>CreGCS<sup>ff</sup> T cells. Shown are means ± SEM, n = 6 per group.

GSL composition of unsorted thymocytes and CD4- and CD8-positive T cells (73). However, gangliosides unlikely represent the iNKT selecting endogenous ligands as mice deficient for ganglioside-synthesizing enzymes were shown to have normal iNKT cell populations (56, 74). These findings pinpoint to GlcCer (and not its downstream metabolites gangliosides) as a
GSL present on DP thymocytes and of importance in iNKT cell selection.

In summary, our results demonstrate in vivo that GCS-dependent GSL, in particular GlcCer, influence the homeostatic iNKT cell development.

ETHICS STATEMENT

Animal experiments were performed in compliance with the German guidelines on animal protection and approved by the committee (Regierungspräsidium Karlsruhe).

AUTHOR CONTRIBUTIONS

SP initiated the study and wrote the manuscript. ZP, MR, RJ, DK, RS, and SP performed and evaluated experiments. H-JG provided conceptualization. ZP, MR, RJ, DK, RS, and SP performed and evaluated experiments. H-JG provided conceptualization. ZP, MR, RJ, DK, RS, and SP performed and evaluated experiments. H-JG provided conceptualization.

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SUPPLEMENTARY MATERIAL

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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