Transcription factor Bcl11b controls selection of invariant natural killer T-cells by regulating glycolipid presentation in double-positive thymocytes

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Invariant natural killer T cells (iNKT cells) are innate-like T cells important in immune regulation, antimicrobial protection, and anti-tumor responses. They express semi-invariant T cell receptors, which recognize glycolipid antigens. Their positive selection is mediated by double-positive (DP) thymocytes, which present glycolipid self-antigens through the noncanonical MHC class I-like molecule CD1d. Here we provide genetic and biochemical evidence that removal of the transcription factor Bcl11b in DP thymocytes leads to an early block in iNKT cell development, caused by both iNKT cell extrinsic and intrinsic defects. Specifically, *Bcl11b*-deficient DP thymocytes failed to support *Bcl11b*-sufficient iNKT precursor development due to defective glycolipid self-antigen presentation, and showed enlarged lysosomes and accumulation of glycosphingolipids. Expression of genes encoding lysosomal proteins with roles in sphingolipid metabolism and glycolipid presentation was found to be altered in *Bcl11b*-deficient DP thymocytes. These include cathepsins and Niemann–Pick disease type A, B, and C genes. Thus, Bcl11b plays a central role in presentation of glycolipid self-antigens by DP thymocytes, and regulates directly or indirectly expression of lysosomal genes, exerting a critical extrinsic role in development of iNKT lineage, in addition to the intrinsic role in iNKT precursors. These studies demonstrate a unique and previously undescribed role of Bcl11b in DP thymocytes, in addition to the critical function in positive selection of conventional CD4 and CD8 single-positive thymocytes.

invariant natural killer T-cell development | lysosomal storage disorder | transcriptional control of iNKT lineage | iNKT cell selection

Invariant natural killer T cells (iNKT cells) play important roles in the immune response against several microbes, including *Mycobacterium*, *Sphingomonas*, *Borelia burgdorferi*, and *Leishmania donovani*, and participate in tumor rejection and allergy (1, 2). The vast majority of iNKT cells bear semiinvariant T-cell receptors (TCRs), composed of a Vα14–Jα18 chain paired with Vβ chains of limited diversity, including Vβ7, -8, and -2, and bind glycolipid antigens presented by the MHC class I-like molecule CD1d (3). iNKT cells also express natural killer (NK) cell receptors, such as NK1.1, NKG2A, NKG2D, and members of the Ly-49 family. Thymic selection of iNKT precursors is mediated by cortical double-positive (DP) thymocytes, which present endogenous glycolipid self-antigens generated by lysosomal processing of sphingolipids (4). It has been suggested that the selecting ligand for iNKT cells is isoglobotrihexosylceramide (iGb3; ref. 5). However, mice lacking the iGb3 synthase present normal development and function of iNKT cells (6), and in humans, the iGb3 synthase is nonfunctional because of inactivating mutations (7), therefore making the identity of the self-selecting lipids unclear. Lysosomal enzymes require sphingolipid activator proteins (SAPs), which include saposins A–D (8) and Gm2 activator protein (Gm2a; ref. 9). Saposins are generated from the precursor prosaposin through cleavage by the lysosomal proteases cathepsins, especially cathepsin D (10). SAPs also participate in the lipid loading on CD1d (11).

Humans and mice with specific mutations in critical genes for sphingolipid processing present lysosomal storage disorders, with accumulation of unmetabolized substrates, affecting multiple organs, and often presenting neurodegeneration and iNKT cell defects (reviewed in ref. 12). Such diseases include Tay–Sachs, with mutations in β-hexosaminidase A gene; Sandhoff, with mutations in β-hexosaminidase A/B genes; Fabry, with mutations in α-galactosidase gene; and GM1 gangliosidosis, with mutations in β-galactosidase gene (*Glb1*). In addition, mutations in Niemann–Pick disease type C1 (*Npc1*) and C2 (*Npc2*) genes, encoding lysosomal proteins, cause accumulation of glycosphingolipids and cholesterol. Mutations in the acid sphingomyelinase gene (*Smpd1*) are associated with Niemann–Pick disease types A and B, with accumulation of sphingomyelin, gangliosides, and cholesterol (13), whereas mutations in prosaposin gene cause accumulation of multiple sphingolipids (reviewed in ref. 12).

Selection of iNKT precursors also requires engagement of TCR by the self lipid-loaded CD1d, as well as homotypic, and possibly heterotypic, interactions between SLAM family members (14). After selection, thymic iNKT precursors are $NK1.1^{-}HSA^{hi}CD44^{low}$. They next down-regulate HSA and further up-regulate CD44, followed by up-regulation of NK1.1 and DX5, in addition to several other NK receptors, to become mature iNKT cells (15).

Several transcription factors have been described to play critical roles in iNKT development. T-bet and IRF-1 are important for the maturation and homeostasis (16–18), and Runx1 and Rorgt are required for the overall development of iNKT cells (19). Ets-1 controls their survival (20), and Erg-2 is involved in development and maturation (21). NF-κB is required for the transition from $NK1.1^{-}$ to $NK1.1^{+}$ mature iNKT cells (22); PLZF controls the innate iNKT cell phenotype (23, 24); and c-Myc controls the precursor proliferation (25).

Bcl11b is a C_2H_2 zinc finger transcriptional regulator (26, 27) expressed in T cells (28–33), including iNKT (this work). It plays a critical role in T cell commitment (34–36), thymocytes selection and survival (30), and expansion of mature $CD8^{+}$ T cells during the immune response (37). A recent report indicated that the removal of Bcl11b resulted in absence of iNKT cells in thymus and spleen (38); however, no information was provided in regard to the mechanisms causing the pronounced reduction in the iNKT cell numbers. In the present study, we investigated in depth the role of

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Bcl11b in iNKT lineage development and demonstrated by genetic means that Bcl11b plays both an iNKT cell extrinsic and intrinsic role: (*i*) Bcl11b is necessary in the DP thymocytes to support the selection of thymic iNKT precursors; and (*ii*) Bcl11b is also intrinsically required for the iNKT precursors. We further focused on the role of Bcl11b in glycolipid self-antigen presentation by DP thymocytes and showed that multiple defects in the lysosomal genes—important for glycolipid processing, trafficking, and loading on CD1d—contribute to the unique defect.

Results

Conditional Removal of *Bcl11b* at DP stage of T-Cell Development Hampers the Generation of iNKT Cells, Despite That Vα14–Jα18 TCR Is Rearranged. Bcl11b is expressed in iNKT cells both in the thymus and periphery (Fig. S1*A*), and its removal was found to cause absence of these cells in the thymus and spleen (38). We further evaluated the iNKT cells of *Bcl11b*$^{F/F}$CD4Cre mice and found a profound reduction of iNKT cells in the liver; both the CD4$^+$ and CD4$^-$ iNKT populations were affected (Fig. 1 *A* and *B*). In terms of absolute numbers, there was an ∼20-fold reduction in the liver compared with wild type (WT; Fig. S1*B*).

The absence of the CD1dT-PBS57-binding cells above background in the thymus (Fig. 1*C*) suggests a very early iNKT developmental block, likely at positive selection stage. The study of Kastner et al. (38) did not provide any insight in regard to the mechanisms responsible for the iNKT developmental block in the absence of *Bcl11b*; therefore, we further set up systems to study the mechanism(s). First, the absence of the iNKT thymic population could be caused by a failure in the Vα14–Jα18 TCR rearrangements. However, this scenario was not the case, as Vα14–Jα18 mRNA levels were similar to WT (Fig. S1*C*). This result was expected, as we previously demonstrated that both early and late TCR-α rearrangements occurred in *Bcl11b*-deficient DP, despite their reduced survival (30).

***Bcl11b*-Deficient DP Thymocytes Are Unable to Support the Selection of *Bcl11b*-Sufficient iNKT Precursors.** The selection of iNKT precursors is mediated by DP thymocytes, which present glycolipid self-antigens loaded on CD1d, as opposed to conventional T cells, which are selected by thymic cortical epithelial cells, which present peptide self-antigens (4). Considering that *Bcl11b*-deficient thymocytes present alterations in expression of many genes—beyond those implicated in positive selection and survival (refs. 30 and 38 and this study)—we hypothesized that other functions of DP thymocytes may be affected. In this regard, the absence of iNKT cells in the thymus of *Bcl11b*$^{F/F}$CD4Cre mice could be caused by a defect in the ability of DP thymocytes to present endogenous glycolipids, necessary for the positive selection of iNKT precursors, in addition to intrinsic defects in the iNKT precursors.

We therefore set up two different bone marrow chimeras to separately address the potential alterations in the ability of the *Bcl11b*-deficient DP thymocytes to present glycolipid self-antigens, as opposed to potential intrinsic alterations in the *Bcl11b*-deficient progenitors. In the mixed-chimera system, the b2m$^{-/-}$ DP thymocytes are able to generate iNKT precursors; however, they cannot present glycolipids and therefore are provided *in trans* with TCR-α$^{-/-}$ DP thymocytes, which are able to present glycolipids but are deficient in generation of iNKT precursors (Fig. 2 *A*, *C*, and *E*). Neither donor bone marrow alone is able to generate iNKT cells, thus making it unnecessary to use different congenic markers.

Specifically, to investigate whether *Bcl11b*-deficient DP thymocytes are able to support the development of normal iNKT precursors, we generated chimeras from *Bcl11b*$^{F/F}$CD4Cre/TCR-α$^{-/-}$ or *Bcl11b*-WT/TCR-α$^{-/-}$ bone marrow with b2m$^{-/-}$ bone marrow at a 50:50 ratio, in irradiated b2m$^{-/-}$ recipient mice (Fig. 2 *A*–*D*). Although the DP thymocytes derived from *Bcl11b*-WT/TCR-α$^{-/-}$ bone marrow efficiently supported the development of iNKT precursors derived *in trans* from b2m$^{-/-}$ bone marrow (Fig. 2 *A* and *B*), DP thymocytes derived from *Bcl11b*$^{F/F}$CD4Cre/TCR-α$^{-/-}$ bone marrow failed to support the development of iNKT precursors derived from b2m$^{-/-}$ bone marrow (Fig. 2 *C* and *D*). Specifically, a pronounced reduction in the numbers of CD1dT-PBS57$^+$ cells was observed both in the thymus (Fig. 2 *D* vs. *B*) and in the liver, in both the CD4$^+$ and CD4$^-$ populations (Fig. 2 *D* vs. *B*). The small number of *Bcl11b*-deficient CD1dT-PBS57$^+$ cells in the thymus was close to the background staining levels with the unloaded CD1d tetramer (Fig. S2). These results suggest a block at developmental stage 0. Furthermore, 80:20 ratio *Bcl11b*$^{F/F}$CD4Cre/TCR-α$^{-/-}$:b2m$^{-/-}$ bone marrow chimeras, which favors presenters vs. precursors, did not improve the generation of iNKT cells above background (Fig. S3 *A* and *B*).

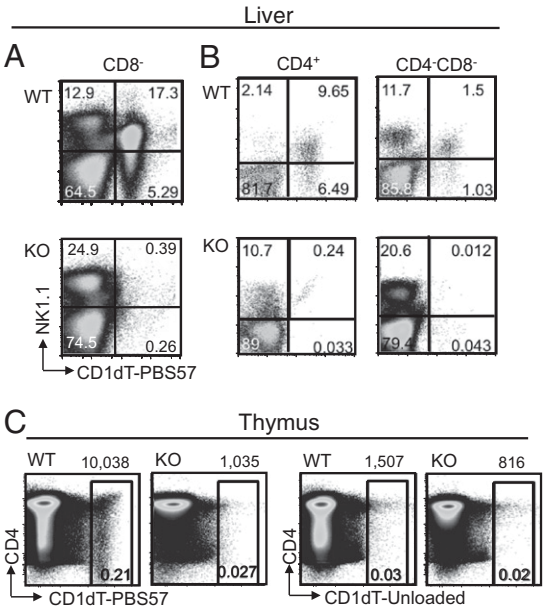

Fig. 1. Removal of *Bcl11b* in DP thymocytes results in pronounced reduction of iNKT cell populations in liver and thymus, although Vα14–Jα18 TCR is normally rearranged. (*A*) Frequencies of total CD1dT-PBS57$^+$ NK.1.1$^+$ cells in livers of *Bcl11b*$^{F/F}$CD4Cre (KO) and control (WT) mice. (*B*) Frequencies of CD4$^+$ and CD4$^-$ CD1dT-PBS57$^+$ NK.1.1$^+$ cells in livers of *Bcl11b*$^{F/F}$CD4Cre (KO) and control mice (WT). *A* and *B* present data representative of nine independent experiments. (*C*) Frequencies of CD1dT-PBS57$^+$ cells vs. CD1dT-unloaded$^+$ cells in the thymi of *Bcl11b*$^{F/F}$CD4Cre (KO) and control (WT) mice.

These results provide genetic evidence that *Bcl11b* plays a critical role in the ability of DP thymocytes to support the iNKT precursor selection, extrinsically controlling iNKT cell lineage development.

***Bcl11b*-Deficient iNKT Precursors Failed to Develop Even in Conditions When Presented Glycolipid Self-Antigens by *Bcl11b*-Sufficient DP Thymocytes.** To determine whether the altered iNKT cell development is also caused by a cell intrinsic defect in the iNKT cell precursors, we generated 50:50 ratio mixed chimeras from *Bcl11b*$^{F/F}$CD4Cre/b2m$^{-/-}$ bone marrow, which provides iNKT precursors, in combination with TCR-α$^{-/-}$ bone marrow, which provides *in trans* DP thymocytes, able to present glycolipid but not iNKT precursors (Fig. 2*E*). Although the precursors derived from *Bcl11b*-WT/b2m$^{-/-}$ bone marrow reconstituted the iNKT lineage in the presence of TCR-α$^{-/-}$ DP thymocytes (Fig. 2*B*), the chimeras derived from *Bcl11b*$^{F/F}$CD4Cre/b2m$^{-/-}$ bone marrow failed to reconstitute the iNKT lineage in the presence of TCR-α$^{-/-}$ DP thymocytes (Fig. 2*F*), genetically demonstrating that *Bcl11b*-deficient iNKT precursors are intrinsically altered. Specifically, the CD1dT-PBS57$^+$ cells were almost absent in the thymus and in the liver in both the CD4$^+$ and CD4$^-$ populations (Fig. 2*F*). The small number of *Bcl11b*-deficient CD1dT-PBS57$^+$ cells in the thymus was at the level of the background staining with unloaded CD1d tetramer (Fig. S4), therefore likely corre-

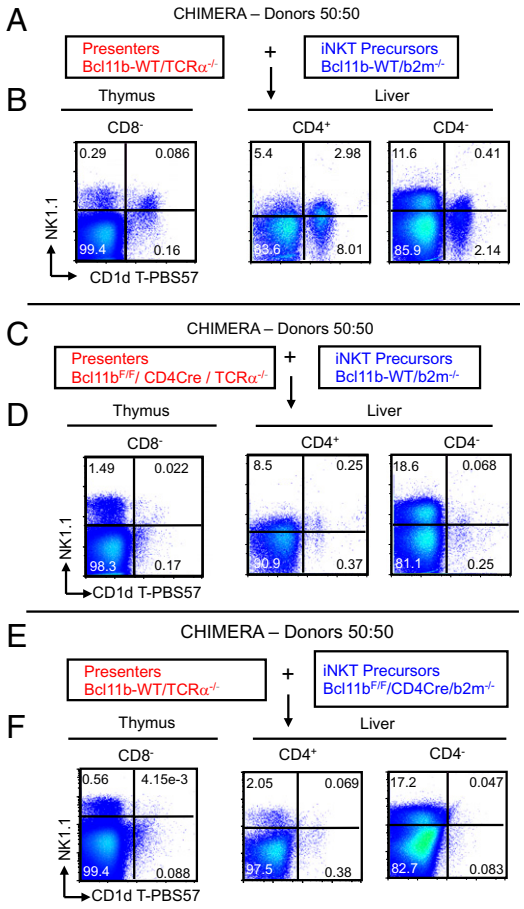

Fig. 2. *Bcl11b*-deficient DP thymocytes fail to support the development of normal iNKT precursors, and Bcl11b-deficient precursors cannot generate iNKT cells, even when presented glycolipid by normal DP thymocytes. (*A*, *C*, and *E*) Mixed chimera model used in this study to interrogate the ability of *Bcl11b*-deficient DP thymocytes to present glycolipid (presenters) vs. intrinsic alterations in *Bcl11b*-deficient iNKT precursors. The iNKT precursors are derived from b2m$^{-/-}$ bone marrow (50%), which generate DP thymocytes unable to present glycolipids. DP thymocytes able to present glycolipids (presenters), but unable to generate iNKT precursors, are provided *in trans* from TCR-α$^{-/-}$ bone marrow (50%). Chimeras were generated in lethally irradiated b2m$^{-/-}$ recipient mice. (*B*) iNKT thymocytes and liver CD4^{+} and CD4^{-} iNKT cell frequencies in chimeras generated as described in *A*. (*D*) iNKT thymocytes and liver CD4^{+} and CD4^{-} iNKT cells frequencies in chimeras generated from *Bcl11b*$^{F/F}$CD4Cre/TCR-α$^{-/-}$ bone marrow (KO presenters) provided *in trans* with normal iNKT precursors derived from b2m$^{-/-}$ bone marrow (WT precursors), as described in *C*. (*F*) iNKT thymocytes and liver CD4^{+} and CD4^{-} iNKT cell frequencies in chimeras generated from *Bcl11b*$^{F/F}$CD4Cre/b2m$^{-/-}$ bone marrow (KO precursors) provided *in trans* with DP thymocytes able to present glycolipids derived from TCR-α$^{-/-}$ bone marrow (WT presenters), as described in *E*. Data are representative of three independent chimera experiments.

sponding to stage 0. These results demonstrate that *Bcl11b*-deficient iNKT precursors have cell-autonomous alterations and are unable to develop, even when they are provided with *Bcl11b*-sufficient DP thymocytes that are able to support their selection.

Together with the data presented above, these results demonstrate that Bcl11b plays a dual role in iNKT development, independently controlling the iNKT precursor generation, as well as the function of DP thymocytes to support the iNKT precursor positive selection, by endogenous glycolipid presentation.

Inability of *Bcl11b*-Deficient DP Thymocytes to Support the Selection of iNKT Precursors Is Not Due to Alterations in the CD1d Expression, Internalization, or Recycling. The inability of *Bcl11b*-deficient DP thymocytes to support the iNKT precursor selection raised the question of whether *Bcl11b*-deficient DP thymocytes have alterations in CD1d. Our results demonstrate that the surface levels of CD1d, as well as the mRNA levels, were similar in *Bcl11b*-deficient and WT DP thymocytes (Fig. S5 *A* and *B*). Not only the surface level of CD1d is important for glycolipid presentation, but also its internalization and trafficking through the late endosomal/lysosomal compartment. Our data show that CD1d was internalized with similar kinetics in *Bcl11b*-deficient and WT DP thymocytes (Fig. S5*C*), indicating that its intracellular trafficking through the endosomal/lysosomal compartment was not altered. These results together indicate that the failure of *Bcl11b*-deficient DP thymocytes to support the iNKT precursor development is not due to defective CD1d expression, internalization, or trafficking through the late endosomal/lysosomal compartment.

***Bcl11b*-Deficient Thymocytes Have a Defect in the Presentation of Endogenous Glycolipid Ligands Loaded Through the Endosomal/Lysosomal Pathway.** The failure of *Bcl11b*-deficient DP thymocytes to support the selection of iNKT precursors raised the question of whether they are able to present glycolipid self-antigens to iNKT cells. We used a CD1d-restricted iNKT cell line, DN32.2D3 hybridoma, which expresses the Vα14–Jα18/Vβ8 TCR and is activated by glycolipid ligands loaded through the endosomal/lysosomal pathway (39). Whereas WT thymocytes activated the hybridoma cells, *Bcl11b*-deficient thymocytes induced IL-2 production by DN32.D3 hybridoma cell line sixfold less efficiently compared with WT (Fig. 3*A*). These results suggest that *Bcl11b*-deficient thymocytes have a defect in presentation of endogenous glycolipid ligands loaded through the endosomal/lysosomal pathway.

***Bcl11b*-Deficient DP Thymocytes Present Defects both in Glycolipid Processing and Loading in the Lysosomal Compartment.** Based on the results presented above, we hypothesized that *Bcl11b*-deficient DP thymocytes have an altered ability to generate selecting endogenous iNKT ligand(s) and/or have defects in CD1d-mediated glycolipid loading. To address these hypotheses, we first provided the thymocytes with the digalactosylceramide [α-GalCer(α1→2) GalCer; DiGalCer], which needs both processing and loading through the lysosomal compartment for surface presentation. Provision of DiGalCer to *Bcl11b*-deficient thymocytes activated the DN32.D3 NKT hybridoma cells to some extent but failed to reach the WT levels at the highest concentrations (Fig. 3*B*), suggesting that these cells present defects in lysosomal processing and/or loading of glycolipid.

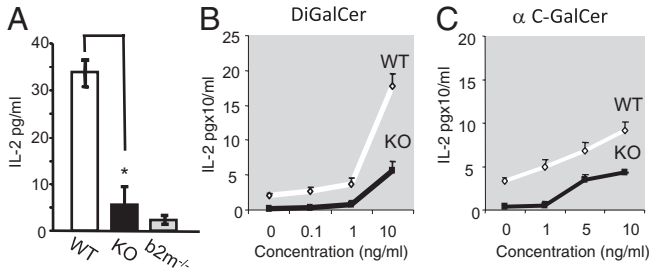

Fig. 3. *Bcl11b*-deficient thymocytes have defects in presentation of endogenous glycolipid ligands loaded through the endosomal/lysosomal pathway, exhibiting both altered glycolipid processing and loading. (*A*) IL-2 production by DN32.D3 hybridoma cells cocultured with thymocytes from *Bcl11b*$^{F/F}$CD4Cre (KO; black), control (WT; white), and b2m$^{-/-}$ (gray) mice. Data are representative of three independent experiments. *$P < 0.05$. (*B* and *C*) IL-2 production by DN32.D3 hybridoma cells cocultured with thymocytes from *Bcl11b*$^{F/F}$CD4Cre (KO; filled rectangles) and controls (WT; open diamonds) mice, in the presence of DiGalCer (*B*) or α-C-GalCer (*C*). Error bars represent SD. Data are representative of three independent experiments.

We then addressed the question of whether provision of glycosphingolipids that do not necessitate lysosomal processing, but require loading in the lysosomal compartment for surface presentation, rescues the inability of *Bcl11b*-deficient thymocytes to activate DN32.D3 hybridoma NKT cells. For this experiment, we used α-C-galactosylceramide (α-C-GalCer), a more stable analog of α-GalCer (3). Provision of α-C-GalCer to *Bcl11b*-deficient thymocytes improved the activation of DN32.D3 hybridoma cells, but the activation remained lower compared with WT (Fig. 3*C*), suggesting that the lipid loading in the late endosomal/lysosomal compartment of *Bcl11b*-deficient thymocytes presents alterations. Nevertheless, *Bcl11b*-deficient thymocytes loaded with 10 ng/mL DiGalCer presented a more pronounced defect as compared with WT (approximately fourfold less efficient activation; Fig. 3*B*) than *Bcl11b*-deficient thymocytes loaded with 10 ng/mL α-C-GalCer (approximately twofold less efficient activation; Fig. 3*C*).

In conclusion, when both processing and loading were tested, the activation of hybridoma cells was more severely affected compared with when only the loading was tested, suggesting that *Bcl11b*-deficient thymocytes present defects in both lysosomal processing and loading. However, we cannot exclude the possibility that *Bcl11b*-deficient DP thymocytes have additional defects beyond glycolipid processing, trafficking, and loading.

***Bcl11b*-Deficient Thymocytes Present Intracellular Accumulation of Several Glycolipid Species and Free Cholesterol and Enlarged Late Endosomal/Lysosomal Compartment.** Specific mutations in critical genes implicated in complex lipid processing are often associated with accumulation of unmetabolized substrates (reviewed in ref. 12). To determine whether *Bcl11b*-deficient thymocytes present alterations in complex lipid degradation, we evaluated the lipid composition of *Bcl11b*-deficient thymocytes by HPLC and found accumulation of several sphingolipid species, including lactosylceramide (LacCer), galactosylceramide (GalCer), glucosylceramide (GluCer), and sphingomyelin (Fig. 4*A*). In addition, we found increased binding of the fluorescent cholera toxin B to G_{M1} and increased levels of asialo-G_{M1} (G_{A1}; Fig. 4 *B* and *C*), indicating that ganglio-glycosphingolipid intermediates also accumulate in the absence of Bcl11b. We also evaluated the free cholesterol by filipin staining and found its levels elevated (Fig. 4*D*).

We further examined whether the accumulation of the sphingolipid species in *Bcl11b*-deficient DP thymocytes translates into morphological alterations of the late endosomal/lysosomal compartment, as this usually occurs when such species are accumulated in the lysosomes. The results indicate higher levels of lysotracker in the *Bcl11b*-deficient thymocytes compared with WT, suggestive of enlarged lysosomal compartment (Fig. 4 *E* and *F*). Collectively, these results demonstrate that Bcl11b removal in DP thymocytes results in accumulation of unmetabolized substrates and free cholesterol, with enlargement of endosomal/lysosomal compartment, likely causing the defective glycolipid antigen presentation.

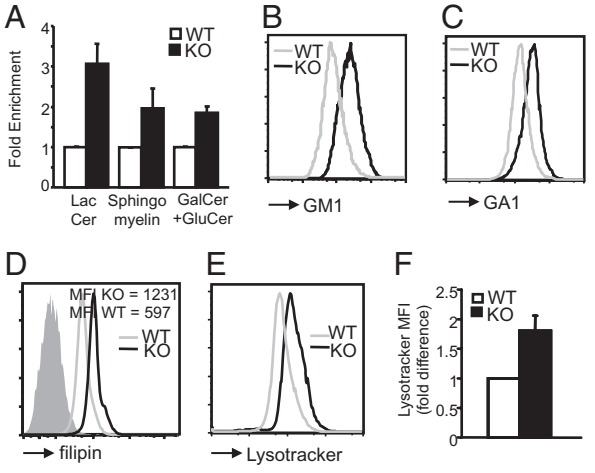

Fig. 4. *Bcl11b*-deficient thymocytes present accumulation of glycolipids and cholesterol and enlarged lysosomal compartment. (*A*) Enrichment of LacCer, GalCer/GluCer, and sphingomyelin in *Bcl11b*$^{F/F}$CD4Cre (KO; filled bars) vs. control (WT; open bars) thymocytes evaluated by HPLC analysis of lipid extracts. The experiment is representative of three independent pairs of mice. *$P < 0.05$. (*B* and *C*) Histograms of *Bcl11b*$^{F/F}$CD4Cre (KO; black traces) and control (WT; gray traces) thymocytes stained with fluorescent cholera toxin, which binds G_{M1} (*B*), and fluorochrome-conjugated anti-G_{A1} antibodies (*C*). (*D*) Histograms of filipin-stained thymocytes from *Bcl11b*$^{F/F}$CD4Cre (KO; black) and control (WT; gray) mice, analyzed by flow cytometry. Data are representative of three independent pairs of mice. (*E*) Histograms of *Bcl11b*$^{F/F}$CD4Cre (KO; black) and control (WT; gray) thymocytes stained with Lysotracker. (*F*) Graph represents the lysotracker mean fluorescence intensity (MFI) of the data in *E*. Data are representative of three independent experiments. *$P < 0.05$.

Absence of *Bcl11b* Results in Alterations of mRNAs Encoding Proteins with Critical Role in Sphingolipid Processing and Glycolipid Trafficking and Loading on CD1d in DP Thymocytes. Considering that a large number of genes were found to present altered expression in the absence of Bcl11b in DP thymocytes (30, 38), we reevaluated the previously conducted microarray analysis (30) and searched for genes with potential relevance for glycolipid metabolism and glycolipid presentation to iNKT precursors, which could explain the defects of *Bcl11b*-deficient DP thymocytes. Two functional clusters of genes were generated: (group I) genes with role in glycolipid trafficking and loading (Fig. 5*A*), and (group II) genes with role in sphingolipid metabolism (Fig. 5*B*). Altered levels of these mRNAs in the absence of Bcl11b were also confirmed by quantitative RT-PCR (qRT-PCR; Fig. 5*C*). Within the glycolipid trafficking and loading group, we found down-regulation of *Npc1* and *Npc2* gene expression (Fig. 5 *A* and *C*). Npc1 is involved in glycolipid trafficking, and Npc2 is important for glycolipid loading on CD1d. Both genes have been shown to play a critical role in iNKT cell development, and their absence resulted in lysosomal accumulation of glycosphingolipids and cholesterol (40, 41). As shown above, *Bcl11b*-deficient DP thymocytes presented accumulation of glycolipids and cholesterol (Fig. 4 *A*–*D*) and alterations in loading (Fig. 3 *B* and *C*).

Within the sphingolipid metabolism cluster group (group II), we identified several genes and organized them based on their biochemical function (Fig. 5*B*). mRNA levels for two lysosomal proteases, cathepsin D (Ctsd) and cathepsin L (Ctsl), were found to be down-regulated (Fig. 5 *B* and *C*). Ctsd cleaves prosaposin, generating saposins (10). Saposins support the lysosomal hydrolases in the process of sphingolipid degradation and also play a critical role in glycolipid loading (11). Ctsl is a critical regulator of endogenous iNKT cell ligand presentation through CD1d (42). Thus, alterations in these genes can contribute to the defects described above.

The next three genes within the sphingolipid metabolism cluster encode enzymes with critical role in complex lipid degradation (Fig. 5 *B* and *C*): (*i*) β-galactosidase (*Glb1*) catalyzes the hydrolysis of G_{M1} and G_{A1}; (*ii*) acid sphingomyelinase (Smpd1) converts sphingomyelin to ceramide; and (*iii*) arylsulfatase A (ArsA) is responsible for the desulfation of 3-0-sulfogalactosyl–containing sphingolipids. Defects in *Glb1, Smpd1, and ArsA* genes result in accumulation of unmetabolized sphingolipid substrates and cholesterol (43–45). Importantly, *Bcl11b*-deficient DP thymocytes presented accumulation of sphingolipids, such as sphingomyelin, G_{M1} and G_{A1} gangliosides, and cholesterol (Fig. 4 *A*–*D*).

Collectively these results show that the absence of Bcl11b directly or indirectly causes deregulated expression of multiple genes required for complex lipid degradation and glycolipid loading, which consequently results in alterations in glycolipid presentation to iNKT precursors.

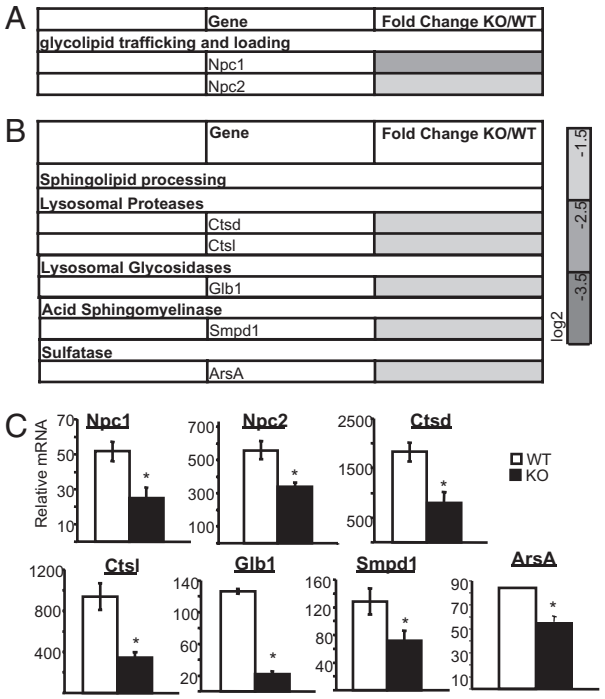

Fig. 5. DP thymocytes from *Bcl11b*$^{F/F}$CD4Cre mice present abnormal mRNA levels encoding proteins with role in sphingolipid processing and glycolipid trafficking and loading on CD1d. RNA extracted from DP thymocytes of *Bcl11b*$^{F/F}$CD4Cre (KO) and control (WT) mice was evaluated by microarray analysis using the Agilent platform. Only results for specific genes with roles in glycolipid metabolism and loading on CD1d are presented. Genes were grouped in the indicated clusters based on functional annotation by using DAVID (Version 6.7) analysis tool (48) and KEGG database (http://www.genome.jp/kegg/pathway.html). (*A*) Cluster I comprises genes with role in glycolipid trafficking and loading. (*B*) Cluster II contains genes involved in sphingolipid metabolism. Data are representative of five independent arrays, each with one pair of mice. (*C*) Relative mRNA levels of the indicated genes in *Bcl11b*-deficient (KO) vs. WT DP thymocytes evaluated by qRT-PCR after normalization to actin. Data are representative of three independent experiments. *$P < 0.05$.

Discussion

In this study, we investigated in-depth the developmental defects in iNKT cell lineage caused by removal of Bcl11b at the DP stage of T cell development. We show that the thymi and liver of Bcl11b$^{F/F}$ CD4Cre mice lack iNKT cells. Chimera experiments demonstrated a unique and previously undescribed role of Bcl11b in presentation of endogenous glycolipid self-antigens by DP thymocytes and an independent role of Bcl11b, intrinsic to iNKT precursors. Further characterization indicated accumulation of several glycolipid intermediates and free cholesterol, associated with enlarged lysosomal compartment, and failure of DP thymocytes to process and load glycolipids, despite the fact that the CD1d levels were normal, as were its internalization and recycling. Critical lysosomal genes, including *Npc1* and *Npc2*, presented reduced expression, which could contribute to the accumulation of cholesterol and glycosphingolipids, as reported for Niemann–Pick disease type C (40, 41). Moreover, the reduced levels of *Npc2* gene could also contribute to the defective loading (41). In addition, reduced expression of acid sphingomyelinase gene could contribute to the increased levels of sphingomyelin and cholesterol, similar to what was observed in Niemann–Pick disease types A and B (13). Altered expression of these three genes, with the consequences presented above, is likely to cause enlarged lysosomes and to interfere with the ability of DP thymocytes to support iNKT cell development through glycolipid presentation.

In addition, the lysosomal protease Ctsd presented reduced expression levels in the absence of Bcl11b. This enzyme is required for cleavage of prosaposin to saposins. Thus, such reduction could also contribute to the alterations in sphingolipid degradation and accumulation of GluCer, GalCer, and LacCer, because saposins A and C act in synergy to stimulate β-glucosylceramidase and β-galactosylceramidase (8). Moreover, the reduced expression of Ctsd may account for the defective glycolipid loading on CD1d, as saposins also participate in this process (11). *Ctsl* gene down-regulation may contribute to the glycolipid loading defect, as observed in the *Ctsl* knockout mice (42). The accumulation of G_{M1} and G_{A1} gangliosides may be a consequence of the reduced expression of the gene encoding Glb1, which is also likely to impair the iNKT selection process, as shown for GM1 gangliosidosis (12). We do not know whether Bcl11b directly or indirectly controls expression of these genes.

Given the increasing importance of lipid metabolites as active participants in signal transduction in apoptosis and survival, perhaps the increased apoptosis of Bcl11b-deficient thymocyte, only partially rescued by provision of Bcl2 (30), may be, at least in part, due to deregulated lipid biosynthesis and metabolism.

Data presented here show that Bcl11b is a unique transcription factor that controls the selection of iNKT precursors, by regulating glycolipid antigen presentation in DP thymocytes, specifically glycolipid processing, trafficking, and loading. To this end, given its high expression in neurons and the important role of sphingolipids in CNS, it is tempting to speculate that, in addition to the defective neuronal development observed in germline *Bcl11b* knockout mice, perhaps some genes with critical roles in sphingolipid metabolism may also be altered in the CNS in the absence of Bcl11b and may contribute, at least in part, to the early lethal neuronal defects reported in the germ-line *Bcl11b* knockout mice (46).

Multiple transcription factors have been shown to play important roles in iNKT cell development, as indicated above. However, in contrast to all these transcription factors, the results presented here demonstrate that Bcl11b has a critical extrinsic role in iNKT development, in glycolipid presentation mediated by DP thymocytes, by regulating expression of genes with crucial role in glycolipid metabolism and loading. We cannot exclude additional defects beyond glycolipid processing and loading that might further impede *Bcl11b*-deficient DP thymocytes to support selection of iNKT precursors.

Our studies also demonstrate that Bcl11b plays an additional intrinsic role in iNKT precursors, independent of the defect in glycolipid presentation by DP thymocytes. The absence of precursors in *Bcl11b*$^{F/F}$CD4Cre mice precluded at the moment their further investigation; however, systems are currently being developed to further study these defects.

Materials and Methods

Mice. *Bcl11b*$^{F/F}$CD4Cre mice were as described (30). B2m$^{-/-}$ and TCR-α$^{-/-}$ mice were purchased from Jackson Laboratory. Experiments were conducted with 5- to 7-wk-old mice, unless specified. All mice were kept under specific pathogen-free conditions in the Animal Resource Facility of Albany Medical College, and animal procedures were conducted as approved by the Institutional Animal Care and Use Committee.

Bone Marrow Chimeras. We generated 50:50 and 80:20 mixed bone marrow chimeras by lethally irradiating Rag2gc$^{-/-}$ or b2m$^{-/-}$ recipient mice, followed by i.v. injection of 1×10^7 donor bone marrow cells. Eight weeks after reconstitution, mice were euthanized, and single cell suspensions from thymi, spleens, and livers were stained and analyzed by FACS.

Flow Cytometry. Staining with CD1dT-PBS57 or CD1dT-unloaded control (1:100) was performed on ice for 1 h, followed by addition of antibodies for surface staining. Intranuclear staining for Bcl11b was conducted after fixation and permeabilization with 1% Triton X-100 (33). Free cholesterol staining was performed with 0.05 mg/mL filipin for 2 h after fixation. Flow cytometry analysis was conducted on FACSCalibur, FACS Canto, and LSR II flow

cytometers (Becton Dickinson), and data were analyzed by using FlowJo software (Tree Star, Inc).

Antigen Presentation Assays. We incubated 5×10^4/well DN32.D3 NKT hybridoma cells with 5×10^5/well thymocytes in 100 μL/well RPMI for 24 h, and IL-2 was evaluated by ELISA. In subsequent experiments, the thymocytes were pulsed with the indicated amounts of α−C-GalCer or DiGalCer for 2 h, before the addition of NKT hybridoma cells.

Evaluation of Lipids by HPLC. Total thymocyte extracts were prepared from *Bcl11b*$^{F/F}$CD4Cre and control mice were in tissue extraction buffer (0.25 M sucrose, 25 mM KCl, 50 mM Tris, and 0.5 mM EDTA, pH 7.4). The equivalent of 1 mg of protein was used for lipid extraction followed by HPLC analysis at the Lipidomics Core Facility of the Medical University of South Carolina.

Statistical Analysis. Differences between different groups of mice or samples were analyzed by the two-tailed Student *t* test and expressed as mean ± SD. $P \leq 0.05$ was considered significant for all analyses.

Supporting Information. Additional material, including primer sequences, antibodies, and reagents, is provided in *SI Materials and Methods*.

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