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NKT cells are a unique subset of T cells that recognize glycolipid Ags presented in the context of CD1d molecules. NKT cells mount strong antitumor responses and are a major focus in developing effective cancer immunotherapy. It is known that CD1d molecules are constantly internalized from the cell surface, recycled through the endocytic compartments, and re-expressed on the cell surface. However, little is known about the regulation of CD1d-mediated Ag processing and presentation in B cell lymphoma. Prosurvival factors of the Bcl-2 family, such as Bcl-xL, are often upregulated in B cell lymphomas and are intimately linked to sphingolipid metabolism, as well as the endocytic compartments. We hypothesized that Bcl-xL can regulate CD1d-mediated Ag presentation to NKT cells. We found that overexpression or induction of Bcl-xL led to increased Ag presentation to NKT cells. Conversely, the inhibition or knockdown of Bcl-xL resulted in the loss of CD1d trafficking to lysosome-associated membrane protein 1+ compartments. Rab7, a late endosomal protein, was upregulated and CD1d molecules accumulated in the Rab7+ late endosomal compartment. These results demonstrate that Bcl-xL regulates CD1d-mediated Ag processing and presentation to NKT cells by altering the late endosomal compartment and changing the intracellular localization of CD1d. The Journal of Immunology, 2014, 193: 2096–2105.

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Abbreviations used in this article: BH3, Bcl-2 homology 3; ER, endoplasmic reticulum; α-GalCer, α-galactosylceramide; HSA, human serum albumin; LAMP1, lysosome-associated membrane protein 1; PM, plasma membrane; shRNA, short hairpin RNA.

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The process of CD1d-mediated Ag presentation is complex and begins with the synthesis of the CD1d α-chain in the endoplasmic reticulum (ER) (12). Here chaperons like calnexin, calreticulin, and Erp57 ensure that it is properly folded (13). The Ag binding groove of CD1d is occupied by a self-lipid Ag thought to be loaded by the microsomal triglyceride transfer protein (14, 15). After association with β₂-microglobulin, the CD1d molecule follows the secretory pathway from the ER to the Golgi and reaches the plasma membrane (PM). To present an activating endogenous Ag to NKT cells, CD1d molecules recycle from the PM to endocytic compartments because of the presence of a tyrosine-based targeting motif (Yxxφ, where Y is tyrosine, x is any amino acid, and φ is a hydrophobic amino acid) (16, 17). This is analogous to the invariant chain for MHC class II molecules. In fact, invariant chain associates with CD1d, but the Yxxφ motif is necessary for the proper trafficking of the CD1d molecules to the endocytic compartments (18). Following internalization from the PM, adaptor proteins AP2 and AP3 direct CD1d molecules to the endocytic compartment, also known as MHC class II compartment, where MHC class II molecules are normally loaded with peptide Ags (19, 20). Once in the endocytic recycling compartment, the stabilizing self-lipid is exchanged for other lipid Ags with the help of saposins (21). These loaded CD1d molecules are then re-expressed on the PM and can be recognized by canonical Vα14Jα18 NKT cells. The localization of CD1d to cholesterol-rich lipid rafts is important for efficient Ag presentation, especially in the presence of low concentrations of Ags, and the disruption of these lipid rafts leads to reduced Ag presentation (22, 23). The complex multistep process of CD1d-mediated Ag processing and presentation has several potential levels of control, yet very few endogenous regulatory factors have been identified. Prominent among these are the MAPKs, protein kinase Cθ, and Rho kinases (24–26). In this study, we sought to identify a target that regulates CD1d-mediated Ag presentation and is relevant to tumor growth and survival.
Antiangiogenic Bcl-2 family members are known to be expressed at high levels in lymphomas and other malignancies, and allow cells to evade apoptotic signals and attain a neoplastic state (27). Bcl-xL is a potent antiangiogenic factor and exerts its antiauxotic function by heterodimerizing with other Bcl-2 family members and preventing the permeabilization of the mitochondrial outer membrane. Bcl-xL can also mediate its prosurvival function by causing the retrotranslocation of the proapoptotic factor Bax from the mitochondria to the cytosol (28). Bcl-xL also prevents the formation of ceramide channels, which contribute to the release of cytochrome c from the mitochondria (29). Although Bcl-xL was traditionally considered a mitochondrial apoptosis regulator, recent evidence shows that this protein is also present in other compartments such as the ER (30). Bcl-xL can also affect the intracellular endosomal compartments, which are critical for CD1d-mediated Ag processing and presentation (31). Furthermore, Bcl-xL has been reported to interact with various trafficking-related proteins such as calnexin, Rab7, ER Golgi intermediate compartment 1, and vesicle-associated membrane protein 3, underscoring the multifaceted effects of Bcl-xL (32).

To examine the role of Bcl-xL on CD1d-mediated Ag processing and presentation, we conducted a series of overexpression, induction, and knockdown studies. In addition, we examined the effect of pharmacological agents known to disrupt Bcl-xL function and assessed intracellular trafficking to endosomal compartments. Interestingly, we found that the mechanism of regulation is independent of surface CD1d expression, costimulatory molecules, and CD1d recycling. After Bcl-xL knockdown, alterations in intracellular trafficking of CD1d were observed. In addition, the expression of Rab7, a late endosomal protein, was increased and there was expansion of the late endosomal compartment. CD1d molecules were lost from the early endosomes and lysosomes, and increased in the late endosomal compartment. These data show that in the absence of Bcl-xL, the expanded late endosomal compartment acts as a CD1d depot and alters Ag presentation, ultimately resulting in loss of NKT cell activation.

Materials and Methods

Cell lines, Ags, and other reagents

L-CD1d, L-CD1d-DR4, and LMTK-CD1d are mouse fibroblast cell lines transfected with mCD1d1 or mCD1d1 and HLA-DR4 (kindly provided by Dr. Randy Brutkiewicz, Indiana University School of Medicine, Indianapolis, IN) and were cultured in DMEM with 10% FBS and 2 mM L-glutamine. In addition, selection agents G418 sulfate (500 μg/ml) from Mediatech (Manassas, VA), and Zeocin (200 μg/ml) and puromycin (5 μg/ml) from Santa Cruz Biotechnology (Santa Cruz, CA) were added as required. Unless otherwise specified, all materials for cell culture were purchased from Life Technologies (Carlsbad, CA). WEHI-231 and WEHI-231/Bcl-xL cells were kindly provided by Dr. Gregory Carey (University of Maryland) and cultured in RPMI 1640 with 10% FBS, 100 mM sodium pyruvate, 10 mM nonessential amino acid solution, 1× vitamin solution, and 50 μM 2-ME. The mouse T NKT cell hybridomas DN32.D3 and N38-3C3, as well as the type II NKT cell hybridomas N37-1A12, have been described and were cultured in IMDM supplemented with 5% FBS and vitamin solution, 2 mM L-glutamine, and 2 mM t-glutamine. In addition, selection agents G418 sulfate (500 μg/ml) from Mediatech (Manassas, VA), and Zeocin (200 μg/ml) and puromycin (5 μg/ml) from Santa Cruz Biotechnology (Santa Cruz, CA) were added as required. Unless otherwise specified, all materials for cell culture were purchased from Life Technologies (Carlsbad, CA).

Overexpression and knockdown of Bcl-xL

LMTK-CD1d cells were transfected with vector alone or vector encoding Bcl-xL, kindly provided by Dr. Mark Williams (University of Maryland). Transfection was carried out using Neofectin one-step transfection reagent from NesBioLab (Cambridge, MA) as per the manufacturer’s directions. Stable transfectants were then cultured in dual selection medium containing G418 sulfate (500 μg/ml) and Zeocin (200 μg/ml), and passaged every 3 d. Overexpression of Bcl-xL was confirmed by Western blotting.

Bcl-xL knockdown was attained by lentiviral vectors purchased from Santa Cruz Biotechnology. These replication incompetent lentiviral particles contained a pool of three to four short hairpin sequences targeted to the Bcl-2I gene that encodes Bcl-xL, or the Bcl-2 gene and polybranched transduction was carried out as per the manufacturer’s instructions. Stable transductants were cultured in dual-selection medium containing G418 sulfate (500 μg/ml) and puromycin (5 μg/ml), and were passaged every 3 d. Bcl-xL knockdown was confirmed by Western blotting.

Mice

Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at ~6 wk of age. All performed procedures were approved by the University of Maryland Institutional Animal Care and Use Committee. After euthanasia, spleens were removed and a single-cell suspension of splenocytes was prepared using a 70-μm nylon mesh from Fisher Scientific (Waltham, MA). B cells were isolated using a mouse B cell enrichment kit from Stem Cell Technologies (Vancouver, BC, Canada) as per the manufacturer’s instructions. Purity was >95% as determined by flow cytometry after staining with FITC-conjugated Ab to B220 (CD45R) from Biolegend (San Diego, CA). Enriched splenic B cells were suspended in RPMI 1640 with 10% FBS and other supplements as described earlier. The liver was processed and liver mononuclear cells were obtained as previously described (35). NKT cell percentage was determined by flow cytometry after staining with a FITC-conjugated Ab to the TCR β-chain from Biolegend (San Diego, CA) and allopurinol-resistant NKT cell hybridomas were obtained by an LSR II from BD Biosciences (San Jose, CA) and analyzed using FCS Express Version 3 from De Novo Software (Los Angeles, CA).

Flow cytometry

Cells were stained in PBS containing 0.5% BSA and 2 mM EDTA for 30 min at 4°C with a PE-conjugated Ab to CD1d (clone 1B1) from BD Biosciences (San Jose, CA). Data were collected on an LSR II from BD Biosciences (San Jose, CA) and analyzed using FCS Express Version 3 from De Novo Software (Los Angeles, CA).

NKT cell hybridoma assay

L-CD1d or LMTK-CD1d cells (5 × 10^4/well) were cocultured with NKT cells (5 × 10^3/well) for 20 h at 37°C in IMDM supplemented with 5% FBS and L-glutamine. Primary splenic B cells were treated with 10 μg/ml anti-CD40 Ab (clone 1C10) from eBioscience (San Diego, CA), washed with complete medium, and cocultured (10^6 B cells/well) with liver mononuclear cells (2 × 10^5/well) for 24 h at 37°C in complete medium. For Bcl-xL/Bcl-2 inhibition studies, L-CD1d-DR4 cells were treated with ABT-263 or ABT-199 for 4 h, washed twice with PBS and once with complete medium, and cocultured with NKT cells. For the peptide Ag presentation assay, L-CD1d-DR4 cells were loaded with HSA overnight, treated with ABT-263 or ABT-199 for 4 h, washed twice with PBS and once with complete medium, and cocultured with 17.9 T cell hybridomas. After the coculture, supernatants were harvested and IL-2, IFN-γ, or IL-4 levels were measured by ELISA. Mouse IL-2 ELISA kit from BD Biosciences (San Jose, CA) and mouse IFN-γ and IL-4 ELISA kits from Biolegend (San Diego, CA) were used. Data were graphed using Prism 5.02 from GraphPad (La Jolla, CA).

Western blotting

Cells were lysed using lysis buffer containing NaCl (150 mM), Tris-Cl (50 mM), EDTA (8.5 mM), sodium azide (0.02%), nonylphenoxypolyethoxyethanol (0.1%), and complete mini protease inhibitor cocktail tablet from Roche Applied Science (Indianapolis, IN) as directed by the manufacturer, prepared in water. Proteins were resolved by electrophoresis on a 4–12% gradient polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using the iBlot transfer system. All gels, equipment, buffers, and other materials were from Life Technologies (Carlsbad, CA) and were used as per the manufacturer’s instructions. Membranes were probed with Abs to Bcl-xL (clone 54H6) from Cell Signaling Technology (Beverly, MA) or Rab7 from Santa Cruz Biotechnology. GAPDH levels were detected on the same membrane using a mouse Ab (clone 1C10) from eBioscience. Dylight-800–conjugated anti-rabbit secondary Ab was purchased from Thermo Scientific (Waltham, MA). Membranes were scanned using the Odyssey Imaging System from Li-COR Biosciences (Lincoln, NE).
Confocal microscopy

Cells were added to a Lab Tek II Chamber slide from Thermo Scientific (Waltham, MA), allowed to adhere overnight and fixed in 1% paraformaldehyde in PBS for 15 min at room temperature. Nonspecific binding sites were blocked by incubating cells in PermWash buffer from BD Biosciences (San Jose, CA) with 5% rat serum for 1 h at 4°C. The cells were then incubated with Abs to CD1d (clone I6H6), kindly provided by Dr. Randy Brutkiewicz (Indiana University School of Medicine), CD107a (lycosome-associated membrane protein 1 [LAMP1]) from eBioscience (San Diego, CA), EEA1 from Cell Signaling Technology (Beverly, MA), or Rab7 from Santa Cruz Biotechnology at 4°C overnight. After washing, Alexa Fluor 546-conjugated anti-mouse IgG, allophycocyanin-conjugated anti-rabbit IgG, and Alexa Fluor 647-conjugated anti-rat IgG from Life Technologies (Carlsbad, CA) were added and incubated for 1 h at room temperature in the dark. Slides were then mounted using Vectashield mounting medium containing DAPI from Vector Laboratories ( Burlingame, CA). Images were acquired using a 40× lens on the LSM 510, a laser-scanning microscope from Carl Zeiss Microscopy (Oberkochen, Germany) using the Zen 2009 software and processed using the Zeiss LSM Image Browser Version 4.2.0.121. Colocalization was quantified using the same software from five different images, and Pearson’s correlation for colocalization was represented as percent of control. In addition, Rab7 staining was quantified by pixel number from five different images and represented as percent of control.

Statistical analyses

All experiments were repeated at least three times. Two-tailed Student t test, one-way ANOVA, or two-way ANOVA was used as appropriate. Specific experimental groups were compared with controls using the Bonferroni posttest. A p value <0.05 was considered significant. All analyses were performed using Prism 5.02 by GraphPad (La Jolla, CA); ***p < 0.001, **p < 0.01, *p < 0.05.

Results

Overexpression of Bcl-xL in APCs leads to increased NKT cell responses

Bcl-xL is a prosurvival member of the Bcl-2 family of proteins. In this study, we hypothesized that Bcl-xL affects the ability of an APC to present glycolipid Ags to NKT cells, independent of its regulation of apoptosis. To determine whether the overexpression of Bcl-xL has an effect on CD1d-mediated Ag presentation, we used WEHI-231, a classical and well-characterized murine B cell lymphoma cell line (36), and compared it with a WEHI-231-derived cell line stably transfected with murine Bcl-xL. Overexpression of Bcl-xL protein was first confirmed by Western blotting (Fig. 1A). Next, the WEHI-231 controls and Bcl-xL transfectants were cocultured with two canonical Vα14Jα18 NKT cell hybridomas, DN32.D3 and N38-3C3, and IL-2 production was measured in the coculture supernatant (Fig. 1B, 1C). In the absence of exogenously added Ags, the WEHI-231 control, as well as Bcl-xL transfected cell lines, failed to activate NKT cells. These data suggest that the endogenous Ag presented by CD1d molecules in these cells does not activate NKT cells. However, when the potent NKT cell agonist, α-GalCer, was added to the coculture, Bcl-xL-transfected cells were able to stimulate NKT cells, unlike the untransfected controls. These data demonstrate that CD1d molecules expressed on the cell surface of WEHI/Bcl-xL cells are functional, but these cells do not present an activating endogenous ligand. Furthermore, the presentation of an exogenous Ag is enhanced following the overexpression of Bcl-xL (Fig. 1B, 1C).

To characterize the potential role of Bcl-xL in CD1d-mediated Ag presentation, we used LMTK cells as an in vitro APC system. LMTK cells, transfected with CD1d (LMTK-CD1d), are known to present an endogenous activating Ag to NKT cells (37). Furthermore, LMTK-CD1d cells can be cocultured with a panel of mouse NKT cell hybridomas, which produce high levels of IL-2 after activation, providing a straightforward system to study CD1d-mediated Ag presentation to NKT cells with minimal confounding factors. To directly test whether Bcl-xL plays a role in Ag presentation to NKT cells, we transfected LMTK-CD1d cells with an empty control plasmid or a plasmid carrying the Bcl2l1 gene, which encodes Bcl-xL, resulting in the overexpression of Bcl-xL protein as seen by Western blotting (Fig. 1G). There were no apparent differences in cell morphology, growth, or proliferation (Supplemental Fig. 1A, 1C). Also, transfection with Bcl-xL did not dramatically alter surface CD1d expression as analyzed by flow cytometry (Fig. 1H). These LMTK-CD1d cells transfected with Bcl-xL or the respective control were cocultured with a panel of three NKT cell hybridomas. Although DN32.D3 and N38-3C3 are canonical type I NKT cells, N37-1A12 is a type II NKT cell hybridoma (33, 34). We found that the overexpression of Bcl-xL in LMTK-CD1d cells elicited significantly higher response from all three hybridoma lines tested (Fig. 1D–F). From these data, it is evident that Bcl-xL can affect NKT cell activation because high levels of Bcl-xL elicit stronger NKT cell responses. Furthermore, we used CD1d blocking Abs and found that IL-2 production was completely lost after CD1d blockade in the control-transfected, as well as Bcl-xL–transfected LMTK-CD1d cells (Supplemental Fig. 2A–C). This indicates that the regulation by Bcl-xL is specific to CD1d-dependent Ag presentation to NKT cells.

Induction of Bcl-xL by CD40 stimulation in B cells leads to increased NKT cell activation

We found that the overexpression of Bcl-xL in APCs leads to enhanced Ag presentation to NKT cells. Next, we wanted to induce Bcl-xL using a biological stimulus and determine whether Ag presentation to NKT cells was affected. One of the most well-known aspects of B cell stimulation with anti-CD40 Ab is the upregulation of Bcl-xL, and it has been reported extensively in WEHI-231 cells, primary mouse B cells, and also in human B cells (38–41). We treated WEHI-231 cells with anti-CD40 Ab (clone 1C10) and confirmed the induction of Bcl-xL protein by Western blotting (Fig. 2A). We found that treatment with anti-CD40 Ab did not alter surface CD1d expression (Fig. 2B). WEHI-231 cells treated with anti-CD40 Ab elicited higher NKT cell activation compared with cells treated with isotype control Ab (Fig. 2C, 2D). This shows that the induction of Bcl-xL in a B cell lymphoma cell line leads to increased NKT cell responses. These results are consistent with the data obtained from overexpression of Bcl-xL (Fig. 1B–F) and show that increased Bcl-xL levels lead to a corresponding increase in CD1d-mediated Ag presentation to NKT cells.

Bcl-xL-mediated regulation of Ag presentation in primary mouse B cells

To eliminate the possibility of cell line–specific effects and validate the results in primary mouse cells, we used freshly isolated splenic B cells as APCs and liver mononuclear cells as a source of primary NKT cells. Approximately 40% of T cells in the liver are NKT cells, as compared with the thymus and spleen (1–2%). Also, liver NKT cells are the only subset known to mediate antitumor effector functions (42). To assess the effect of Bcl-xL induction on Ag presentation to NKT cells, we treated splenic B cells with anti-CD40 Ab for 24 h and used Western blotting to ensure the up-regulation of Bcl-xL protein (Fig. 3A). After treatment, cells were washed and cocultured with liver mononuclear cells in the presence or absence of α-GalCer. NKT cell activation was assessed by measuring IFN-γ or IL-4 in the supernatant by ELISA (Fig. 3B, 3C). There was no detectable cytokine production in the absence of α-GalCer, demonstrating that the observed responses were NKT cell specific. In the presence of α-GalCer, anti-CD40–treated
B cells led to increased IFN-γ production by NKT cells as compared with their untreated or isotype control–treated counterparts (Fig. 3B). However, IL-4 production was unchanged after treatment with anti-CD40 Ab (Fig. 3C). Despite the use of total liver mononuclear cells, the observed responses were NKT cell specific because all cytokine levels were detectable only in cocultures containing NKT cell Ags. In addition, we analyzed the cells by flow cytometry after treatment and found no changes in the surface expression of MHC class I, CD1d, CD80, or CD86 (Fig. 3D–G). Together, these data show that Bcl-xL plays a role in CD1d-mediated Ag processing and presentation to NKT cells in an ex vivo system using primary mouse B cells and NKT cells.

**Pharmacological inhibition of Bcl-xL leads to reduced NKT cell responses**

To study the effect of Bcl-xL inhibition on CD1d-mediated Ag presentation to NKT cells, we used small-molecule inhibitors that bind the Bcl-2 Homology 3 (BH3) domain of Bcl-xL. BH3–BH3 interactions between proteins of the Bcl-2 family are critical for their function. We treated L-CD1d cells with two small-molecule BH3 mimetics, ABT-263 and ABT-199 (43, 44), which are functional inhibitors. ABT-263 targets both Bcl-xL and Bcl-2, whereas ABT-199 is Bcl-2 specific. Pretreatment of L-CD1d-DR4 cells with ABT-263 led to decreased CD1d-mediated Ag presentation to NKT cells (Fig. 4A). In contrast, pretreatment with ABT-199 had no effect on Ag presentation to NKT cells. These data demonstrate that BH3 domain-based functional inhibition of Bcl-xL, but not Bcl-2, leads to reduced CD1d-mediated Ag presentation to NKT cells. Because the Ag presentation pathway of CD1d is very similar to classical MHC class II and involves the lysosomal MHC class II compartment or MIIC, we sought to determine the effect of Bcl-xL inhibition of peptide Ag presentation to classical CD4⁺ T cells (45). L-CD1d-DR4 cells loaded with HSA were treated with ABT-263 or ABT-199, washed extensively, and cocultured with the 17.9 T cell hybridoma line (Fig. 4B). Similar to CD1d-mediated Ag presentation, we found that the presentation of HSA was reduced after treatment with ABT-263 but was unaffected by treatment with ABT-199. Because ABT-263 and ABT-199 can induce apoptosis at higher concentrations, cell viability was determined by Annexin V–PI staining, where Annexin V⁺ PI⁺ cells were considered apoptotic and the remainder of cells were considered viable (single positive cells were minimal). There were no differences in viability between...
treated and untreated groups, indicating that our selected treatment concentrations were not sufficient to induce cell death and the role of Bcl-xL in CD1d-mediated Ag presentation is independent of apoptosis (Fig. 4C). Finally, there were no evident changes in cell-surface expression of CD1d or HLA-DR after treatment with ABT-263 or ABT-199 (Fig. 4D).

Stable knockdown of Bcl-xL causes inhibition of CD1d-mediated Ag presentation to NKT cells

L-CD1d cells express high levels of Bcl-xL; therefore, it is a good system to investigate the effects of Bcl-xL knockdown on CD1d-mediated Ag processing and presentation. We used lentiviral particles carrying a pool of short hairpin RNA (shRNA) sequences targeting the Bcl2l1 gene, which encodes the Bcl-xL protein. After transduction, stable cell lines were generated and Bcl-xL or Bcl-2 knockdown was confirmed by Western blotting (Fig. 5A). There were no apparent differences in cell morphology, growth, or proliferation after stable knockdown (Supplemental Fig. 1B, 1D). Also, there were no evident changes in surface CD1d expression after transduction and selection of stable integrants (Fig. 5B). L-CD1d cells transduced with a scrambled sequence or with shRNA to knockdown Bcl-xL or Bcl-2 were cocultured with mouse NKT cell hybridomas (B) DN32.D3 or (C) N38-3C3 in the presence or absence of an exogenous Ag for 20 h. IL-2 was measured in the supernatant by ELISA. ***p < 0.001.

Bcl-xL regulates intracellular CD1d trafficking

Bcl-xL knockdown results in a significant reduction in CD1d-mediated Ag presentation to NKT cells; however, the levels of surface CD1d expression are comparable with the controls. The overexpression or knockdown of Bcl-xL altered the activation of N37-1A12, a type II NKT cell hybridoma. These NKT cells do not require CD1d recycling for Ag presentation, suggesting that this is not the mechanism by which Bcl-xL mediates its regulation. We therefore hypothesized that Bcl-xL alters the intracellular trafficking of CD1d molecules. To determine the precise mechanism of regulation, we first analyzed the intracellular trafficking of CD1d by anti-CD40 mAb treatment in WEHI-231 cells results in increased NKT cell responses. (A) Western blotting for Bcl-xL (upper panel) and GAPDH (loading control, lower panel) after treatment of WEHI-231 cells with anti-CD40 Ab (1C10) for 24 h (10 μg/ml) or with an isotype control Ab. (B) Cell-surface CD1d expression on WEHI-231 cells treated with anti-CD40 Ab or the corresponding controls was determined by flow cytometry. (C and D) Medium alone or WEHI-231 cells treated with anti-CD40 Ab were cocultured with mouse NKT cell hybridomas (B) DN32.D3 or (C) N38-3C3 in the presence or absence of an exogenous Ag for 20 h. IL-2 was measured in the supernatant by ELISA. ***p < 0.001.

FIGURE 2. Induction of Bcl-xL by anti-CD40 mAb treatment in WEHI-231 cells results in increased NKT cell responses. (A) Western blotting for Bcl-xL (upper panel) and GAPDH (loading control, lower panel) after treatment of WEHI-231 cells with anti-CD40 Ab (1C10) for 24 h (10 μg/ml) or with an isotype control Ab. (B) Cell-surface CD1d expression on WEHI-231 cells treated with anti-CD40 Ab or the corresponding controls was determined by flow cytometry. (C and D) Medium alone or WEHI-231 cells treated with anti-CD40 Ab were cocultured with mouse NKT cell hybridomas (B) DN32.D3 or (C) N38-3C3 in the presence or absence of an exogenous Ag for 20 h. IL-2 was measured in the supernatant by ELISA. ***p < 0.001.

FIGURE 3. Induction of Bcl-xL by anti-CD40 mAb treatment in primary mouse B cells increases CD1d-mediated Ag presentation to NKT cells. (A) Splenic mouse B cells were treated with 10 μg/ml anti-CD40 Ab (1C10) or isotype control for 24 h, and Bcl-xL protein was detected by Western blotting (upper panel). GAPDH was used as the loading control (lower panel). (B and C) Treated B cells or medium alone were cocultured with primary murine liver mononuclear cells with or without α-GalCer. (B) IFN-γ and (C) IL-4 were measured in the supernatant by ELISA. (D–G) Flow cytometry was performed to determine cell-surface expression of (D) MHC class I (H-2Kb), (E) CD1d, (F) CD80, or (G) CD86 after treatment of primary mouse B cells with anti-CD40 Ab. ***p < 0.001.
localization of CD1d in L-CD1d cells after Bcl-xL knockdown. Under normal conditions, CD1d is localized in the lysosomal compartment, as seen by its colocalization with LAMP1 (CD107a) (16). We used confocal microscopy to determine the intracellular location of CD1d after Bcl-xL knockdown. As expected, control L-CD1d cells (scramble) showed strong colocalization of CD1d with LAMP1 (Fig. 6A). Interestingly, after Bcl-xL knockdown (shBcl-xL), this colocalization was substantially reduced (Fig. 6A, 6B). These data show that after Bcl-xL knockdown, CD1d molecules are lost from the LAMP1+ compartment. Such an alteration is known to affect CD1d-mediated Ag processing and presentation, indicating that this is at least one mechanism by which Bcl-xL regulates Ag presentation to NKT cells.

**FIGURE 4.** Pharmacological inhibition of Bcl-xL inhibits CD1d-mediated Ag presentation to NKT cells, as well as MHC class II-mediated Ag presentation to classical CD4+ T cells. (A) L-CD1d-DR4 cells were treated with vehicle alone (DMSO), ABT-263, or ABT-199 for 4 h at a concentration of 10 or 20 μM, washed extensively, and cocultured with NKT cell hybridomas. IL-2 in the supernatant was measured by ELISA. (B) L-CD1d-DR4 cells were loaded with HSA overnight and treated with vehicle alone, ABT-263, or ABT-199 as indicated earlier. After treatment, cells were washed extensively and cocultured with 17.9 T cell hybridomas. IL-2 was measured in the supernatant by ELISA. (C) Annexin V–PI staining was used to determine the percentage of viable cells (cells that were not positive for Annexin V and PI) after treatment with ABT-263 or ABT-199. (D) Flow cytometry shows surface expression of CD1d or HLA-DR after treatment of L-CD1d-DR4 cells with ABT-263 or ABT-199. ***p < 0.001.

**FIGURE 5.** Knockdown of Bcl-xL or Bcl-2 leads to decreased CD1d-mediated Ag presentation to NKT cells. (A) Western blotting showing Bcl-xL protein levels (upper panel) or Bcl-2 (middle panel) after transduction of L-CD1d cells with lentiviral vectors carrying shRNA sequences targeting Bcl-xL or Bcl-2. GAPDH is the loading control (lower panel). (B) Flow cytometry shows surface CD1d expression on L-CD1d cells transduced with a scramble shRNA sequence or sequence targeting Bcl-xL or Bcl-2. (C and D) Medium alone or L-CD1d cells after knockdown of Bcl-xL or Bcl-2 were cocultured with (C) DN32.D3 or (D) N37-1A12 NKT cell hybridomas, and IL-2 released in the supernatant was measured by ELISA. **p < 0.01, ***p < 0.001.
Bcl-xL causes expansion of the Rab7⁺ late endosomal compartment

We found that Bcl-xL knockdown leads to the loss of CD1d from the LAMP1⁺ compartment (Fig. 6A, 6B). We further sought to determine an alternative intracellular compartment where CD1d molecules accumulate after Bcl-xL knockdown. We found that similar to the LAMP1⁺ compartment, there was minimal colocalization of CD1d with EEA1, a marker for early endosomes in the control (yellow), whereas this localization pattern is lost after Bcl-xL knockdown. Original magnification ×400. (B) Quantification of CD1d and LAMP1 colocalization in scramble control or Bcl-xL knockdown cells. *p < 0.05.

FIGURE 6. Loss of CD1d molecules from the LAMP1⁺ lysosomal compartment after Bcl-xL knockdown. (A) Confocal microscopy was used to determine the intracellular localization of CD1d. L-CD1d cells transduced with a scrambled shRNA sequence (scramble, upper row) or an shRNA sequence targeting Bcl-xL (shBcl-xL, lower row) were fixed, permeabilized, and stained with Abs to CD1d (red) and LAMP1 (green), a marker of the lysosomal compartment. DAPI was used to visualize the nucleus. The merge (rightmost panels) shows that CD1d is localized in the LAMP1⁺ late endosomal/lysosomal compartment in the control (yellow), whereas this localization pattern is lost after Bcl-xL knockdown. Original magnification 3400. (B) Quantification of CD1d and LAMP1 colocalization in scramble control or Bcl-xL knockdown cells. *p < 0.05.

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FIGURE 7. Reduced expression of CD1d in early endosomes after Bcl-xL knockdown. (A) Confocal microscopy on control or Bcl-xL-deficient L-CD1d cells stained with Abs against CD1d (red) or EEA1 (green), a marker for early endosomes. The rightmost panels show colocalization of CD1d with EEA1, which is indicative of its presence in the early endosomal compartment. As compared with the control, Bcl-xL knockdown cells show less localization of CD1d in the early endosomes (yellow). Original magnification ×400. (B) Quantification of CD1d and EEA1 colocalization in scramble control or Bcl-xL knockdown cells. **p < 0.01.
early endosomes, indicating that CD1d molecules might have accumulated in a different intracellular compartment, impairing Ag presentation after Bcl-XL knockdown.

A previous report has shown that Bcl-xL can bind to Rab7, which is a Ras-related GTP binding protein present in late endosomes (46). Rab7 is critical to endosome biogenesis and the endocytic pathway. We found that there was greater Rab7 staining after Bcl-xL knockdown as compared with the scramble control (Fig. 8A, 8B). The increased Rab7 staining indicates an expansion of the late endosomal compartment, whereas the probability of colocalization of CD1d and Rab7 remained unchanged, suggesting that after Bcl-xL knockdown, CD1d molecules may be accumulating in the late endosomes (Fig. 8A–C). We also examined Rab7 protein levels by Western blotting and found that after Bcl-xL knockdown, Rab7 protein was increased as compared with the scramble (Fig. 8D). Thus, knocking down Bcl-xL leads to upregulation of Rab7 and an expansion of the late endosomal compartment. Furthermore, this expanded endosomal compartment acts as a depot for CD1d molecules, resulting in impaired trafficking to the lysosomal compartment, which is critical for Ag presentation (Supplemental Fig. 3). The results indicate that under normal conditions, Bcl-xL regulates the endosomes and limits the size of the late endosomal compartment. When Bcl-xL is reduced, this regulation is lost, leading to upregulation of Rab7 and expansion of the late endosomal compartment (Supplemental Fig. 3).

Discussion

We have identified a novel function for Bcl-xL in CD1d-mediated Ag processing and presentation through its regulation of the late endosomal compartment. Because of the current interest in using NKT cell–based cancer therapy (47), and with the knowledge that lymphoma cells have an alteration in CD1d-mediated Ag presentation compared with healthy B cells (48), the identification of such a regulatory factor is not only an advancement in our understanding of the mechanisms that form the basis of NKT cell–mediated tumor recognition, but also have direct translational impact by allowing the development of more effective NKT cell–based antitumor immunotherapy.

We present evidence of altered CD1d trafficking as a mechanism for Bcl-xL–mediated regulation of Ag presentation. Bcl-xL was also found to regulate peptide Ag presentation by MHC class II molecules in a similar manner, altering their ability to present Ag to classical T cells. This could potentially affect the antitumor immune response and the tumor microenvironment because CD4+ T cells are critical in orchestrating a cytokine milieu that could significantly alter the effectiveness of an antitumor response.

In this study, we show that the overexpression of Bcl-xL in APCs leads to increased activation of NKT cells. Furthermore, the knockdown of Bcl-xL using shRNA led to decreased Ag presentation to NKT cells, indicating that Bcl-xL plays a role in the regulation of CD1d-mediated Ag processing and presentation. After the induction of Bcl-xL in a B cell lymphoma cell line, as well as in primary mouse B cells by treatment with anti-CD40 Ab, there was increased NKT cell activation. Finally, we used pharmacological inhibition using ABT-263, an inhibitor of Bcl-xL and Bcl-2, and found that there was decreased Ag presentation to NKT cells, as well as classical CD4+ T cells, whereas the selective inhibition of Bcl-2 using ABT-199 had no effect. ABT-263 and ABT-199 are BH3 mimetics, and functionally inhibit Bcl-xL and/or Bcl-2 by binding to its BH3 domain and preventing dimerization with other BH3-containing proteins. This type of inhibition is distinct from shRNA-mediated knockdown, which results in an overall reduc-

FIGURE 8. Accumulation of CD1d molecules in the Rab7+ late endosomal compartment after Bcl-xL knockdown. (A) Confocal microscopy on control or Bcl-xL knockdown L-CD1d cells stained with Abs to CD1d (red) and Rab7 (green), a late endosomal marker. The rightmost panels show that CD1d is more heavily colocalized with Rab7 (yellow) in the Bcl-xL knockdown cells as compared with the control. (B) Quantification of Rab7 staining based on pixel number in scramble control or Bcl-xL knockdown cells. (C) Quantification of colocalization of CD1d and Rab7 in scramble control or Bcl-xL knockdown cells. (D) Western blotting shows that Rab7 protein is upregulated after Bcl-xL, but not Bcl-2, knockdown (upper panel), and GAPDH is shown as the loading control (lower panel). Original magnification ×400.
tion in BcL-XL or BcL-2 protein expression. We have discovered that although knocking down BcL-XL or BcL-2 led to reduced Ag presentation, selective BH3-dependent inhibition of BcL-XL and BcL-2, but not BcL-2 alone, led to reduced Ag presentation to NKT cells. This suggests that BcL-XL and BcL-2 regulate CD1d-mediated Ag presentation through different mechanisms. Our studies demonstrate that BcL-XL--mediated regulation is dependent upon its BH3 domain and involves increased Rab7 and altered trafficking of CD1d molecules through the endocytic compartments. However, the ability of BcL-2 to regulate CD1d-mediated Ag presentation was not dependent upon the BH3 domain, and there was no evidence of Rab7 upregulation after BcL-2 knockdown. These findings highlight an important functional difference between the structurally similar BcL-XL and BcL-2 proteins.

Surface CD1d expression was unchanged after BcL-XL overexpression, induction, inhibition, or knockdown in WEHI-231 cells, mouse fibroblast cell lines LMTK-CD1d and L-CD1d, as well as primary mouse B cells. Therefore, we hypothesized that BcL-XL regulates the intracellular CD1d-mediated Ag processing and presentation. In line with this, we found that after BcL-XL knockdown, CD1d molecules were lost from the LAMPI+ compartment and accumulated in the Rab7+ late endosomal compartment. A previous study has reported a direct interaction between BcL-XL and Rab7 (32). However, we do not know whether this interaction is sufficient to explain the upregulation of Rab7 after BcL-XL knockdown. It is also possible that BcL-XL causes expansion of the late endosomal compartment through an indirect mechanism, and the upregulation of Rab7 is a consequence of this expansion (46). Further studies may reveal more intermediates through which BcL-XL mediates its regulation on CD1d-mediated Ag presentation. Another issue that warrants further investigation is the finding that intracellular CD1d trafficking is altered after BcL-XL knockdown, without significant changes in surface CD1d expression (Fig. 5). To ascertain these results, we performed a time-course study of CD1d cell-surface expression, as well as examined CD1d-expressing CalCeR complexes, and found no striking differences, indicating that the observed effects cannot be attributed to subtle temporal changes in CD1d expression (Supplemental Fig. 2D, 2E). It is well established that the CD1d-mediated Ag presentation pathway through the endocytic compartments requires trafficking through the low pH lysosomal compartment or MIIC for proper Ag presentation. The requirement for accessory factors available only in the LAMP1+ compartment, or the presence of inhibitory factors in the late endosomes, can be a potential explanation for the observed results. The involvement of other enhancing or inhibitory factors requires further investigation and can lead to an important advancement in our understanding of the regulation of Ag presentation pathways, ultimately resulting in the development of strategies to modulate Ag presentation not only in cancer, but also in any other setting where boosting of the immune response is desired.

In summary, we have found that BcL-XL plays a role in the regulation of CD1d-mediated Ag presentation to NKT cells through alterations in CD1d trafficking. Cells in which BcL-XL expression is reduced have high Rab7 expression and an expanded late endosomal compartment. This indicates that BcL-XL has more general effects on the endocytic pathway even under nonapoptotic conditions and can change CD1d trafficking and affect Ag presentation to NKT cells. Our study reveals a novel function of BcL-XL, which is a major target for the development of therapeutics (49), and may help in designing better immuno-therapy.

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Disclosures

The authors have no financial conflicts of interest.

References


