Published in final edited form as:

J Immunol. 2014 September 1; 193(5): 2096–2105. doi:10.4049/jimmunol.1400155.

Bcl-xL regulates CD1d-mediated antigen presentation to NKT cells by altering CD1d trafficking through the endocytic pathway

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Abstract

Natural killer T (NKT) cells are a unique subset of T cells that recognize glycolipid antigens presented in the context of CD1d molecules. NKT cells mount strong anti-tumor 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, very little is known about the regulation of CD1d-mediated antigen processing and presentation in B cell lymphoma. Pro-survival 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 antigen presentation to NKT cells. We found that over-expression or induction of Bcl-xL led to increased antigen presentation to NKT cells. Conversely, the inhibition or knockdown of Bcl-xL led to decreased NKT cell activation. Furthermore, knockdown of Bcl-xL resulted in the loss of CD1d trafficking to LAMP1+ 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 antigen processing and presentation to NKT cells by altering the late endosomal compartment and changing the intracellular localization of CD1d.

Introduction

NKT cells are a unique subset of T cells that recognize lipid antigens presented by CD1d, an MHC class I-like molecule (1-3). Once activated, NKT cells can mediate direct cytotoxicity and also rapidly produce large amounts of cytokines such as IFN- γ and IL-4. One of the most striking and well-established functions of NKT cells is their anti-tumor effect, mediated directly by cytotoxicity, as well as indirectly by cytokine production leading to the recruitment and activation of other cell types (4-6). However, the precise mechanisms that underlie the recognition of tumors by NKT cells, in the absence of an exogenous activating antigen like the prototypical α -Galactosylceramide (α -GalCer), remain poorly understood.

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Conflicts of Interest

The authors declare no conflict of interest.

In contrast to the MHC restriction of classical T cells, NKT cells are CD1d-restricted (7, 8). Mice possess *CD1d1* and *CD1d2* genes, however, antigen presentation to NKT cells is dependent upon CD1d1 molecules (referred to as CD1d). The CD1d molecule is structurally similar to MHC class I with a three domain α chain that associates with β_2 -microglobulin (β_2m), but unlike the classical MHC class I molecule, CD1d has a hydrophobic antigen binding groove (9, 10). Also, in contrast to the ubiquitous expression of MHC class I, CD1d is mainly expressed on dendritic cells, macrophages, B cells and T cells (11).

The process of CD1d-mediated antigen presentation is complex and begins with the synthesis of the CD1d α chain in the ER (12). Here chaperons like calnexin, calreticulin and Erp57 ensure that it is properly folded (13). The antigen binding groove of CD1d is occupied by a self lipid antigen thought to be loaded by the microsomal triglyceride transfer protein (MTTP) (14, 15). After association with β_2m , the CD1d molecule follows the secretory pathway from the ER to the Golgi and reaches the plasma membrane (PM). In order to present an activating endogenous antigen to NKT cells, CD1d molecules recycle from the PM to endocytic compartments due to the presence of a tyrosine based targeting motif (Yxx ϕ 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 (Ii) for MHC class II molecules. In fact, Ii associates with CD1d but the Yxx ϕ 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 MIIC, where MHC class II molecules are normally loaded with peptide antigens (19, 20). Once in the endocytic recycling compartment, the stabilizing self lipid is exchanged for other lipid antigens with the help of saposins (21). These loaded CD1d molecules are then re-expressed on the PM and can be recognized by canonical V α 14J α 18 NKT cells. The localization of CD1d to cholesterol-rich lipid rafts is important for efficient antigen presentation, especially in the presence of low concentrations of antigens and the disruption of these lipid rafts leads to reduced antigen presentation (22, 23). The complex multi-step process of CD1d-mediated antigen processing and presentation has several potential levels of control, yet very few endogenous regulatory factors have been identified. Prominent among these, are the mitogen-activated protein kinases (MAPK), PKC δ and Rho kinases (24-26). In this study we sought to identify a target that regulates CD1d-mediated antigen presentation and is relevant to tumor growth and survival.

Anti-apoptotic 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 anti-apoptotic factor and exerts its anti-apoptotic function by heterodimerizing with other Bcl-2 family members and preventing the permeabilization of the mitochondrial outer membrane. Bcl-xL can also mediate its pro-survival function by causing the retro-translocation of the pro-apoptotic 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

antigen 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 (ERGIC1) and vesicle associated membrane protein 3 (VAMP3), underscoring the multifaceted effects of Bcl-xL (32).

To examine the role of Bcl-xL on CD1d-mediated antigen 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. Following Bcl-xL knock down, 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 antigen presentation, ultimately resulting in loss of NKT cell activation.

Materials and Methods

Cell lines, antigens and other reagents

L-CD1d, L-CD1d-DR4 and LMTK-CD1d, are mouse fibroblast cell lines transfected with *mCD1d1* or *mCD1d* and *HLA-DR4* (kindly provided by Dr. Randy Brutkiewicz, Indiana University School of Medicine) and were cultured in DMEM with 10% FBS and 2 mM L-glutamine. Additionally, selection agents G418 sulfate (500 µg/ml) from Mediatech Inc. (Manassas, VA), 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, Baltimore) and cultured in RPMI 1640 with 10% FBS, 100 mM sodium pyruvate, 10 mM non-essential amino acid solution, 1x vitamin solution and 50 µM 2-mercaptoethanol. The mouse type I 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 2 mM L-glutamine (33, 34). 17.9 CD4⁺ T cell hybridomas were kindly provided by Dr. Janice Blum (Indiana University School of Medicine) and cultured in RPMI supplemented with 10% FBS, 50 µM 2-mercaptoethanol and 2 mM L-glutamine. α-Galactosylceramide purchased from Enzo Life Sciences (New York City, NY) was used at a final concentration of 100 ng/ml Human Serum Albumin was purchased from Sigma-Aldrich (St. Louis, MO) and used at 10 µM. ABT-263 and ABT-199 used for the inhibition of Bcl-xL and/or Bcl-2 were a kind gift from Dr. Gregory Carey (University of Maryland, Baltimore).

Over-expression 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, Baltimore). Transfection was

carried out using Neofectin one-step transfection reagent from NeoBioLab (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 three days. Over-expression of Bcl-xL was confirmed by Western blotting.

Bcl-xL knockdown was attained using lentiviral vectors purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These replication incompetent lentiviral particles contained a pool of 3-4 short hairpin sequences targeted to the *Bcl2l1* gene that encodes Bcl-xL, or the *Bcl-2* gene and polybrene based 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 three days. 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 approximately 6 weeks of age. All performed procedures were approved by the University of Maryland Baltimore, 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, Canada) as per the manufacturer's instructions. Purity was greater than 95% as determined by flow cytometry after staining with FITC conjugated antibody to B220 (CD45RO) from BioLegend (San Diego, CA). Enriched splenic B cells were suspended in RPMI 1640 with 10% FBS, and other supplements as described above. 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 antibody to the TCR beta chain from BioLegend (San Diego, CA) and APC conjugated CD1d tetramers loaded with α-GalCer, graciously provided by the NIH Tetramer Core Facility at Emory University (Atlanta, GA).

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 antibody 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 (5×10^5 per well) were co-cultured with NKT cells (5×10^4 per well) for 20 hours at 37°C in IMDM supplemented with 5% FBS and L-glutamine. Primary splenic B cells were treated with 10 µg/ml of anti-CD40 antibody (clone 1C10) from eBioscience (San Diego, CA), washed with complete medium and cocultured (10^6 B cells per well) with liver mononuclear cells (2×10^5 per well) for 24 hours 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 hours, washed twice with PBS and once with complete medium, and cocultured with NKT cells. For the peptide antigen presentation assay, L-CD1d-DR4

cells were loaded with HSA overnight, treated with ABT-263 or ABT-199 for 4 hours, washed extensively and cocultured with 17.9 T cells hybridomas. Following 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%), NP-40 (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 PVDF 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 antibodies to Bcl-xL (clone 54H6) from Cell Signaling Technology (Beverly, MA) or Rab7 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). GAPDH levels were detected on the same blot as the test protein using an antibody (clone 14C10) from Cell Signaling Technology (Beverly, MA). Dylight-800 conjugated anti-rabbit secondary antibody was purchased from Thermo Scientific (Waltham, MA). Membranes were scanned using the Odyssey Imaging System from Li-COR Biosciences (Lincoln, Nebraska).

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 minutes at room temperature. Non-specific binding sites were blocked by incubating cells in PermWash buffer from BD Bioscience (San Jose, CA) with 5% rat serum for 1 hour at 4°C. The cells were then incubated with antibodies to CD1d (clone 1H6), kindly provided by Dr. Randy Brutkiewicz (Indiana University School of Medicine, Indianapolis, IN), CD107a (LAMP1) from eBioscience (San Diego, CA), EEA1 from Cell Signaling Technology (Beverly, MA) or Rab7 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) at 4°C overnight. After washing, AlexaFluor-546 conjugated anti-mouse IgG, APC conjugated anti-rabbit IgG and AlexaFluor 647 conjugated anti-rat IgG from Life Technologies (Carlsbad, CA) were added and incubated for 1 hour 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 40x 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 5 different images, and Pearson's correlation for colocalization was represented as percent of control. Additionally, Rab7 staining was quantified by pixel number from 5 different images and represented as percent of control.

Statistical analyses

All experiments were repeated at least three times. Two-tailed student's t test, one-way analysis of variance (ANOVA) or two-way ANOVA were used as appropriate. Specific experimental groups were compared with controls using the Bonferroni post test. p value less than 0.05 was considered significant. All analyses were performed using Prism 5.02 by GraphPad (La Jolla, CA). *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Results

Over-expression of Bcl-xL in antigen presenting cells leads to increased NKT cell responses

Bcl-xL is a pro-survival member of the Bcl-2 family of proteins. Here, we hypothesized that Bcl-xL affects the ability of an antigen presenting cell to present glycolipid antigens to NKT cells, independent of its regulation of apoptosis. To determine if the overexpression of Bcl-xL has an effect on CD1d mediated antigen presentation, we utilized WEHI-231, a classical and well-characterized murine B cell lymphoma cell line (36), and compared it to a WEHI-231-derived cell line stably transfected with murine Bcl-xL. Over-expression 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 V α 14J α 18 NKT cell hybridomas- DN32.D3 and N38-3C3 and IL-2 production was measured in the co-culture supernatant (Fig. 1B and C). In the absence of exogenously added antigens, the WEHI-231 control, as well as Bcl-xL transfected cell lines, failed to activate NKT cells. These data suggest that the endogenous antigen presented by CD1d molecules in these cells does not activate NKT cells. However, when the potent NKT cell agonist, α -Galactosylceramide, was added to the co-culture, 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 antigen is enhanced following the over-expression of Bcl-xL (Fig. 1B and C).

To characterize the potential role of Bcl-xL in CD1d-mediated antigen presentation we used LMTK cells as an *in vitro* antigen presenting cell system. LMTK cells, transfected with CD1d (LMTK-CD1d), are known to present an endogenous activating antigen 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 following activation, providing a straightforward system to study CD1d-mediated antigen presentation to NKT cells with minimal confounding factors. In order to directly test if Bcl-xL plays a role in antigen presentation to NKT cells, LMTK-CD1d cells were transfected with an empty control plasmid or a plasmid carrying the *Bcl2l1* gene, which encodes Bcl-xL, resulting in the over-expression of Bcl-xL protein as seen by Western blotting (Fig. 1G). There were no apparent differences in cell morphology, growth or proliferation (Fig. S1A-D). 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. While 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 over-expression of Bcl-xL in LMTK-CD1d cells elicited significantly higher response from all three hybridoma lines tested (Fig. 1D, E and F). From these data, it is evident that Bcl-xL can affect NKT cell activation since high levels of Bcl-xL elicit stronger NKT cell responses. Furthermore, we used CD1d blocking antibodies and found that IL-2 production was completely lost following CD1d blockade in the control transfected as well as Bcl-xL transfected LMTK-CD1d cells (Fig. S2A-C). This indicates that regulation of Bcl-xL is specific to CD1d-dependent antigen presentation to NKT cells.

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

We found that the over-expression of Bcl-xL in antigen presenting cells leads to enhanced antigen presentation to NKT cells. Next, we wanted to induce Bcl-xL using a biological stimulus and determine if antigen presentation to NKT cells was affected. One of the most well-known aspects of B cell stimulation with anti-CD40 antibody is the upregulation of Bcl-xL, and 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 antibody (clone 1C10) and confirmed the induction of Bcl-xL protein by Western blotting (Fig. 2A). We found that treatment with anti-CD40 antibody did not alter surface CD1d expression (Fig. 2B). WEHI-231 cells treated with anti-CD40 antibody elicited higher NKT cell activation compared to cells treated with isotype control antibody (Fig. 2C and D). 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 over-expression of Bcl-xL (Fig. 1B-F) and show that increased Bcl-xL levels lead to a corresponding increase in CD1d-mediated antigen presentation to NKT cells.

Bcl-xL-mediated regulation of antigen presentation by 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 antigen presenting cells and liver mononuclear cells as a source of primary NKT cells. Approximately 40% of T cells in the liver are NKT cells, as compared to the thymus and spleen (1-2%). Also, liver NKT cells are the only subset known to mediate anti-tumor effector functions (42). To assess the effect of Bcl-xL induction on antigen presentation to NKT cells, splenic B cells were treated with anti-CD40 antibody for 24 hours and Western blotting was used to ensure the upregulation of Bcl-xL protein (Fig. 3A). Following 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 and C). 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 to their untreated or isotype control treated counterparts (Fig. 3B). However, IL-4 production was unchanged after treatment with anti-CD40 antibody (Fig. 3C). In spite of the use of total liver mononuclear cells, the observed responses were NKT cell specific as all cytokine levels were detectable only in cocultures containing NKT cell antigens. In addition, we analyzed the cells by flow cytometry following 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 antigen 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

In order to study the effect of Bcl-xL inhibition on CD1d-mediated antigen 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, while ABT-199 is Bcl-2 specific. Pre-treatment of L-CD1d-DR4 cells with ABT-263 led to decreased CD1d-mediated antigen presentation to NKT cells (Fig. 4A). In contrast, pre-treatment with ABT-199 had no effect on antigen 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 antigen presentation to NKT cells. Since the antigen 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 antigen presentation to classical CD4⁺ T cells (45). L-CD1d-DR4 cells loaded with human serum albumin (HSA) were treated with ABT-263 or ABT-199, washed extensively and cocultured with 17.9 T cell hybridoma line (Fig. 4B). Similar to CD1d-mediated antigen presentation, we found that the presentation of HSA was reduced following treatment with ABT-263 but was unaffected by treatment with ABT-199. Since 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 antigen presentation is independent of apoptosis (Fig. 4C). Finally, there were no evident changes in cell surface expression of CD1d or HLA-DR following treatment with ABT-263 or ABT-199 (Fig. 4D).

Stable knock-down of Bcl-xL causes inhibition of CD1d-mediated antigen 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 antigen processing and presentation. We used lentiviral particles carrying a pool of 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 following stable knock down (Fig. S1A and D). Also, there were no evident changes in surface CD1d expression following 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 NKT cell hybridomas. The knockdown of Bcl-xL or Bcl-2 led to decreased NKT cell activation as indicated by a significant reduction in IL-2 production by both DN32.D3 and N37-1A12 NKT cell hybridomas (Fig. 5C and D).

Bcl-xL regulates intracellular CD1d trafficking

Bcl-xL knockdown results in a significant reduction in CD1d-mediated antigen presentation to NKT cells; however, the levels of surface CD1d expression are comparable to the controls. The over-expression 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 antigen 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 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 (lysosome associated membrane protein 1, CD107a) (46). We used confocal microscopy to determine the intracellular location of CD1d following 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 and B). These data show that following Bcl-xL knockdown, CD1d molecules are lost from the LAMP1⁺ compartment. Such an alteration is known to affect CD1d-mediated antigen processing and presentation, indicating that this is at least one mechanism by which Bcl-xL regulates antigen presentation to NKT cells.

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 and B). We further sought to determine an alternative intracellular compartment where CD1d molecules accumulate following Bcl-xL knockdown. We found that similar to the LAMP1⁺ compartment, there was minimal colocalization of CD1d with EEA1, a marker for early endosomes, following Bcl-xL knockdown (Fig. 7A and B). This shows that CD1d molecules are lost from the LAMP1⁺ lysosomes as well as the EEA1⁺ early endosomes, indicating that CD1d molecules might have accumulated in a different intracellular compartment, impairing antigen 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 (47). Rab7 is critical to endosome biogenesis and the endocytic pathway. We found that there was greater Rab7 staining after Bcl-xL knockdown as compared to the scramble control (Fig. 8A and B). The increased Rab7 staining indicates an expansion of the late endosomal compartment, while the probability of colocalization of CD1d and Rab7 remained unchanged, suggesting that following Bcl-xL knockdown, CD1d molecules may be accumulating in the late endosomes (Fig. 8 A, B and C). We also examined Rab7 protein levels by Western blotting and found that following Bcl-xL knockdown, Rab7 protein was increased as compared to the scramble (Fig. 8D). Thus, knocking down Bcl-xL leads to up-regulation 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 antigen presentation (Fig. S3). 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 (Fig. S3).

Discussion

We have identified a novel function for Bcl-xL in CD1d-mediated antigen processing and presentation through its regulation of the late endosomal compartment. Due to the current interest in using NKT cell based cancer therapy (48), and with the knowledge that lymphoma cells have an alteration in CD1d-mediated antigen presentation compared to healthy B cells (Sun *et al.*, manuscript in preparation), 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 anti-tumor immunotherapy.

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

In this study, we show that the over-expression of Bcl-xL in antigen presenting cells leads to increased activation of NKT cells. Furthermore, the knock-down of Bcl-xL using shRNA led to decreased antigen presentation to NKT cells, indicating that Bcl-xL plays a role in the regulation of CD1d-mediated antigen processing and presentation. Following 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 antibody, 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 antigen presentation to NKT cells as well as classical CD4⁺ T cells, while 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 a complete reduction in Bcl-xL or Bcl-2 protein expression. We have discovered that while knocking down Bcl-xL or Bcl-2 led to reduced antigen presentation, selective BH3-dependent inhibition of Bcl-xL and Bcl-2, but not Bcl-2 alone, led to reduced antigen presentation to NKT cells. This suggests that Bcl-xL and Bcl-2 regulate CD1d-mediated antigen 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 compartment. However, the ability of Bcl-2 to regulate CD1d-mediated antigen presentation was not dependent upon the BH3 domain and there was no evidence of Rab7 upregulation following 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 over-expression, 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 antigen processing and presentation. In line with this, we found

that following Bcl-xL knock down, CD1d molecules were lost from the LAMP1⁺ 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 if this interaction is sufficient to explain the upregulation of Rab7 following Bcl-xL knockdown. It is also possible that Bcl-xL causes expansion of the late endosomal compartment through an indirect mechanism, and the up-regulation of Rab7 is a consequence of this expansion (47). Further studies may reveal more intermediates through which Bcl-xL mediates its regulation on CD1d-mediated antigen presentation. Another issue that warrants further investigation is the finding that intracellular CD1d trafficking is altered following 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:α-GalCer complexes, and found no striking differences, indicating that the observed effects cannot be attributed to subtle temporal changes in CD1d expression (Fig. S2D and E). It is well established that the CD1d-mediated antigen presentation pathway through the endocytic compartments requires trafficking through the low pH lysosomal compartment or MIIC for proper antigen 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 antigen presentation pathways, ultimately resulting in the development of strategies to modulate antigen 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 antigen 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 non-apoptotic conditions and can change CD1d trafficking and affect antigen 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 immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH/NCI K01 CA131487, R21 CA162273, R21 CA162277, and P30 Tumor Immunology and Immunotherapy Program to T.J. Webb. The authors would like to acknowledge the NIH Tetramer Core Facility at Emory University (Atlanta, GA) for kindly providing antigen loaded CD1d tetramers for this study.

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Figure 1.

Over-expression of Bcl-xL results in enhanced CD1d-mediated antigen presentation to NKT cells. **(A)** Western blot showing Bcl-xL protein levels (upper panel) in WEHI-231 or WEHI-231 following Bcl-xL overexpression. GAPDH (lower panel) is the loading control. **(B-C)** Medium alone, WEHI-231 (Ctrl) or WEHI-231/Bcl-xL (Bcl-xL) cells were cocultured with **(B)** DN32.D3 or **(C)** N38-3C3 NKT cell hybridomas with or without an exogenous antigen and IL-2 in the supernatant was measured by ELISA. **(D-F)** Medium alone, LMTK-CD1d cells over-expressing Bcl-xL or the corresponding empty vector controls were cocultured with mouse NKT cell hybridomas **(D)** DN32.D3, **(E)** N37-1A12 or **(F)** N38-3C3 for 20 hours and IL-2 in the supernatant was measured by ELISA. **(G)** Western blot showing Bcl-xL protein levels (upper panel) in LMTK-CD1d cells transfected with empty vector (ctrl) or Bcl-xL. GAPDH is the loading control (lower panel). **(H)** Flow cytometry shows surface CD1d expression on LMTK-CD1d cells transfected with Bcl-xL (open histogram) or vector alone (shaded histogram).

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 antibody (1C10) for 24 hours (10 μ g/ml) or with an isotype control antibody. **(B)** Cell surface CD1d expression on WEHI-231 cells treated with anti-CD40 antibody or the corresponding ctrls was determined by flow cytometry. **(C-D)** Medium alone or WEHI-231 cells treated with anti-CD40 antibody were cocultured with mouse NKT cell hybridomas (B) DN32.D3 or (C) N38-3C3 in the presence or absence of an exogenous antigen for 20 hours. IL-2 was measured in the supernatant by ELISA.

Figure 3.

Induction of Bcl-xL by anti-CD40 mAb treatment in primary mouse B cells increases CD1d-mediated antigen presentation to NKT cells. (A) Splenic mouse B cells were treated with 10 μ g anti-CD40 antibody (1C10) or isotype control for 24 hours and Bcl-xL protein was detected by Western blotting (upper panel). GAPDH was used as the loading control (lower panel). (B-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) CD1d, (E) MHC Class I (H-2K^b), (F) CD80 or (G) CD86 after treatment of primary mouse B cells with anti-CD40 antibody.

Figure 4.

Pharmacological inhibition of Bcl-xL inhibits CD1d-mediated antigen presentation to NKT cells as well as MHC Class II-mediated antigen presentation to classical CD4⁺ T cells. (A) L-CD1d-DR4 cells were treated with vehicle alone (DMSO), ABT-263 or ABT-199 for 4 hours 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 Human Serum Albumin (HSA) overnight and treated with Vehicle alone, ABT-263 or ABT-199 as indicated above. 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) following treatment with ABT-263 or ABT-199. (D) Flow cytometry shows surface expression of CD1d or HLA-DR following treatment of L-CD1d-DR4 cells with ABT-263 or ABT-199.

Figure 5.

Knockdown of Bcl-xL or Bcl-2 leads to decreased CD1d-mediated antigen 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, 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.

Figure 6.

Loss of CD1d molecules from the LAMP1⁺ lysosomal compartment following 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 antibodies to CD1d (red), and LAMP1 (green), a marker of the lysosomal compartment. DAPI was used to visualize the nucleus. The merge (rightmost panels) show that CD1d is localized in the LAMP1⁺ late endosomal/lysosomal compartment in the control (yellow) while this localization pattern is lost after Bcl-xL knockdown. (**B**) Quantification of CD1d and LAMP1 colocalization in scramble control or Bcl-xL knockdown cells.

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 antibodies 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 to the control, Bcl-xL knockdown cells show less localization of CD1d in the early endosomes (yellow). **(B)** Quantification of CD1d and EEA1 colocalization in scramble control or Bcl-xL knockdown cells.

Figure 8.

Accumulation of CD1d molecules in the Rab7⁺ late endosomal compartment following Bcl-xL knockdown. **(A)** Confocal microscopy on control or Bcl-xL knockdown L-CD1d cells stained with antibodies 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 knock down cells as compared to 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 following Bcl-xL but not Bcl-2 knockdown (upper panel) and GAPDH is shown as the loading control (lower panel).